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# Use of *Bacillus subtilis* starters during red sorghum malting:

## a contribution to sorghum malting and brewing microbiology and biochemistry

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Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

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**Jean-Claude Bwanganga Tawaba (2013)**. Use of *Bacillus subtilis* starters during red sorghum malting: a contribution to sorghum malting and brewing microbiology and biochemistry. PhD thesis, University of Liege, Gembloux Agro-Bio Tech (Belgium), 145 p., 29 tables, 35 figures.

### *Summary*

*Bacillus subtilis* was used to inhibit mould growth during red sorghum malting. Improved conditions for achieving good malt properties were studied and mathematical models are proposed for the induction and the repression phases of $\alpha$- and $\beta$-amylase synthesis. The problems associated with the hydrolysis of $\beta$-glucans and the biocontrol steeping effect on $\beta$-glucanase activities are discussed. The effect of the biocontrol treatment and that of phytohormones produced by the bacterial strain used on the synthesis of specific red sorghum enzymes are elucidated. Gibberellic acid and abscisic acid diffusion and cross-talk as factors affecting the synthesis of red sorghum malt $\alpha$- and $\beta$-amylase activities are also discussed. The production of 3-indole acetic acid (IAA) by *B. subtilis* S499 was also a focus in this study and the determination of the conditions for improving the production of indole-3-acetic acid are determined.

Keywords: *Bacillus subtilis*, Mould biocontrol, sorghum malting

**Jean-Claude Bwanganga Tawaba (2013)**. Utilisation des starters de *Bacillus subtilis* lors du maltage du sorgho rouge : contribution à la microbiologie et à la biochimie du maltage et du brassage du sorgho. Thèse de doctorat ; Université de Liège, Gembloux Agro-Bio Tech, (Belgique), 145 p., 29 tableaux, 35 figures.

### *Résumé*

*Bacillus subtilis* a été utilisé pour lutter contre le développement des moisissures lors du maltage du sorgho rouge. Les conditions d'amélioration des propriétés du malt sont étudiées et des modèles mathématiques proposés pour les phases d'induction et de répression de synthèse des enzymes clés. La problématique liée à l'hydrolyse des $\beta$-glucanes et l'effet du biocontrôle sur les activités $\beta$-glucanasiques sont abordés. L'effet du biocontrôle et des phytohormones, produites par la souche bactérienne utilisée, sur la synthèse des enzymes est élucidé. La diffusion et, l'interaction entre l'acide abscissique et l'acide gibbérellique comme facteurs affectant la synthèse des enzymes du malt sont aussi abordées dans ce travail. La production de l'acide 3-indole acétique (AIA) par *B. subtilis* S499 a été suivie et les conditions pour améliorer la production de cette phytohormone ont été déterminées.
Mots clés : *Bacillus subtilis*, biocontrôle des moisissures, maltage du sorgho

Faut-il tuer Dieu mais se soumettre aux législateurs, ou bien vivre libre dans les bois en continuant à craindre les esprits ? L'autonomie pratique et matérielle ne semble pas une conquête moins noble que l'autonomie spirituelle et intellectuelle.

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## Abbreviations and acronyms

AA: Alpha-amylase
ABA: Abscisic Acid
AIA: Acide 3-indole acétique
ANOVA: Analysis Of Variance
BA: Beta-amylase
BGlu: Beta-glucanase
BSA: Bovine serum albumin
*BSP*: *Bacillus subtilis* population
CE: Cathechin Equivalent
d. b.: Dried basis
DF: Dilution Factor
CFU: Colony-Forming Unit
*DI*: Durée d'incubation
EBC: European Brewery Convention
EDTA: Ethylenediaminetetraacetic acid
GA: Gibberellic Acid
GAE: Gallic Acid Equivalent
GC: Germination Capacity
*GD*: Germination Duration
GE: Germination Energy
GLM: General Linear Model
GLMLL: General Linear Model with Logarithm Link
*GT*: Germination Temperature
HSD: Honestly Significant Difference
IAA: Indolacetic Acid
N-RSL: Not Renewed Steeping Liquor
MaxID: Maximal Inhibitory Dilution
MES: 2-(N-morpholino) ethanesulfonic acid
minID: Minimal Inhibitory Dilution
$2^{nd}$ OPM: $2^{nd}$ Order Polynomial Model
PCA: Plate Count Agar
PDA+C: Potato Dextrose Agar with Chloramphenicol
PNP$\beta$-G3: p-nitrophenyl-$\beta$-D-maltotrioside
RBGlu: Residual beta-glucans
RMSE: Root Mean Square Error
RP-HPLC-DAD-MS: Reverse Phase High-Performance Liquid Chromatography-Photodiode Array Detector-Mass Spectrometry
RSL: Renewed Steeping Liquor
R-sq: R square
R-sq (adj): Adjusted R square
SNK: Student-Newman-Keuls
SSE: Sum of Square Error
ST: Steeping Treatment
TGW: Thousand Grain Weight
TML: Total Malting Loss
Tryp: tryptophane
w.b.: Wet basis
W-4-PM: Weibull 4-parameter Model

## List of publications

### Published peer-reviewed papers

1. Bwanganga, T.J-C., Béra, F. and Thonart, P., 2012. Optimizing red sorghum malt quality when *B. subtilis* is used during steeping to control mould growth. *Journal of the Institute of Brewing* 118 (3), 295-304.
2. Bwanganga, T.J-C., Béra, F. and Thonart, P., 2013. Modelling the *β*-amylase activity during red sorghum malting when *B. subtilis* is used to control mould growth. *Journal of Cereal Science* 57, 115-119.
3. Bwanganga, T.J-C., Destain, J., Malumba, K.P., Béra, F. and Thonart, P., 2013. Effect of the use of dilute alkaline prior to *Bacillus subtilis*-based biocontrol steeping and germination conditions on red sorghum malt *β*-glucanase activities and residual *β*-glucans. *Journal of Cereal Science*. 58 (1), 148-155.

### Peer-reviewed papers accepted for publication

**1.** Bwanganga, T.J-C., Ba, K., Malumba, K.P., Destain, J., Béra, F. and Thonart, P. (in press). Vers une intégration du sorgho comme matière première pour la brasserie moderne. *BASE*.

**2.** Bwanganga, T.J-C., Pondo, K.B., Malumba, K.P., Destain, J., Béra, F. and Thonart, P. (in press). Suitability of the Weibull 4-parameters model to predict the induction phase of α-amylase production during red sorghum malting when a steep in dilute NaOH is used prior to a resteep in a *Bacillus subtilis*-S499 based treatment. *Journal of the Institute of Brewing*.

## Table of Contents

## List of tables

## List of Figures

## List of equations

## General introduction

In 2006, Professor J.R.N. Taylor published an interesting review on the uses of sorghum (Taylor *et al*., 2006) exposing the progress and focus for the integration of sorghum in the modern brewing industry. Is it possible to malt sorghum in good conditions in order to obtain a sorghum malt as a substitute to barley malt, allowing the production of beer with an acceptable quality in a cost-effective manner? Is the current state of the understanding of the changes taking place, as well as during sorghum malting and brewing, reassuring for the industrial use of sorghum? Is there a better future for the use of sorghum as a raw material in the modern food industry in general?

The use of sorghum in the modern brewing industry poses a number of problems, namely: (1) the difficulty of lautering, this is due, among others, to the fact that sorghum grains have no protective husks; (2) the difficulty of achieving both the foam stability and the colloidal stability especially when it comes to making Pils-type beers; (3) the need to add exogenous enzymes, the enzyme content of sorghum varieties are generally poor; (4) the difference of saccharification temperature between sorghum and barley starches which makes the simultaneous brewing of sorghum and barley malt difficult (Ogbonna, 1992; Hallgren, 1995; Kunze, 1999; Ogbonna, 2011); (5) mould growth and mycotoxin production, etc. According to Singh and Bandyopadhyay (2000), the loss of production due to mould varies from 30 to 100% depending on the cultivar, time of flowering and prevailing weather conditions from flowering to harvest.

If these difficulties are not all solved, it is due to the emerging interest which sorghum is granted by the modern brewing industry and is not due to a disadvantage of using sorghum grain as compared with barley, nor the more acute performance demanded by the use of sorghum in brewing. Today, enzyme technology knowledge, for example, should allow the use of sorghum as the raw grain in brewing and solve the problem of its high levels of phenolic compounds by adding the required enzymes for timely brewing; the development in lautering technology (e.g. MEURA 2001 filter) should allow easy lautering of sorghum-based worts; the problems related to beer stability are solvable by the use of both good brewing methods and appropriate technological additions; the control of mould growth is possible today as chemical treatments and / or biocontrol have been used to combat mould growth during sorghum malting.

Although sorghum has some problems when used in brewing – these problems are related to the fact that initially, the brewer wanted to use it without much additional investment; the reasons brewers became interested in sorghum are numerous:

- sorghum is a gluten-free cereal (good raw material for the manufacture of gluten-free beer);

- some varieties of sorghum have *α*-amylase levels higher than barley (Aniche and Palmer, 1990);
- for African brewers the increasing price of barley malt is one of the constraints which is driving the greatest possible effort to enable the use of local products, namely sorghum (Kunze, 1999) − sometimes price increases of around 70% are experienced in a few weeks (E-malt website, 2007), etc.

This study addresses four main issues:

- can *B. subtilis* be used to control mould growth under sorghum malting conditions?
- how to improve malt properties so as to meet the brewer's expectations when *B. subtilis* is used as starters?
- how to improve the conditions of the use of this bacterium to make the most profit in terms of sorghum malt properties?
- what are the phenomena which underlie the effects of the use of this bacterium and how to improve the efficiency of the treatments?

The overall objective of this work is the use of sorghum malt, as a substitute or supplement to barley malt, in modern brewing. That said, the fight against mould growth, determining the conditions for obtaining sorghum malts with interesting characteristics for brewing use (suitable enzyme pool, good modification, etc.) and the effect of the interaction between *B. subtilis* and malted grain were the focus of this study. The starting point of this project is the fight against mould growth, which is of major concern to the brewer and maltster; consequently a series of measures have been developed to enhance the use of this biocontrol (*B. subtilis* starters).

This study aims to contribute to the understanding of the underlying biochemical and physiological phenomena which play a role in obtaining sorghum malt with good qualities when *B. subtilis* starters are used to inhibit mould growth. In fact, malting is foremost "diverting the grain intelligence with contours hitherto poorly circumscribed for the benefit of the maltster". This is where the role of the maltster becomes important, namely to direct all physiological and biochemical changes, especially those taking place in the non-living part of the grain, i.e. the endosperm before "the poor little heir" − the seed − is fully aware of everything that is rightfully availables: the use of energy reserves of grain.

The purpose of malting grain is also to select members of its microbial ecosystem, to choice molecules that must be eliminated and the downtime of all the physical and biochemical transformations (...)." Malting therefore, also changes the biotic and abiotic environment of the grain, not to ensure good growth of the embryo, but rather to assure that the grain metabolism is either wholly or mainly directed towards the synthesis of the enzymes needed for brewing.

In this work we present: generalities which include an overview of malting, the role of the microbial ecosystem of grain and a review on the malting and brewing of sorghum. The results are focused on the use of *B. subtilis* as a biocontrol of mould during red sorghum malting. The search for optimal soaking and germination conditions, along with an understanding of the biochemical and physiological phenomena that underlie the effect of the use of *B. subtilis* on the properties of malts are presented. A general discussion and conclusion, and some perspectives are also presented.

The first step in this study was obtaining the culture of *B. subtilis* at a density allowing the development of mould biocontrol. Malting was then conducted in three stages: soaking, germination and kilning, with some variations depending on the treatment in question and the study objectives (see details in each chapter). A series of treatments were conducted to enhance the properties of the sorghum and to elucidate the effect of the use of *B. subtilis* on the characteristics of the malted grain. The problem of the hydrolysis of $\beta$-glucans, the synthesis of phytohormones (by *B. subtilis*) and their application conditions are studied; improvement of the synthesis of specific enzymes associated with the grain-*Bacillus* interactions are addressed and indole 3-acetic acid production by *B. subtilis* was also enhanced.

# Chapter I. Sorghum as brewing adjuncts - general information

## *1.1. Malting cereal: an overview*

Beer production can be divided into three major milestones, namely: malting, brewing and fermentation steps; packaging is also a very important step in the modern brewery in terms of investment, working time and labour, etc.

Beer production implies the modification of the grain to obtain malt, the extraction of malt to obtain fermentable wort and the fermentation of the wort to produce beer. This is made possible by controlling the conditions of the synthesis and functioning of hydrolytic enzymes (during malting and brewing), and those of the anaerobic working of yeast (during fermentation). Indeed, for producing beer, it is necessary to provide yeast with sugars and a range of nutrients. In raw grain, sugars are largely stored as starch and some other nutrients that may be used by the yeast are in unavailable forms (proteins, etc.). The key to brewing is to extract the maximum possible sugars in their fermentable form, to dissolve the amount of nitrogen required for the development of yeast, foam formation and stability, and development softness, etc. All of this requires functioning enzymes, which may be more or less influenced by the pretreatment suffered by the proteins and starches of the grain, because the malting ability of the grain is the result of various interactions, complex by nature, between a number of biochemical and structural parameters which determine the efficiency of the use of the endosperm reserves and nutrient availability.

The raw grain must be transformed into malt via a process in which the enzymes for hydrolysis of the grain constituents are synthesized and / or activated. This process begins by soaking, allowing the grain moisture to increase about 12% to about 45%, thus initiating the germination of seeds. The maltster must create conditions of temperature, pH and aeration, for good seed germination. When the physical and biochemical changes taking place during this process meet the requirements of the maltster, the germination is stopped by drying the green malt (kilning), which reduces the rate of the initiated biochemical reactions, stops the growth of the malt germ and provides the particular colour and characteristics of the malt by developing desirable flavour compounds and removing undesirable volatiles. This heat treatment, which reduces the moisture of grain to about 4% or less, also allows easy removal of rootlets. Enzymes produced during germination are, so to speak, partially inactivated, because the conditions necessary for their function: pH, moisture, temperature, etc. no longer exist. Portions of enzymes developed in the green malt are also destroyed during kilning depending on the temperature scales used and the temperature sensitivity of the enzyme in question. The malt obtained contains more or less hydrolytic enzymes (amylases, proteases, glucanases, etc.), is more or less modified and is then milled before the conditions for hydrolysis and extraction of the grain components are created during brewing (extraction, lautering, boiling and hopping). Hopped wort is

pitched with yeast which transforms, under anaerobic conditions, the fermentable sugars into alcohol and the other components of the wort into any series of molecules that give the beer its specific profile.

During malting, the increase in grain moisture is a prerequisite for germination. It is thus necessary to achieve optimum moisture for germination, and create the temperature and aeration conditions needed to trigger germination. During steeping, the water enters the grain and diffuses, bringing with it a multitude of molecules with various differing functions. Although classical models are often used for grain rehydration equating the grain in a homogeneous body or not, spherical and ellipsoidal in others, etc., the movement of water in the grain is unique. Indeed, the water in the grain initially follows a preferential path from the basal end (the side of the embryo) towards the apical end. When considering the rehydration of grain, malting can be divided into five phases (Figure 1): the first 3 phases, which are rehydration phases cover soaking and germination (see Bewley and Black, 1994; Bewley, 1997) and the last two phases, which are drying phases, cover the kilning (see De Clerck, 1962).

Phase 1: This is the imbibition phase during which the rehydration is simply due to the low water potential of the grain (-100 to -200 MPa) (Chong *et al.*, 2002). During this physical diffusion phase, the volume of grain increases (Chong *et al.*, 2002) and the rehydration rate may be strongly influenced by the temperature and the nature of the steeping liquor.

Phase 2: This is the so-called active metabolism and hydrolysis phase during which water absorption is a function of metabolic needs − the internal osmotic potential (Chong *et al.*, 2002). According to De Clerk (1962), this phase begins when grain moisture is around 40%. As mentioned above, along with the diffusion of water is one of a series of molecules, some of which are signalling molecules (phytohormones, for example). Indeed, the germination is controlled by the balance between the activators and inhibitors of germination (Chong *et al.*, 2002), modulated by several biotic and abiotic factors (Ma and Berkowitz, 2012). During germination, these phytohormones are perceived by specific receptors in specialized cells. This perception is followed by complex and tangled transduction pathways and amplification, only now beginning to be understood (Hopkins, 2003). Finally, the target cell receives a very specific signal. During this second phase, phytohormones (endogenous and / or exogenous) diffuse into the grain, from the embryo to the aleurone layer (via the apoplastic and / or symplastic pathway), trigger the synthesis and / or activation of enzymes (glucanases, proteases, amylases, etc.) which hydrolyse the content of the grain, making the diffusion of enzymes and phytohormones in the endosperm easier (Bruggeman *et al.*, 2001).

Phase 3: Begins with the emergence of the radicle and plumule (the so-called visible germination phase). During this phase, the additional water needed to break the constraints of the embryo expansion exerted by adjacent cells, is only possible if the water potential of the steeping solution is

below -0.2 to -0.3 MPa (Chong *et al.*, 2002). This is important because it can greatly affect the percentage of pre-germination, even when the conditions of temperature and aeration are met.

Phase 4: This is the desiccation phase (germination and / or enzymatic phase of kilning), during which the germination and enzymatic reactions initiated during the early stages continue but are reduced (grain moisture is high enough and temperature is compatible for germination and enzymatic reactions to occur). It is therefore conceivable that the duration of this phase depends on the kilning temperature of the green malt and the intrinsic characteristics of the grain (moisture, modification degree, etc.).

Phase 5: The chemical phase of kilning: "coup de feu" (De Clerck, 1962). The grain moisture does not allow the continuation of germination and enzymatic reactions, but several chemical and physical reactions may occur during this phase (DMS degradation, Maillard reactions, oxidation of polyphenols, etc.), (see Lewis and Bamforth, 2006).

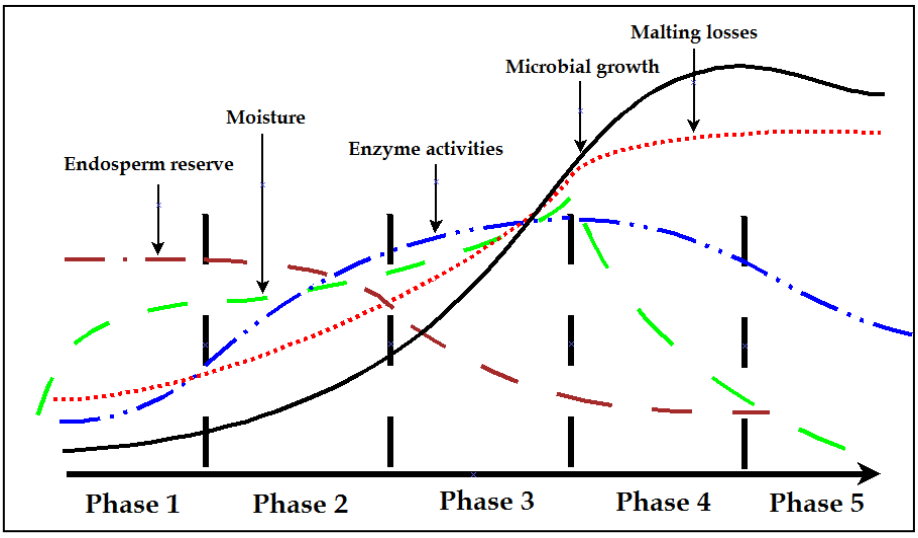



**Figure 1. Five malting phases**

### *1.2. The grain-microbe interactions: role of the phytohormones produced by members of the grain microbial ecosystem*

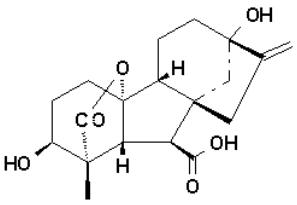
Sorghum grain can be infected by several fungal species such as: *Acladium conspersum, Acremonium strictum, Alternaria (A. alternate, A. brassicicola, A. longipes, A. longissima, A. tenuissima), Aspergillus (A. candidus, A. flavus, A. niger), Bipolaris (B. australiensis, B. halodes, B. maydis, B. sacchari, B. spicifera, B. zeicola), Botrytis cinerea, Chaetomium oryzae, Cladosporium (C. oxysporum, C. sphaerospermum), Colletotrichum graminicola, Curvularia (C. affinis, C. clavata, C. eragrostidis, C. fallax, C. geniculata, C. harveyi, C. lunata, C. lunata* var *aeria, C. ovoidea, C. pallescens, C. trifolii, C. tuberculata), Epicoccum nigrum, Exserohilum (E. rostratum, E. turcicum, Fusarium (F. moniliforme, F. semitectum), Gloecercospora sorghi, Gonatobotrys simplex, Nigrospora*

*oryzae, Penicillium (P. citrinum, P. griseofulvum), Periconia macrospinosa, Phoma sorghina, Rhizopus stolonifer, Spadicoides obovata, Torula graminis* and *Trichothecium roseum* (Navi *et al.*, 1999).

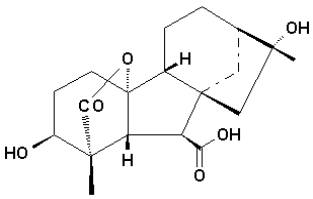
Some grain colonizing microbes have positive effects during malting, in the sense that they can improve seed germination and hence the processability of the malt, but the presence of others during malting is rather disadvantageous (production of mycotoxins, reduction of the filtration rate, premature yeast flocculation, etc.) (Laitila *et al.*, 2007, Just *et al.*, 2011) − hence the need to make a choice among the members of the microbial ecosystem of the grain. The maltster has several ways to combat undesirable microorganisms; carefully selected microbial cultures can be used as starters during soaking to modify the malting microbial ecosystem dynamic. Often used in a high density population, these microbes are perceived by the grain during germination as a biotic stress and cause metabolic interactions and a variety of informational flow, expressed in an obscure language, understood by the microorganisms and germinated grain. The interactions between these two metabolically active groups, i.e. microorganisms on one side and the grain on the other side, affect the quality of the malt and beer (Petters *et al.*, 1988; Laitila *et al.*, 2007; Just *et al.*, 2011); the exact mechanisms by which microorganisms influence malt properties are not fully known.

Indeed, during malting the conditions for grain germination must be met to ensure proper germination. These conditions also allow the growth of several members of the microbial ecosystem, whose role in grain germination is not to be overlooked.

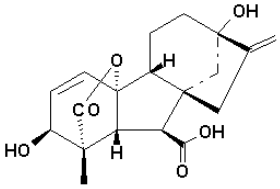
Germination is the stage during which the majority of physical, chemical and biochemical changes take place in the grain. This stage is strongly influenced by the grain microbial ecosystem (Just *et al.*, 2011) and is under hormonal control (Hopkins, 2003). Indeed, plant hormones, which are grouped into five classes, namely auxins, gibberellins, cytokinins, abscisic acid and ethylene (see http://www.plant-hormones.info) are not only produced by plants, but also by certain microorganisms (bacteria and fungi) (Takahashi *et al.*, 1955; Atzorn *et al.*, 1988).



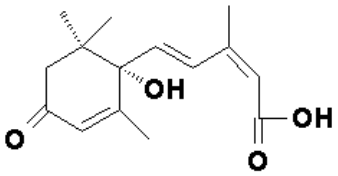
Gibberellin $A_1$;
Molar mass = 348.4 g $mol^{-1}$;
Molecular formula = $C_{19}H_{24}O_6$



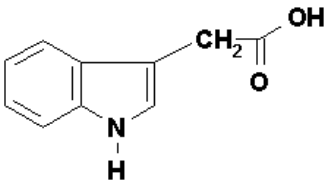
Gibberellin $A_2$;
Molar mass = 350.42 g $mol^{-1}$;
Molecular formula = $C_{19}H_{26}O_6$



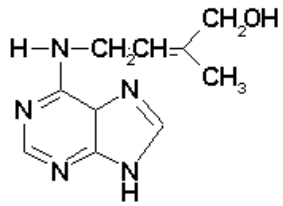
Gibberellin $A_3$;
Molar mass = 346.38 g $mol^{-1}$;
Molecular formula = $C_{19}H_{22}O_6$



Abscisic acid;
Molar mass = 264.32 g $mol^{-1}$;
Molecular formula = $C_{15}H_{20}O_4$



Indole acetic acid ;
Molar mass = 175.184 g.$mol^{-1}$;
Molecular formula = $C_{10}H_9NO_2$



Zeatin;
Molar mass = 219,2431 g·$mol^{-1}$;
Molecular formula = $C_{10}H_{13}N_5O$

**$H_2C=CH_2$**: Ethylene; Molar mass = 28,0532 g·$mol^{-1}$; Molecular formula = $C_2H_4$

Gibberellins have been identified as the secondary metabolites of a rice pathogen, namely *Gibberella fujikuroi* (bakanae disease) (Takahashi *et al.*, 1955). Several other microorganisms are recognized as gibberellin producers; some moulds such as: *Phaeospheria* sp. L487, *Sphaceloma bidentis*, *Sphaceloma manihiticola*, *Sphaceloma menthea*, *Sphaceloma perseae*, *Sphaceloma rhois* and some bacteria such as: *Rhizobium meliloti*, *Azospirillum lipoferum*, *Azospirillum brasilense*, *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Bacillus pumilus*, *Bacillus licheniformis* and *Bacillus subtilis* (Atzorn *et al.*, 1988; Bottini *et al.*, 1989; Bastián *et al.*, 1998; Bastián *et al.*, 1999; Gutiérrez-Mañero *et al.*, 2001; Sgroy *et al.*, 2009 - to name only a few). Another important family of phytohormones is that of auxins; these phytohormones are synthesized by plants as well as microorganisms. The following microorganisms are known as auxin producers, namely: *Pisolithus tinctorius*, *Azospirillum*, *Pseudomonas syringae*, *Bradyrhizobium*, *Agrobacterium*, *Rhizobium*, *Herbaspirillum*, *Gluconocetobacter*, *Erwinia herbicola*, *Klebsiella* and *Cyanobacteria* (see http://www. plant-hormones.info for details). Other plant hormones are also synthesized by microorganisms: cytokinins by *Azotobacter chroococcum* (Arshad and Frankenberger, 1991) and *Bacillus subtilis* (Sgroy *et al.*, 2009), ethylene by *Acremomium falcate* (Arshad and Frankenberger,

1991), etc. The phytohormones produced by members of the plant microbial ecosystem can be absorbed by the plant cells and affect plant metabolism and physiology. Some microorganisms (fungi and bacteria) of the malted barley grains ecosystem have been recognized as phytohormone (GA3, IAA and abscisic acid) producers (Tuomi *et al.*, 1995). Microorganisms can thus divert plant intelligence by secreting these signalling molecules which affect plant growth. This strategy aims to seduce the plant cells so as to make the necessary nutrients for microbial growth available (see Zhao, 2010).

### *1.3. Vers une intégration du sorgho comme matière première pour la brasserie moderne*

***Résumé***

Malgré la nature somme toute récente de l'utilisation du sorgho en brasserie, la somme des études réalisées sur l'utilisation de cette céréale en malterie et en brasserie est loin d'être rudimentaire. Les connaissances sur la biochimie du maltage du sorgho ne font que s'accumuler. Malgré que l'intérêt de l'utilisation du sorgho en brasserie ne soit plus à démontré, les possibilités et les limites d'utilisation de ce cette céréale en brasserie moderne ne sont pas clairement élucidées - le maltage et toutes les transformations prenant place lors de ce process étant loin d'être totalement connus. Cette revue présente une somme des connaissances sur les propriétés du sorgho intéressant aussi bien le malteur que le brasseur : composition du grain, biochimie et physiologie du maltage, problématiques du brassage, les rudimentaires travaux de fermentation, etc. Les avancées et difficultés rencontrées lors du maltage du sorgho sont ainsi présentées en fonction des attentes du brasseur.
Mots clés : malt, bière, sorgho, maltage, brassage

***Abstract***

Despite the relatively recent use of sorghum in brewing, the accumulated information from studies on the use of this cereal in malting and brewing is far from basic; the biochemistry of sorghum malting consolidates this knowledge. Despite the interest bestowed in demonstrating the use of sorghum in brewing, the possibilities and limitations of using this cereal in the modern malting and brewing industries are not fully elucidated; in addition, all the transformations involved in these processes are far from completely understood. This review provides a wealth of knowledge on the properties of sorghum, which is of interest to the maltster and the brewer, such as: grain composition, malted grain biochemistry and physiology, brewing and fermentation of sorghum, etc. The progress and difficulties encountered during sorghum malting are presented according to the brewer's expectations.
Key words: malt, beer, sorghum, malting, brewing

### 1.3.1. Introduction

Le sorgho [*sorghum bicolor* (L.) Moench] est une céréale bien connue, dont l'aire d'origine possible serait entre l'Ouest de l'Ethiopie et l'Est du Tchad (Murty *et al.,* 2001). Il est la cinquième céréale mondiale en termes de production après le blé, le riz, le maïs et l'orge, avec 55 à 60 millions de tonnes produites par an – production qui était évaluée à exactement 55721588 tonnes en 2010 (http://faostat.fao.org). La difficulté de cultiver de façon rentable l'orge de brasserie sous les tropiques pousse de plus en plus les brasseurs travaillant en Afrique à penser à d'autres céréales comme compléments ou substituts au malt d'orge. En effet, au début de l'implantation des brasseries modernes en milieux tropicaux, cette céréale ne faisait pas partie des principales cultures de la région tropicale. La plupart des programmes publics de développement de l'agriculture étaient axés sur la production d'autres sources d'amidon comme le riz, le maïs et le manioc. Avec la perturbation climatique, la sécheresse croissante, le sorgho devint de plus en plus important et fut intégré comme ressource pouvant assurer la sécurité alimentaire de nombreuses populations. L'utilisation du sorgho en brasserie, malgré que somme toute récente, prend un regain d'intérêt, aussi bien pour la production de bières sans gluten que pour sa richesse en polyphénols, malgré toutes les difficultés rencontrées.

Les limites de l'utilisation du sorgho en brasserie sont nombreuses : méthodes de purification trop performantes pour retirer l'enveloppe du grain (traitements chimiques plus ou moins délicats utilisant des produits comme la soude caustique, le formaldéhyde, l'acide chlorhydrique), la contamination fongique impliquant la production des mycotoxines (les conditions des pays chauds étant favorables au développement des moisissures et à la production des mycotoxines) ; la faible digestibilité de ses constituants majeurs caractérisée par les températures d'empesage élevées de ses amidons, la possibilité de formation des ponts disulfures pour les protéines riches en résidus soufrés rendant difficile la digestibilité des protéines, et, partant, la solubilisation de l'azote ; les faibles activités *β*-amylasique et *β*-glucanasique du malt ; des teneurs élevées en *β*-glucanes faiblement déstructurés, ayant un impact sur le rendement d'extraction, la filtrabilité du moût et la stabilité ultérieure de la bière ; la présence d'énormes quantités de polyphénols avec un effet non négligeable sur le travail des enzymes, et la formation de trouble aussi bien au chaud qu'au froid (Beta *et al.,* 2000 ; Murty *et al.,* 2001 ; Taylor et al., 2006).

Cette revue présentera, et de façon non exhaustive, un aperçu sur les connaissances actuelles sur les constituants majeurs et les activités enzymatiques du sorgho pouvant jouer un rôle sur la processabilité de ses malts. Dans cette revue, l'état des connaissances actuelles sur les propriétés du sorgho ayant trait à son utilisation comme matière première en malterie et brasserie est discuté. L'objectif de ce travail est de montrer que les connaissances scientifiques actuelles sur le sorgho ne sont plus rudimentaires et devraient permettre son utilisation comme substitut ou complément au malt d'orge en

brasserie moderne. C'est ainsi qu'après chaque description d'une caractéristique, les attentes des malteurs et des brasseurs sont brièvement présentées.

### 1.3.2. Des constituants majeurs

#### *1.3.2.1. De l'amidon du sorgho*

L'amidon est de loin le constituant le plus abondant du grain de sorgho (65 à 85% matière humide) (Dicko *et al.*, 2006). Il est insoluble dans l'eau à l'état natif et est stocké sous forme de granules constituées de deux polymères principaux, à savoir l'amylose et l'amylopectine. Chez le sorgho, comme d'ailleurs chez les autres céréales, la teneur en amylose varie en fonction de la variété. Boudries *et al.* (2009) ont trouvé des teneurs moyennes en amylose de l'ordre de 24,8% (sorgho rouge) et 27,2% (sorgho blanc) – valeurs qui dépassent les limites proposées par Dicko *et al.* (2006). Signalons ici l'existence des variétés cireuses (waxy sorghum) dont l'amidon ne contient pas, ou très peu, d'amylose (Myer *et al.*, 1985). Les granules d'amidon du sorgho ont des diamètres compris entre 5 et 25 µm, avec une valeur moyenne autour de 15 µm (Dicko *et al.*, 2006).

Les propriétés physicochimiques, thermiques et rhéologiques des amidons sont tributaires des diverses interactions qui ont lieu avec les autres constituants (lipides, eau, protéines, etc.) (Sang *et al.*, 2008). La teneur en amylose, par exemple, affecte considérablement la gélatinisation et la rétrogradation de l'amidon, la viscosité de la pâte et la digestibilité de l'amidon par les amylases (Boudries *et al.*, 2009). Il est aussi connu, par exemple, que la formation des complexes amylose-lipides réduit l'accessibilité des liaisons osidiques aux sites des amylases (Tester *et al.*, 2006). La température d'empesage des amidons de sorgho (67 à 81°C pour les variétés cultivées en Inde) est bien supérieure à celle de l'amidon du malt d'orge (51 à 60°C) (Dufour *et al.*, 1992). Comme l'ont montré Adebowale *et al.* (2005), les traitements thermiques à humidité intermédiaire (Heat Moisture treatment) induisent une diminution de la capacité de gonflement et la solubilité des amidons de sorgho. Plusieurs auteurs ont soutenu le fait que même chauffé jusqu'à 98 à 100°C, la digestibilité de l'amidon du sorgho reste faible. Les interactions entre amidons et protéines du sorgho peuvent diminuer la digestibilité aussi bien des uns que des autres (Duodu *et al.*, 2002). Et, inversement, un traitement aux protéases peut être préconisé avant la liquéfaction de l'amidon pour améliorer la digestibilité de ce dernier (Pérez-Carrillo *et al.*, 2007).

Les brasseurs attendent de l'amidon qu'il soit liquéfié, empesé sans former une solution pâteuse salissant les vaisseaux et totalement saccharifié lors du brassage. Tout ceci passe par un travail qui commence au trempage des grains et doit normalement et complètement s'arrêter en chaudière d'ébullition.

#### *1.3.2.2. Des protéines du sorgho*

Les protéines représentent 7 à 15 % du poids sec du grain de sorgho (Dicko *et al.*, 2006), mais des valeurs encore plus petites ou plus grandes ont déjà été proposées (Lasztity, 1996). Les protéines des grains peuvent être groupées en 4 classes en fonction de leur solubilité ; il s'agit : des albumines (solubles dans l'eau), des globulines (solubles dans des solutions salines diluées), des glutélines (extractibles dans les solutions acides ou basiques diluées) et les prolamines (solubles dans l'alcool) - les prolamines étant les plus abondantes des protéines du sorgho (kafirines) (Hamaker *et al.*, 1995). En fonction de leur solubilité, de leur structure et de leur composition en acides aminés, les prolamines sont subdivisées en sous-classes (*α*-prolamines, *β*-prolamines, γ-prolamines et *δ*-prolamines). Les prolamines du sorgho forment des structures cylindriques qui sont rendues rigides par la présence des *γ*-prolamines qui cimentent en quelque sorte les unités de la sous-classe « *α* » rendant difficile le travail enzymatique. La sous-classe *α*-prolamine (22-28 kDa) serait essentiellement localisée à l'intérieur de la structure protéique et constituerait au-delà de 66 à 84% (Lasztity, 1996) de protéines totales du sorgho. Les *β*-prolamines (19-20 kDa) (7 à 8%) et *γ*-prolamines (27-28 kDa) (9 à 12%) (Lasztity, 1996) seraient présentes à l'extérieure de la structure formant une espèce de ciment pour les briques que sont les *α*-prolamines (Watterson *et al.*, 1993 ; Mazhar *et al.*, 1995). Les *δ*-prolamines (13 kDa) riches en méthionine (Belton *et al.*, 2006) seraient en très petites quantités dans le sorgho et très peu caractérisées (Lasztity, 1996). Les *α*-kafirines du sorgho auraient une faible teneur en cystéine alors que les *β* et γ-kafirines contiendraient des teneurs en cystéine respectivement autour de 5 et 7 % (% molaire) (Shull *et al.*, 1992), ce qui peut être à l'origine du développement des arômes soufrés. Selon Hamaker *et al.* (1995) il conviendrait mieux de classifier les protéines du sorgho en kafirines et non-kafirines pour permettre de mieux opposer d'un côté les kafirines, prolamines de nature homogène, et de l'autre les non-kafirines impliquées dans les fonctions cellulaires diverses.

Plusieurs facteurs affectent la digestibilité des protéines du sorgho. Des pourcentages de digestibilité de 96% (digestibilité in vitro par la pepsine) ont été obtenus après cuisson et traitement, par des agents réducteurs comme le bisulfite de sodium, de la farine de sorgho sans tannins condensés (Rom *et al.*, 1992) alors que Arbab *et al.* (1997) ont obtenu, après cuisson de la farine de sorgho riche en tannins condensés, un pourcentage de digestibilité in vitro à la pepsine de 12%. Des facteurs endogènes comme : la formation des ponts disulfures et non disulfures (p.ex. le couplage entre résidus tyrosines avec formation des ponts inter-peptides), l'hydrophobicité des kafirines et des changements dans la structure secondaire des protéines et exogènes comme : la structure du grain, la présence des composés polyphénoliques, l'acide phytique, l'amidon et les autres polysaccharides, influent sur la digestibilité des protéines du sorgho – la présence des ponts disulfures ayant une part plus importante dans la réduction de la digestibilité des protéines du sorgho (Duodu *et al.*, 2003).

Les protéines du sorgho sont riches en résidus prolines et ressemblent ainsi aux protéines salivaires avec lesquelles elles ont une grande affinité (Butler *et al.*, 1984 ; Emmambux *et al.*, 2003). Elles forment avec les composés phénoliques du sorgho (acides phénoliques, flavonoïdes, etc.) des complexes qui contribuent pour beaucoup à la réduction de leur digestibilité. La lysine et la thréonine sont les deux acides aminés limitants dans le sorgho - mais signalons tout de même qu'il existe à l'heure actuelle des variétés à très haute teneur en lysine.

Ce que veulent les brasseurs c'est que les protéines soient hydrolysées au maltage de sorte qu'au cours de l'extraction du grain (brassage) les acides aminés et les peptides nécessaires au maintien du pouvoir tampon du moût, à la croissance de la levure, à la tenue et à la stabilité de la mousse (…) se solubilisent en quantité et qualité nécessaires.

#### *1.3.2.3. Des polyphénols du sorgho*

Le sorgho renferme plusieurs classes de polyphénols. On trouve aussi bien des acides phénoliques de type benzoïque (acide protocatéchique, acide *p*-hydroxybenzoïque, acide gallique, acide vanillique, etc.) que cinnamique (acide férulique, *p*-coumarique et sinapique) (Beta *et al.*, 1999 ; Hahn *et al.*, 1984). Les plus abondants des acides phénoliques seraient l'acide férulique et l'acide *p*-coumarique (Hahn *et al.*, 1984). Ces composés existent soit sous forme libre soit sous forme estérifiée et sont concentrés dans la couche externe du grain (Waniska, 2000; Awika *et al.*, 2004). Selon les mêmes auteurs, ces composés sont indésirables du fait de leurs effets carcinogènes, hépatotoxique, goitrogénique, etc. Il est cependant possible de les éliminer par un simple traitement à la chaleur. Le Sorgho ne contiendrait pas d'acide tannique ni de tannins hydrolysables (Waniska, 2000; Awika *et al.*, 2004), mais contient, par contre, des procyanidines : (+)-catéchine, (-)-épicatéchine, (+)-gallocatéchine, (-) -galloépicatéchine et leurs polymères (Awika *et al.*, 2003). Les sorghos rouges sont en général plus riches en tannins condensés que l'orge (Dykes *et al.*, 2007). Des teneurs en tannins condensés de l'ordre de 0.01 à 3.48 % Equivalent Catéchine (E.C.) ont été obtenues par Earp *et al.* (1981). Des anthocyanidines ont aussi été trouvées dans le sorgho. Les plus abondantes sont les 3-deoxyanthocyanidines, comme par exemple l'apigéninidine et la lutéolinidine (Awika *et al.*, 2004). Ces composés sont très intéressants pour des applications alimentaires du fait de leur stabilité thermique et de la stabilité de leur coloration (Awika *et al.*, 2004). On trouve aussi des flavanones : naringénine (sorgho brun et rouge) (Awika *et al.*, 2004) ; des flavan-4-ols (sorgho blanc et sorgho rouge) et des stilbènes. Le *trans*-resvératrol et le *trans*-picéide ont aussi été découverts dans le sorgho rouge (Bröhan *et al.*, 2011).

Les composés polyphénoliques du sorgho - qui d'un côté se sont montrés capables de protéger les grains contre les attaques fongiques, les insectes et les oiseaux ce qui, sans doute est un avantage agronomique – réduisent la digestibilité enzymatique aussi bien des protéines que des amidons et

autres polysaccharides (Earp *et al.*, 1981 ; Serna-Saldivar and Rooney, 1995). La formation des complexes protéines-tannins qui, pour le cas du sorgho, se ferait majoritairement par des liaisons hydrogènes et par la formation des associations du type hydrophobe permettrait dans les conditions optimales aux tannins du sorgho de précipiter au moins 12 fois, leur poids propre, de protéines (Butler *et al.*, 1984).

Pour les malts utilisables en brasserie, le brasseur attend que la teneur polyphénols soit telle qu'elle rende possible la précipitation du surplus des protéines non hydrolysées ou mieux non solubilisées (responsables de l'instabilité ultérieure de la bière : formation de troubles) et, ce, au brassage majoritairement, à l'ébullition et à la filtration bière. Il faut tout de même aussi un peu de polyphénols pour assurer un certain pouvoir antioxydant à la bière. Certaines bières contiendraient d'ailleurs plus de proantocyanidines que certains vins blancs (USDA, 2004).

#### *1.3.2.4. De l'humidité des grains*

L'humidité des grains est un facteur important, aussi bien dans la conservation et le transport que lors des différents traitements subis par les grains. Les valeurs d'humidité des grains de sorgho seraient comprises entre 8 et 12 % (poids humide) (Dicko *et al.*, 2006). S'il est important de réduire l'humidité des grains pour permettre un bon stockage et une réduction du coût de transport, lors du trempage et de la germination, cette humidité atteint jusque 35 à 40 %, voire un peu plus (Ogbonna *et al.*, 2004). L'activité de l'eau ainsi modifiée permet de créer les conditions nécessaires à la synthèse et à la mobilisation des enzymes hydrolytiques, et aussi à toute une série de réactions biochimiques dont le résultat se résume en ce qu'il convient d'appeler la « désagrégation du grain ». Cette augmentation de l'humidité crée aussi des conditions favorables au développement des bactéries, levures et moisissures ainsi qu'à l'activation des spores. Et, si les conditions de température et d'humidité sont réunies, la production des mycotoxines devient plus que probable.

A la fin de la germination, lorsque toutes les transformations attendues ont eu lieu, l'humidité des grains est réduite par séchage, jusqu'autour de 3,5 – 4 % pour les malts pâles et 1,5 – 2 % pour les malts foncés (Esslinger *et al.*, 2005) si l'on s'en tient aux valeurs obtenues avec le malt d'orge. Cette réduction de l'humidité est importante pour permettre l'arrêt sinon la réduction de la vitesse des réactions biochimiques ayant pris place dans le grain et de faciliter les opérations ultérieures : transport, stockage, mouture, etc. Lors du maltage, ce n'est pas seulement l'humidité finale des grains qui est intéressante mais aussi toute la cinétique de réhumidification des grains. Lors du trempage par exemple, la vitesse de réhumidification est tributaire du traitement utilisé (Bwanganga *et al.*, 2012).

#### *1.3.2.5. Des lipides*

Les lipides représentent, dans les grains de sorgho, entre 1.5 à 6 % matière humide (Dicko *et al.*, 2006). Parmi les problèmes majeurs auxquels l'industrie brassicole moderne est confrontée figurent ceux liés à la stabilité de la bière et du développement des flaveurs indésirables lors du vieillissement. Contrairement au vin qui s'améliore en vieillissant, le vieillissement de la bière s'accompagne de l'apparition des flaveurs qui altèrent la qualité de celle-ci (Vanderhaegen *et al.*, 2006). L'un des points focaux est l'apparition des composés du type (E)-2-nonénal (par exemple le trans-2 nonénal responsable de l'arôme de carton) à travers le mécanisme d'oxydation des lipides, à côté de la formation d'arômes soufrés surtout pour une céréale comme le sorgho dont les protéines (kafirines) contiennent des fractions non négligeables d'acides aminés soufrés (Shull *et al.*, 1992). Les lipides du sorgho, composés majoritairement d'acides gras polyinsaturés (…) (Dicko *et al.*, 2006) ne sont pas à l'abri de l'attaque par les lipoxygénases – enzymes actives aux températures de touraillage du sorgho.

#### *1.3.2.6. Des polysaccharides non amylacés*

A part l'amidon qui est le polysaccharide majoritaire, le grain de sorgho contient toute une série de polysaccharides (arabinoxylanes, $\beta$-glucanes, pentosanes, celluloses, etc.) dont le rôle aussi bien lors du maltage que du brassage n'est pas à négliger. Les différentes proportions de chacun d'entre eux sont présentées dans la revue proposée par Dicko *et al.* (2006). La déstructuration et l'hydrolyse des polysaccharides constituant les parois de l'endosperme sont parmi les objectifs majeurs du maltage et du brassage du sorgho (Taylor *et al.*, 2006). Les $\beta$-(1,3),(1,4)-glucanes – polymères de $\beta$-D-glucose - sont les constituants majeurs de l'enveloppe des graminées; leur décomposition pendant le maltage et leur solubilisation au brassage constituent deux étapes importantes du process en ce sens qu'elles influent sur la viscosité du moût et la stabilité ultérieure de la bière. Les parois de l'endosperme du sorgho sont en grande partie extractibles dans les solutions alcalines (Glennie, 1984). Lors du brassage il est important que ces polysaccharides, les $\beta$-glucanes en l'occurrence, soient hydrolysés à un niveau tel que leur teneur résiduelle n'affecte la filtration du moût et de la bière, la floculation de la levure et la stabilité ultérieure de la bière.

### 1.3.3. De la problématique de l'hydrolyse des constituants du grain

#### *1.3.3.1. Des enzymes hydrolysant l'amidon*

Les enzymes hydrolysant les liaisons glucosidiques sont groupées dans ce que l'on appelle la famille 13 des glucosides hydrolases (Svensson *et al.*, 2002). Ces enzymes hydrolyseraient la liaison glucosidique suivant un mécanisme classique de substitution nucléophile. Deux mécanismes sont proposés dans la littérature (une substitution nucléophile d'ordre 1 et une substitution nucléophile d'ordre 2) (Takashi *et al.*, 2005). Le Pouvoir Diastasique (Diastatic Power : DP) est une expression de

l'activité enzymatique (quantité et type d'enzymes impliquées). Les enzymes dont l'activité collective convient d'être résumée en DP sont : *α*-amylase, *β*-amylase, *α*-glucosidase et limite dextrinase (Evans *et al.*, N.d). L'*α*-amylase existe sous deux formes dans le sorgho, à savoir l'isoforme 1 et l'isoforme 2 lesquelles ont des masses moléculaires comprises entre 41500 – 42700 Da (Mundy, 1982). Mundy (1982) a en effet trouvé, par analyse immunoélectrophorétique et par électrodiffusion une identité entre l'isoforme 1 du sorgho (qui n'existe que sous forme de trace dans les grains crus) et l'isoforme 2 de l'orge, aucune identité n'étant trouvée entre l'isoforme 2 du sorgho et les amylases de l'orge.

La *β*-amylase [*α*-1,4-D-glucan maltohydrolase, EC 3.1.1.2], plus sensible à la température que l'*α*-amylase (Lewis *et al.*, 2006) est une préoccupation majeure lors du maltage du sorgho (reviewed by Taylor *et al.*, 2006). Les facteurs influençant la synthèse de cette enzyme durant le maltage ont été étudiés par Taylor *et al.* (1993) and Bwanganga *et al.* (2013a). Ces résultats ont montré par exemple que, contrairement à l' *α*-amylase) la température de germination influe négativement sur la vitesse de synthèse de l'activité *β*-amylase. Des modèles mathématiques (modèle de Weibull à 4paramètres) ont été proposés (Bwanganga *et al.*, 2013a) pour permettre de trouver un compromis entre ces deux enzymes (*α* et *β*-amylases) dont l'importance sur l'hydrolyse de l'amidon n'est plus à démontrer.

L'*α*-glucosidase (EC 3.2.1.20) catalyse l'hydrolyse du maltose en deux unités glucose, le terme maltase en est le synonyme. Son rôle lors de la germination du grain est important, à savoir la production de glucose à partir du maltose, des oligosaccharides, des dextrines et amidons (Briggs *et al.*, 2004) – le glucose étant facilement assimilable par le germe. Sa présence en quantité élevée dans le malt de sorgho est souvent vue comme un défaut, en ce sens qu'il n'est pas important de transformer le maltose en glucose – le maltose ne posant aucun problème d'assimilation par la levure). Alors que le maltose est le sucre principal dans le moût de malt d'orge, c'est le glucose qui est le principal sucre dans un moût de malt de sorgho – ce qui est souvent attribué à la faible activité *β*-amylasique du moût de malt de sorgho (Agu *et al.*, 1996a) ; mais d'autres auteurs pensent que l'activité *α*-glucosidase élevée des malts des sorghos aurait une contribution non négligeable à cette anomalie (Taylor, 1992). L'hydrolyse du maltose durant le brassage peut aussi être vue comme un moyen de lever l'inhibition créée par l'augmentation de la concentration en maltose sur l'activité *β*-amylase.

La dextrinase limite (EC 3.2.1.41) est une enzyme hydrolysant les liaisons *α*-(1,6) dans les *α* et *β*-dextrines dérivant de l'amylopectine, elle-même, dérivée de l'amidon et d'autres oligo-saccharides des céréales. Les dextrines qui sont des sucres non assimilables par la levure sont importantes pour donner à la bière du corps et son petit caractère sucré.

Un autre problème posé par l'utilisation du sorgho en brasserie est la saccharification incomplète de son amidon, impliquant l'obtention des extraits peu fermentescibles (Taylor *et al.*, 2006). Cette

saccharification incomplète a été attribuée aux températures élevées de gélatinisation des amidons (Dufour *et al.,* 1992), à l'inhibition partielle de l'activité amylasique par d'importantes quantités de tannins naturellement présentes dans diverses variétés de sorgho (surtout les sorghos rouges), au faible pouvoir diastasique endogène (activités $\alpha$ et $\beta$-amylases) des grains de sorgho et à ses parois cellulaires insuffisamment dégradées pendant la germination (Etokakpan *et al.,* 1990) et empêchant un ramollissement de l'endosperme et une digestion optimale des amidons faiblement dispersé. Certaines variétés de sorgho ont des teneurs en $\alpha$-amylase supérieures à celles de l'orge (Aniche *et al.,* 1990).

La $\beta$-amylase n'existerait quasi pas dans les grains crus de sorgho- même pas sous forme liée : pas d'augmentation de la teneur en $\beta$-amylase après traitements par les agents réducteurs ou la papaïne – mais est synthétisée pendant le maltage (Taylor *et al.,* 1993). Plusieurs traitements visant à améliorer la qualité des malts du sorgho sont évalués en termes d'activités d'enzymes précises : $\alpha$-amylase, $\beta$-amylase, etc. Le scénario du brassage est tellement dynamique que l'activité globale des enzymes in situ est loin d'être la somme des activités individuelles (Lewis *et al.*, 2006).

#### *1.3.3.2. Des peptidases du sorgho*

Le deuxième constituant majeur du sorgho c'est sa fraction protéique. Les peptidases (protéases ou enzymes protéolytiques) sont des enzymes qui brisent les liaisons peptidiques des protéines. On parle alors de coupure protéolytique ou de protéolyse. La conversion des protéines en peptides et acides aminés solubles est l'une des transformations les plus importantes qui ont lieu pendant le maltage et le brassage (Evans *et al.,* 1990). La teneur en acides aminés et en peptides dans le moût est l'un des paramètres importants dans l'appréciation de la qualité du moût et même plus important que la teneur en azote soluble total (Agu *et al.*, 1996a). L'utilisation des réserves protéiques du grain est tributaire de l'activation des enzymes protéolytiques (endopeptidases, exopeptidases et dipeptidases) (Jensen, 1994). Certaines de ces enzymes sont particulièrement thermosensibles (endoprotéases) alors que d'autres peuvent encore travailler à des températures relativement élevées – en l'occurrence les carboxypeptidases A qui sont des exo-enzymes (Lewis *et al.*, 2006). Il est donc crucial, à moins de recourir à l'utilisation des enzymes exogènes, que les endopeptidases travaillent correctement lors du trempage, de la germination et des phases enzymatique et germinative du touraillage.

Les endoprotéases sont des enzymes importantes en brasserie, en ce sens que le rendement au brassage, la fermentabilité du moût, la stabilité de la mousse et la coloration finale de la bière sont affectés par le degré de protéolyse. Aussi, le pouvoir tampon du moût est fortement dépendant de la contribution des acides aminés (Lewis *et al.,* 2006). Un autre problème qui affecte la solubilisation de l'azote c'est la présence des inhibiteurs à faibles poids moléculaires (low-molecular-weight : LMW) des endoprotéinases (Jones, 2001). Selon Jones (2001), ces inhibiteurs forment des complexes avec les

protéinases – complexes qui ne se dissocient qu'après ébullition (100°C), alors qu'à 70°C déjà quasi toute l'activité enzymatique du moût est détruite.

#### *1.3.3.3. Des β-glucanases du sorgho*

Les *β*-glucanases sont des enzymes hydrolysant les *β*-glucanes. Alors que le sorgho grain contient moins de *β*-D-glucanes que l'orge, les malts de sorgho et leurs moûts en contiennent plus que ceux de l'orge (Etokakpan, 1992). Quoique l'absence d'enveloppe pailleuse, la présence d'énormes quantités de polyphénols donnant possibilité à la formation des complexes « protéines-tannins » et la température élevée de gélatinisation de l'amidon du sorgho ont été envisagées comme facteurs à l'origine de cette anomalie - il apparaît que le niveau de *β*-glucane solubilases et de (1,3)-*β*-glucanases pendant le maltage avec une faible activité (1,3),(1,4)-*β*-glucanase (Etokakpan, 1992) soit à l'origine de la teneur élevée en *β*-D-glucanes dans les moûts de sorgho. Plusieurs chercheurs se sont focalisés sur l'étude des *β*-glucanases aussi bien de l'orge que du sorgho. Onwurah *et al.* (N.d.) ont trouvé, dans les malts de sorgho, deux isoformes de *β*-glucanases désignées par Forme I et Forme II – lesquelles formes présentent respectivement les activités exo-*β*-(1,3)- glucanase (libération du glucose) et endo-*β*-(1,4)–glucanase (libération des oligosaccharides principalement). L'activité de la *β*-glucanase augmente après addition d'acide gibbérellique, mais selon Aisien *et al.* (1986), par exemple, il n'y aurait aucune évidence que la réponse de l'acide gibbérellique comme inducteur de la production d'enzymes *α*-amylase, protéase, pentosanases et endo-*β*- glucanases chez l'orge soit équivalente de celle du sorgho. Il a récemment été montré que lorsque *Bacillus subtilis* S499 est utilisé comme starter dans les solutions de trempage, l'activité *β*-glucanase est significativement améliorée (Bwanganga *et al.*, 2012, Bwanganga *et al.*, 2013b).

#### *1.3.3.4. De l'utilisation des enzymes exogènes*

Plusieurs enzymes exogènes sont actuellement utilisées en brasserie. Les *β*-glucanases pour la dégradation des *β*-glucanes et les xylanases pour l'élimination des pentosanes lorsque des grains crus sont utilisés (Lewis *et al.*, 2006). Des protéinases et des amylases sont utilisées lors des brassages hautes densités « high gravity brewing », les amyloglucosidases sont utilisées aussi bien en brassage qu'en fermentation, l'acétoacétate décarboxylase en fermentation pour contourner la production du diacétyle, de la papaïne et la propyl endopeptidase en maturation pour l'élimination des polypeptides à l'origine du trouble dans la bière, glucose oxydase/catalase dans les bières conditionnées pour l'élimination de l'oxygène, etc. (Lewis *et al.*, 2006).

Dans certains pays d'Afrique (Kenya par exemple) voire d'Asie (Japon par exemple) des réductions des taxes allant jusqu'à 50% sont obtenues lors que l'on utilise des grains crus (Goode *et al.*, 2005). Des combinaisons optimales d'enzymes issues de certaines bactéries peuvent donner à partir des

grains crus des moûts de bonne qualité (Goode *et al.*, 2005). Odibo *et al.* (2002) ont obtenu avec des enzymes exogènes des moûts de sorgho, de ces moûts ils ont - par évaporation - obtenu des extraits qui ont pu être conservés pendant une période plus ou moins longue et, ce, dans un état stable que pour pouvoir être utilisés lorsque cela est nécessaire pour le brassage. Signalons tout de même que le maltage est une étape qui ne représente qu'environ 3,5% du coût total de production de la bière (Goode *et al.*, 2005). Le recours aux enzymes exogènes, généralement bactériennes, n'est encore une fois qu'une preuve d'un défaut d'optimisation du maltage : l'idéal pour le brasseur étant de tout tirer de sa matière première.

### 1.3.4. De l'obtention d'un malt de sorgho de qualité

Le maltage est un processus biologique complexe impliquant toute une série de réactions biochimiques et physiologiques qui induisent *in fine* dans les grains : des transformations morphologiques (développement de l'embryon, des radicelles), le développement des enzymes hydrolytiques et des modifications de la structure de la graine (protéolyse, dégradation des parois cellulaires, etc.) (Bamforth *et al.*, 1993). Le maltage du sorgho - comme celui de l'orge - est réalisé en trois étapes principales : le trempage, la germination et le touraillage.

#### *1.3.4.1. Du trempage des grains de sorgho*

Le trempage est une étape très critique lors du maltage du sorgho en ce sens qu'il implique une augmentation de l'humidité des grains (35 à 40% voire un peu plus) (Ogbonna *et al.*, 2004), et partant de l'activité de l'eau, créant ainsi des conditions favorables au développement des bactéries, levures et moisissures ainsi qu'à l'activation des spores. Il existe plusieurs techniques de trempage. Certains essais sont réalisés sans aération, d'autres avec des périodes à aération et sans aération cycliques, d'autres encore avec aération continue durant toute la durée de trempage. Les effets de la durée de trempage, de la température et de l'aération sur la qualité du malt en termes de pouvoir diastasique, d'acides aminés libres (Free Amino Nitrogen : FAN) et d'extrait à l'eau chaude (Hot Water Extract : HWE) ont été étudiés (Dewar *et al.*, 1997a ; Elmaki *et al.*, 1999).

Le couple température - temps de trempage s'est montré capable d'améliorer les qualités du malt ; les optima de pouvoir diastasique, quantité d'acides aminés libres et rendement en extrait des grains ont été obtenus à la température de trempage de 25°C par Dewar *et al.* (1997a). Les conditions de trempage (température et durée) affectent différemment les activités enzymatiques. Il a par exemple été montré qu'à température élevée (35°C), les activités $\alpha$ et $\beta$-amylases augmentent rapidement avec la durée de trempage, atteignent un maximum puis diminuent ; et que la phase de diminution est plus abrupte pour la $\beta$-amylase comparée à l'$\alpha$-amylase pour un cultivar de sorgho rouge utilisé par Bwanganga *et al.* (2012). Des traitements chimiques tels que : l'addition de l'acide chlorhydrique, du

formaldéhyde, de l'hydroxyde de calcium ou de la soude caustique dans les eaux de trempage sont utilisés pour améliorer la vitesse d'absorption de l'eau par les grains de sorgho pendant le trempage (ce qui permet de réduire le temps de maltage), réduire la teneur en polyphénols et augmenter le pouvoir diastasique (Dewar *et al.*, 1997b ; Beta *et al.*, 1999). Le formaldéhyde permettrait de polymériser les composés phénoliques comme les tannins donnant ainsi des résines phénol-formaldéhyde, ce qui entraîne l'inactivation des tannins. Le trempage dans une solution alcaline a aussi été envisagé comme traitement permettant de lutter contre le développement des moisissures pendant le maltage du sorgho (Lefyedi *et al.*, 2006) et selon Lefyedi *et al.* (2007) ce traitement peut être remplacé par l'utilisation des micro-organismes comme *Pedioccocus pentosaceus* et *Saccharomyces* sp. utilisés comme starters dans les eaux de trempage. Les bactéries utilisées par Lefyedi *et al.* (2007) –en l'occurrence *Pediococcus pentosaceus* et *Lactobacillus plantarum* - étant listées parmi les micro-organismes contaminants de la bière (Sakamoto *et al.*, 2003), une souche de *Bacillus subtilis* S499 - non pathogène, déjà utilisée pour lutter contre les champignons au champ (biopesticides), non listée parmi les pathogènes de la bière - a été utilisée pour lutter contre le développement des moisissures lors du maltage d'une variété de sorgho rouge (Bwanganga *et al.*, 2012) et des pourcentage d'inhibition des moisissures atteignant jusque 99,99% ont été obtenus. Lorsque les biocontrôles sont utilisés lors du trempage du sorgho, la solution de trempage doit être bien aérée pour limiter la compétition en oxygène entre la grande population microbienne utilisée et les grains trempés et pour permettre d'éliminer le $CO_2$ produit lors de la respiration des grains (Bwanganga *et al.*, 2012). Cette aération permet d'améliorer la germination des grains et, partant, les propriétés du malt (Bwanganga *et al.*, 2012). D'autres facteurs inhérents au grain peuvent influencer la teneur en eau finale du grain, il s'agit – entre autres - de la dureté et de la grandeur du grain, etc. et avoir un effet sur la synthèse et/ou l'activation des enzymes et, partant, sur le travail des enzymes lors du maltage.

#### *1.3.4.2. De la germination des grains se sorgho*

La deuxième phase du maltage est la germination. La germination des grains trempés se fait en absence de lumière sous atmosphère saturée d'eau. L'humidité des grains, la température, la durée de germination et l'aération sont des facteurs à maîtriser pour l'obtention d'un malt de qualité. Les travaux d'Ahmed *et al.* (1996) ont montré que la durée de germination permettait d'augmenter la teneur en tannins (220, 410 et 400 mg de tannins par 100g de sorgho sont passés, après 5 jours de germination, à 330, 500 et 470 mg/100 respectivement), le pouvoir diastasique et le contenu en acide cyanhydrique. La diminution de la teneur en polyphénols pendant le maltage se ferait donc au

trempage (Nwaguma *et al.*, 1996) et par perte des radicelles au touraillage (Butler, 1982[*] cité par Nwanguma *et al.*, 1996). Une diminution de 71,4% de polyphénols a été obtenue par Iwohua *et al.* (1997) après 6 jours de germination avec le formaldéhyde dans les eaux de trempage. Des diminutions de la teneur en tannins jusque 99% ont par ailleurs déjà été obtenues (Elmaki *et al.*, 1999).

Selon Elmaki et al (1999), les teneurs en protéines brutes, en fibres, en lipides et en cendres diminuaient légèrement avec la durée de germination aussi bien pour le sorgho sans tannins que pour celui riche en tannins. Les protéines du sorgho étant en général pauvres en lysine, il a été démontré que la germination permettait d'augmenter la teneur en lysine (Wu *et al.*, 1980). Selon Agu *et al.* (1997b), la température de germination influe sur le développement des radicelles et sur le développement de l'activité $\beta$-amylasique aussi bien pour le sorgho que pour l'orge. Elle permet en plus d'améliorer l'activité $\alpha$-amylasique, la teneur en azote total et le pourcentage en extrait pour le sorgho mais non pour l'orge (Agu *et al.*, 1997b). La synthèse des arômes soufrés, en l'occurrence le DiMéthyleSulfure (DMS), aussi bien au maltage que lors de la fermentation, est d'un intérêt non négligeable. Cette synthèse passe par un intermédiaire (le S-méthyl méthionine) qui est un dérivé de la méthionine – un acide aminé présent dans le malt comme résultat de l'hydrolyse des protéines du grain. Le management de la synthèse des précurseurs de ces arômes doit en grande partie être fait lors du maltage. On sait par exemple que le malteur peut, en fonction de la teneur en DMS désiré, augmenter ou réduire le durée de la germination pour obtenir un malt plus ou moins désagrégé. A la fin de la germination, on obtient ce qu'on appelle le malt vert qui doit ainsi être séché et conservé pour utilisation.

#### *1.3.4.3. Du touraillage du malt vert*

Le touraillage de l'orge, qui est réalisé dans le souci d'arrêter les transformations biochimiques qui ont pris place dans le malt vert, consiste en un séchage à des températures atteignant jusque 200°C durant lequel l'humidité des grains passe de 41 - 46% (malts pâles) et 48 - 50 % (malts foncés), à 3,5 – 4 % pour les malts pâles et 1,5 – 2 % pour les malts foncés (Esslinger *et al.*, 2005). Des touraillages ont aussi été réalisés à des températures allant de 35 à 50°C (Agu *et al.*, 1996b). Et, il a été démontré que le pouvoir diastasique et l'activité cellulasique des malts de sorgho diminuait avec la température de touraillage quand celle-ci passait de 35 à 45°C (jusque 12,4% pour le DP et 12,6% pour l'activité cellulasique de diminution par rapport au malt vert de sorgho) (Agu *et al.*, 1996b). Le touraillage, comme tout traitement thermique modifie la structuration de l'amylose et de l'amylopectine ainsi que les interactions diverses existant entre l'amidon et les autres composés de la matrice, ce qui, en

[*] Butler, L.G., 1982. Relative degree of polymerisation of sorghum tannin during seed development and maturation. *J Agric Food Chem* **30,** 1090-1094.

somme, détermine les propriétés de l'amidon (Boudries *et al.,* 2009). Ces propriétés permettent de déterminer les applications potentielles de l'amidon et influent sur le travail enzymatique (Boudries *et al.,* 2009).

### 1.3.5. De l'écosystème microbien du maltage

Lors du maltage, un certain nombre de réactions biochimiques prennent place. Ces réactions, qui *grosso modo* sont attribuées au processus biologique sous-jacent qui est la germination, peuvent être modulées par la présence d'une communauté microbienne dont le rôle lors du processus de maltage - malgré que pas totalement connu - n'est plus à démontrer. Selon Laitila (2007), le processus de maltage peut être considéré comme un écosystème impliquant deux groupes métaboliquement actifs, à savoir : les grains en germination et la flore microbienne en présence. Tout changement de cet écosystème microbien, influerait sur le process et, partant, sur les propriétés du produit fini (Laitila, 2007). L'infection des malts par les moisissures du genre *Fusarium,* par exemple, est connue comme étant à l'origine du « gushing » (Sarlin *et al.,* 2005). Il a été montré que de petites quantités de proteines, les hydrophobines, isolées des espèces des moisissures du genre *Fusarium, Nigrospora* et *Trichoderma* agissaient comme facteurs à l'origine du « gushing » dans la bière (Sarlin *et al.,* 2005).

Plusieurs moyens de lutte contre le développement des moisissures et la production des mycotoxines lors du maltage du sorgho ont été préconisés (Lefyedi *et al.,* 2006 ; Lefyedi *et al.,* 2007 ; Bwanganga *et al.,* 2012) – l'effet des traitements chimiques (comme l'utilisation de la soude dans les eaux de trempage) et celui des starters micobiens comme *Lactobacillus plantarum, Pediococcus pentosaceus, Saccharomyces* sp. et *Bacillus subtilis* mis en exergue. Des travaux sur la lutte contre les moisissures et le développement des mycotoxines doivent être faits. Les arguments qui militent en faveur des biocontrôles prennent davantage le dessus (San-Lang *et al.,* 2002) ; car le devenir de nombre des produits chimiques utilisés dans la lutte contre le développement microbien est de plus en plus discuté (dégradabilité, compatibilité environnementale, pollution, etc.).

Notons que l'atteinte des objectifs du malteur pendant le touraillage (rendement au point de vue thermique, toute l'économie du séchage) ne coïncident pas forcément aux attentes du brasseur. L'optimisation du maltage doit tenir compte des cahiers de charge des brasseurs dont le degré de sévérité est parfois extrême, sans oublier toutes les contraintes technologiques imposées par le process.

### 1.3.6. Du brassage du sorgho

Lors du brassage les différentes enzymes développées au maltage sont mises dans les conditions de travail (température, pH, humidité) pour l'obtention d'un moût contenant les substances utiles au travail de la levure. La composition, aussi bien quantitative que qualitative, du moût oriente aussi bien

le métabolisme principal de la levure (utilisation préférentielle de tel ou tel composé…) que le métabolisme secondaire (production d'esters, d'alcools secondaires, d'arôme, etc.) – et, partant, le profil final de la bière (Engan, 1970). Selon qu'il s'agit d'un brassage par décantation ou par infusion, les caractéristiques du moût obtenu avec le malt de sorgho changent. Il a été démontré par Igyor *et al.* (2001) qu'une décantation est meilleure pour le malt de sorgho qu'une infusion qui est plutôt adaptée pour le malt d'orge. Selon ces auteurs la température a une influence non négligeable sur la qualité du moût, et la décantation à 100°C est celle ayant donné le meilleur résultat. Lors du brassage du sorgho à 65°C, l'hydrolyse de l'amidon n'est pas complète, ce qui, selon Agu *et al.* (1998) et Ogul *et al.* (2006), n'est pas forcément un problème d'insuffisance d'activités enzymatiques, mais plutôt serait causé par les facteurs affectant l'empesage de l'amidon.

La teneur en azote soluble dans le moût obtenu avec le malt de sorgho est inférieure à celle obtenue avec le malt d'orge même quand les deux maltages ont été conduits dans les conditions optimales de température (30°C pour le sorgho et 17°C pour l'orge) (Agu *et al.*, 1999). La teneur en azote soluble diminue plus ou moins fortement après l'ébullition, de sorte que, dans certains cas, on obtient avec le sorgho jusque 8.2% de différence entre les ratios SN/TN avant et après ébullition (Bwanganga *et al.*, 2012) – valeur qui est de loin supérieure à celle préconisée pour le moût de malt d'orge (Lewis *et al.*, 2006). Un autre problème qui inquiète souvent les brasseurs utilisant le sorgho est la viscosité élevée du moût qui influe sur sa filtrabilité. Cette viscosité élevée est souvent attribuée à la présence d'énormes quantités de *β*-glucanes dans le moût dues à la faible activité *β*-glucanasique (Agu *et al.*, 1999). Aussi, la température optimale de la *β*-glucanase (37°C pour la forme I et 45°C pour la forme II) est inférieure aux températures de brassage, cette enzyme voit donc son activité fortement réduite pendant le brassage, laquelle activité avait déjà été réduite pendant le touraillage (Woodward *et al.*, 1982).

L'utilisation des enzymes exogènes est devenue pratique courante lors du brassage du sorgho faute d'optimisation des diagrammes de brassage (Goode *et al.*, 2002 ; Ogul *et al.*, 2006) et le sorgho non malté est souvent utilisé au Nigéria pour la production des bières de type européen (Agu *et al.*, 1997a). Des brassages avec 100% de sorgho non malté ont été réalisés avec addition d'enzymes exogènes, et les paliers de 50, 95 et 65°C ont été utilisés (Goode *et al.*, 2002). Des diagrammes de brassage sont proposés dans la revue présentée par Ogbonna (2011).

### 1.3.7. De la fermentabilité des moûts du sorgho

La fermentation est une phase importante de la fabrication de la bière. En brasserie, cette phase, difficile à bien mener, est loin d'être réduite en la seule transformation des sucres fermentescibles en alcool - le profil aromatique de la bière étant plus ou moins dominé par les sous-produits du métabolisme de la levure. Plusieurs facteurs de la fermentation peuvent influer sur le profil final de la

bière : les conditions de température, de pH, de pression, de potentiel redox, d'agitation, la composition du moût (densité, teneur en azote soluble mesurée par le FAN, le profil en acides aminés, les acides amères du houblon, les acides gras en solution, la présence d'ions minéraux, etc.), et la quantité, le type et l'état physiologique de cellules de levure ensemencées. La composition des moûts obtenus avec le sorgho, malté ou non, est différente de celle de moût de malt d'orge classique. Il en résulte par exemple que les bières obtenues avec les moûts de sorgho utilisé comme grains crus avec des enzymes exogènes, contiennent des faibles niveaux d'acétate d'éthyle et des niveaux élevés de 2- et 3-méthyle butanol (Bajomo *et al.*, 1994). Selon Bajomo *et al.* (1994), le profil aromatique est fortement tributaire du pool d'acides aminés (quantité et types), ce profil dépendant lui-même du travail des protéases lors du maltage et du brassage.

### 1.3.8. Conclusions et perspectives

Des travaux visant à démontrer l'aptitude de certaines variétés de sorgho au maltage et au brassage ne font que s'accumuler : de l'établissement de la différence de structure entre le grain de sorgho et celui de l'orge à l'obtention des bières 100% sorgho non malté, passant par tous les traitements visant à améliorer la qualité de la bière finie (Taylor *et al.*, 2006). Tous ces auteurs sont presque unanimes pour affirmer que la température de gélatinisation élevée de l'amidon du sorgho, le rôle inhibiteur de ses protéines et éventuellement des arabinoxylanes, des $\beta$-glucanes sur l'hydrolyse et la gélatinisation de l'amidon, les faibles activités $\beta$-amylasique et $\beta$-glucanasique - sans parler des teneurs élevées en polyphénols, de la faible digestibilité des constituants de la paroi de l'endosperme et de la couche à aleuronne, et des contaminations fongiques - sont les quelques problèmes qui inquiètent encore les brasseurs utilisant le sorgho. Très peu d'études seulement ont été menées sur la fermentation des moûts de sorgho.

Si les solutions optimales à ces problèmes ne sont pas toutes obtenues, la connaissance des mécanismes sous-jacents est loin d'être un mystère pour la science actuelle. Plusieurs scénarii peuvent être envisagés pour réaliser le défi de la substitution du malt d'orge par celui du sorgho en brasserie moderne (…), mais le choix du scénario le plus réaliste ne peut passer que par toute une série d'essais guidés par l'empirisme et la somme des connaissances actuelles.

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# Chapter II. Development of the *Bacillus subtilis* biocontrol of mould during red sorghum malting

## *Présentation du chapitre*

Dans le chapitre qui suit (chapitre 2), les possibilités d'utilisation de *B. subtilis* S499 comme biocontrôle des moisissures dans les conditions de maltage du sorgho rouge ont été étudiées. Cette étude, qui se veut être une mise au point du biocontrôle des moisissures, répond à deux questions majeures, à savoir celle relative à l'efficacité du biocontrôle dans les conditions de maltage (faible quantité des nutriments, interactions diverses avec les grains maltés, etc.) et celles liées à l'effet du biocontrôle sur les propriétés des malts en termes d'activités enzymatiques ($\alpha$-amylase, $\beta$-amylase et $\beta$-glucanases), teneurs en composés polyphénoliques (polyphénols totaux et tannins condensés) et freintes au maltage.

Dans ce travail, l'effet de la dilution d'une culture de *B. subtilis* S499 à $10^8$ cellules/mL sur le développement des moisissures est modélisé et les propriétés des malts ainsi obtenus sont comparées à celles des traitements chimiques usuels – en l'occurrence le trempage en solutions alcalines (NaOH et $Ca(OH)_2$).


## *Abstract*

*Bacillus subtilis* exerts an inhibitory effect on moulds isolated from red sorghum raw grain. The total fungal count reduction time course, affected by the dilution of *B. subtilis* culture, follows a sigmoidal function type. Therefore, one can distinguish three zones: a first zone of high inhibition, where the dilution of *B. subtilis* culture does not greatly affect the steeping treatment on reducing mould growth (DF > minID); a second zone of dilution where the inhibitory effect of the steeping treatment is almost proportional to the dilution of the *B. subtilis* culture (MaxID < DF < minID); and a third zone of dilution where treatment is simply ineffective i.e., no significant reduction in total fungal count upon increasing the concentration of *B. subtilis* (DF < MaxID). Steeping in the biocontrol allows malt production with a low level of fungal contamination, relatively low malting losses and high $\beta$-glucanase levels. When compared with dilute alkaline steeping, the biocontrol treatments result in malts with low $\alpha$- and $\beta$-amylase activities and a relatively high content of total phenolic compounds and condensed tannins. *B. subtilis* dilution has been found to significantly affect kilned malt enzyme activities depending on the type of enzyme studied.


Keywords: *Bacillus-subtilis*-based biocontrol, mould growth inhibition, red sorghum malting

### *2.1. Introduction*

Sorghum is a major crop in Africa, Asia and South America, and malted sorghum is used notably in making products for local consumption, such as beer, infant porridge and non-fermented beverages. During malting, the maltster has to create good grain germination conditions (moisture, aeration, temperature, etc.) in order to take advantage of the underlying transformations (enzyme production, grain modification, reducing the importance of certain substances such as tannins, reduction of undesirable microorganism development, removal of raw flavour, development of malty flavour and colour, etc. (Lewis and Bamforth, 2006)). The importance of microbes during barley malting was elucidated (Laitila, 2007); as was the contribution of the microbial ecosystem on malt properties. Maltsters also recognize the influence of the natural barley grain microflora on grain physiology during malting (Camphnhoui *et al.*, 1998). It is also known that the grain microflora interact with the malted grain both by their presence and metabolic activity (Noots *et al.*, 1999). However, the presence of certain microorganisms during malting is disadvantageous, as they can exert an adverse effect on the quality of both malt and malted products such as beer. The negative role of mould is well known (mycotoxin production during malting and the effect on beer quality and consumer health) (Schapira *et al.*, 1989) and procedures to prevent mould toxin production during malting are currently employed.

During sorghum malting, mould control is of paramount importance in the sense that sorghum malting − unlike that of barley − is conducted at relatively high temperatures (Dewar *et al.*, 1997). In addition, grain sorghum is not dressed; hence the endosperm cell wall is attacked to a varying degree, allowing the microorganisms easy access to nutrients. Sorghum raw grain is recognized to be a susceptible material to invasion by potentially toxigenic fungi (Saubois *et al.*, 1999). Thus, several studies have been conducted on mould control and mycotoxin production during sorghum malting; the use of chemical treatments (diluted acids, dilute alkalines) and the use of biocontrols (lactic bacteria and yeasts) (Agu and Palmer, 1997; Lefyedi and Taylor, 2006, 2007; Rabie and Lübben 1984, − to name only a few).

Biological control of postharvest diseases in general (reviewed by Sharma *et al.*, 2009) and mould biocontrol during malting (sorghum as well as barley), is an environmental and economic challenge, and several studies have already been performed in this direction (Lefyedi and Taylor, 2007; Lowe and Arendt, 2004; van Sinderen and Rouse, 2008).

Beer spoilage microorganisms and hop resistance were presented in a detailed and interesting review (Sakamoto and Konings, 2003), placing several species of lactic acid bacteria at the head of list. It has been shown that the presence of lactic acid bacteria in the brewing environment is not of interest, especially as counting methods and identification of lactic acid bacteria are very expensive (different

auxotrophic profiles, several lactic acid bacteria grow poorly in mainstream media) (Simpson, 1993; Simpson and Fernandez, 1994).

Recently, (Reddy *et al.*, 2010) showed the effect of *B. subtilis* on the inhibition of *Aspergillus flavus* growth and aflatoxin B1 production in sorghum grain, and several other studies have shown the effect of *B. subtilis* on the inhibition of mould growth in the field (Makoto, 2000). Indeed, *B. subtilis* produced a series of lipopeptides with antifungal properties (Nihorimbere *et al.*, 2011; Ongena and Jacques, 2007). Its presence in the brewing environment is not a concern as *B. subtilis* is a non-pathogenic bacterium; is not a potential beer spoilage bacterium (Sakamoto and Konings, 2003) and is easily destroyed in the presence of hops (Teuber and Schmalreck, 1973).

Thus in this work, we have focused specifically on the possibility of using a harmless microbe, *B. subtilis*, to control mould development during red sorghum malting. The inhibitory effect of this strain on various moulds isolated from raw sorghum grain was first evaluated *in vitro*, and − during red sorghum malting − the inhibitory effect of the *B. subtilis* biocontrol was evaluated after using this bacteria as a starter in the steeping liquor. The effect of the dilution of a *B. subtilis* culture on the grain total fungal count was modelled using a sigmoidal type function. Parallel to the monitoring of fungal development, other malt properties were also evaluated (enzymatic activities: $\alpha$- and $\beta$-amylase, $\beta$-glucanases, polyphenol and condensed tannin contents, total malting loss).

## *2.2. Methods*

### 2.2.1. Sorghum grain characteristics

Red sorghum grain was obtained from the Democratic Republic of Congo (DRC) (vernacular cultivar Imbuto ngufi). The thousand grain weight (TGW), germination energy (GE), germination capacity (GC), and moisture content were determined as described in Analytica EBC (2004), EBC method 3.4, 2004, EBC method 3.6.3 and EBC method 3.5.2, respectively.

### 2.2.2. Malting

Sorghum grain was cleaned and then 150 g was steeped without aeration for 16 h and allowed to germinate at 30°C for 72 h. Green malt was dried at 40°C for 48 h and rootlets were hand removed by gentle brushing. Six steeping treatments were tested: **$H_2O$** (16 h in distilled water), **NaOH** (16 h in 0.2% NaOH), $Ca(OH)_2$ (16 h in 0.1% $Ca(OH)_2$), **C+B** (16 h in a *B. subtilis* S499 culture diluted with distilled water to $10^8$ cells/mL), **C** (16 h in distilled water containing *B. subtilis* S499 cells at $10^8$ cells/mL), and **B** (16 h in the cell-free supernatant obtained after centrifuging a culture diluted as in C+B). For C+B, C, and B, *B. subtilis* S499 was first grown on Luria Broth agar at 37°C for 24 h. An inoculating loopful was transferred to 100 mL Landy broth optimized for *B. subtilis* lipopeptide

production and incubated for 16 h. 10 mL was then finally transferred to 350 mL optimized Landy Broth and incubated at 30°C (under rotary shaking at 130 rpm) for 72 h. After cell counting under a microscope in a Burker cell, the culture was diluted with distilled water to $10^8$ cells/mL. This diluted culture was used directly for C+B and centrifuged at 10000×g for 15 min for C and B. The supernatant was used in B and the pellet was washed and resuspended in distilled water (so as to obtain $10^8$ cells/mL) for C. The RP-HPLC-DAD-MS method was used to assay lipopeptide families (Nihorimbere *et al.*, 2011).

The total fungal population was counted on potato dextrose agar supplemented with 0.005% chloramphenicol (PDA+C). The percentage of inhibition was then calculated as:

$I(\%)=(X_0-X)/X_0$ where: $I(\%)$ is the percentage of inhibition, $X_0$ the CFU when using distilled water ($T_0$), and $X$ the CFU of the treatment.

#### 4.1.1. 2.2.3. Microbial growth inhibition tests

*B. subtilis* S499 was grown under conditions optimized for lipopeptide production as described above (see Figure 2). The culture was diluted with water to $10^8$ cells/mL, centrifuged, and the supernatant collected. This cell-free supernatant was used undiluted and at various dilutions in the inhibition tests. 150µL samples were placed in wells of 5 mm diameter cut into PDA+C plates. Each sample was allowed to diffuse into the agar for 4 hours at room temperature before incubation at 30°C. Inhibition was graded as follows: (+) when the strain was inhibited by the undiluted *B. subtilis* culture, (+ +) when it was inhibited by dilution 1/10, (+ + +) when it was inhibited by dilution 1/100, and (+ + + +) when it was inhibited by dilution 1/1000.

Three randomly sampled lots of 100 red sorghum grains were obtained and immersed for 2 min in 70% ethanol followed by 2 min in 0.4% chlorine and then rinsed with sterile distilled water (Pitt and Hocking, 2009). The grains were transferred to pre-sterilized Petri dish humidity chambers (25 grains/dish) under aseptic conditions and were incubated, for 5 days at room temperature with a 12 h light cycle, for observation (see Navi *et al.*, 1999). Infected kernels were soaked in sterile distilled water at 4 °C, diluted before inoculation on PDA+C and incubated at room temperature. Seven morphologically distinct isolated colonies were selected and grown on PDA+C until spore formation (in the dark). The spores were resuspended in distilled water (0.01% Tween) and re- inoculated on PDA+C to produce isolated colonies. Individual colony forming units were placed on PDA+C for the inhibition test. Mould identification was performed as described in Pitt and Hocking (2009) and results were compared with that of the Fusarium interactive key (Agri. and Agri-Food Canada, 1996), Navi *et al.*, (1999), Rafi and Sajjad-Ur-Rahman (2002) and http://www.mycology.adelaide.edu.au.

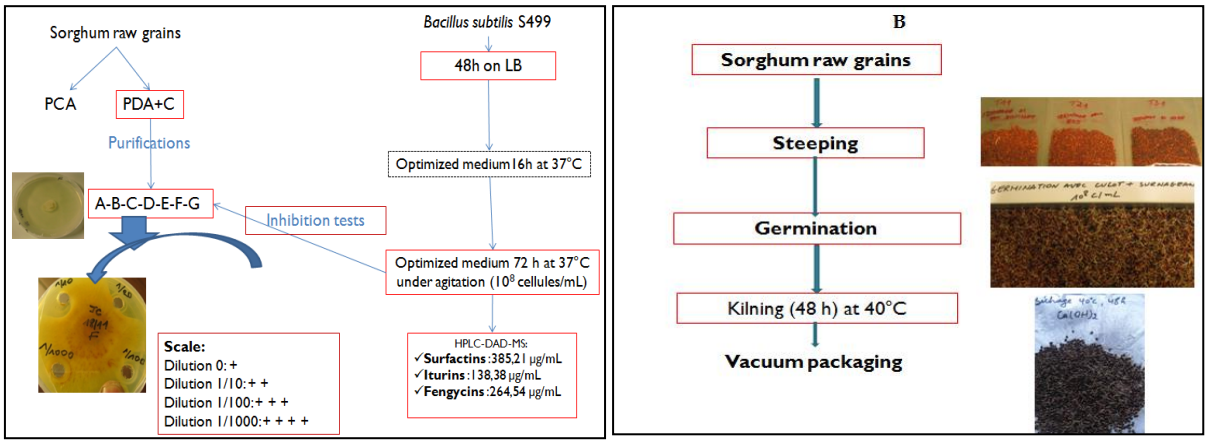


**Figure 2. A. Moulds isolation and inhibition by *Bacillus subtilis*; B. malting steps**

### 2.2.4. The effect of *B. subtilis* population on malt total fungal count

When a biocontrol is used it is important that the bacterial population is at the minimum necessary to obtain the expected response. Thus, before establishing a biocontrol strategy, we must first answer a crucial question: what is the minimum bacterial population required? To answer this question, sorghum grains (10 g) were soaked in 20 mL *B. subtilis* culture (C+B), washed cells (C) or cell-free supernatant (B) and in diluted (1/10, 1/20, etc.) treatments for 16 hours. Malting was conducted as described previously and the kilned malts total fungal count was evaluated in log CFU on PDA+C agar after incubation at 30°C for 48 h. The total fungal count has been described as a function of the steeping liquor dilution, and the time course of the grain total fungal count for steeping treatment C+B has been modelled as follows:

$$y = (\alpha \times \beta)/( \alpha + (\beta - \alpha) \times \exp(\gamma x) \quad \text{Eq. 1.}$$

where y and x represent the total fungal population (log CFU $g^{-1}$ kilned malt), and the logarithm of *B. subtilis* culture dilution. "$\alpha$, $\beta$ and $\gamma$", represent the model kinetic parameters and have been calculated as follows:

1. $\gamma$ was considered equal to 1, which helped to write the equation Eq. 1 as follows:

$$y = (\alpha \times \beta)/( \alpha + (\beta - \alpha) \times \exp(x) \quad \text{Eq. 2.}$$

and calculating the approximate values of $\alpha$ and $\beta$ by plotting the line:

$$y^{-1} = \beta^{-1} + (\alpha^{-1} - \beta^{-1}) \exp(x) \quad \text{Eq. 3.}$$

Eq. 3. is a straight line whose slope and x-intercept are respectively $(\alpha^{-1} - \beta^{-1})$ and $[\alpha / (\alpha - \beta)]$.

2. The values of $\alpha$ and $\beta$ obtained were used as start values of iterations and Minitab 16 was used to obtain the final model (confidence level for all intervals: 95%, the Gauss-Newton algorithm and a convergence tolerance of 0.00001).

From Eq. 1, when $x \to 0$, $y \to y_0 = \alpha$, and when $x \to -\infty$, $y \to y_\infty = \beta$.

The root mean square error (RMSE = $(1/n\sum(\text{experimental data-predicted data})^2)^{1/2}$ has been used to evaluate the goodness of fit.

### *2.2.5.* Sorghum malt properties

All enzymatic assays were performed on extracts of malt flour obtained by grinding kilned malt in an IKA mill followed by sieving (mesh size: 0.5 mm). $\alpha$- and $\beta$-amylase activities were extracted and assayed using Megazyme methods (Ceralpha Method: K-CERA 08/05 and Betamyl-3 Method: K-BETA 10/10 respectively). $\beta$-(1-4)-glucanase extraction was carried out for 15 min at 30°C in a centrifugation tube containing 0.5 g malt flour and 8 mL extraction buffer (50 mM Na-acetate, pH 4.8), with vigorous vortexing every 5 min. The mixture was then centrifuged at 1000×g for 10 min and the supernatant collected. The assay was performed at 40°C in a reaction mixture containing 0.5 mL extract and 0.5 mL 2% carboxymethylcellulose as the substrate for $\beta$-(1-4)-glucanase. The incubation time was 5 min and the reaction was stopped by immersing the tubes in boiling water. The amount of glucose released was then determined by the method of Nelson-Somogyi (Primarini and Yoshiyuki, 2000) and the $\beta$-(1-4)-glucanase activity was expressed in µmoles of glucose released per minute per kg of kilned malt. Megazyme assay procedure for cereal flours and malt endo-(1,3)-$\beta$-glucanase activity and Megazyme S-ABG100 03/11 method were used for $\beta$-(1,3)- and $\beta$-(1,3)-(1-4)-glucanase activities.

Total phenolics were assayed using the method optimized by Georgé *et al.* (2005) without eliminating water-soluble compounds and results are expressed in mg gallic acid equivalents per gram of dried malt (mg GAE $g^{-1}$). Condensed tannins were estimated by the modified vanillin/HCl method of Price *et al.* (1978) and results are expressed in per cent catechin equivalents (% CE).

### *2.2.6.* Statistical analysis

Tukey's HSD tests, statistical computing and graphics were performed with Minitab 16 software.

## *2.3. Results*

The red sorghum used in this study contained: 11.1±0.2 (% wet weight) moisture content, 26.2 g ± 0.1 thousand grain weight, 96.3% ± 0.3 germination energy and a germination capacity of 95.5 % ± 0.5. The raw grain total aerobic count was 6.4±0.1 log CFU/g and the initial fungal plate count was 4.8±0.2 log CFU/g. The protein content was 12.7±0.3g/100 g and the total phenolic compounds and condensed tannins were 8.1±0.1 mg gallic acid equivalent and 0.18±0.0 % catechin equivalent, respectively. Lipopeptide levels in the *B. subtilis* supernatant (RP-HPLC-DAD-MS) were: surfactins: 385.21 µg/mL; iturins: 138.38 µg/mL; fengycins: 264.54 µg/mL.

Kilned malt total fungal count, enzyme activities ($\alpha$-amylase, $\beta$-amylase, $\beta$-(1,4)-, $\beta$-(1,3)-, $\beta$-(1,3)-(1,4)-glucanases), total phenolic compounds, condensed tannins and losses are presented in Table 1.

**Table 1. Red sorghum malts properties as affected by steeping treatment**

| Malt properties | $H_2O$ | NaOH | $Ca(OH)_2$ | C+B | C | B |
|---|---|---|---|---|---|---|
| **Kilned malt Total fungal count (log CFU)** | 4.6±0.1 a | 2.8 ±0.0 c | 3.7±0.0 b | 1.4±0.0 f | 1.9±0.1 d | 1.7±0.0 e |
| ***α*-amylase (U/g)** | 43.7±0.1 e | 229.1±0.6 a | 212.8±0.3 b | 45.0±0.2 e | 60.6±0.5 c | 55.3±0.7 d |
| ***β*-amylase (U/g)** | 23.5±0.6 e | 49.0±0.3 b | 55.8±0.6 a | 28.8±0.6 d | 32.9±0.6 c | 28.4±0.3 d |
| ***β*-(1,4)-glucanase (U/kg)** | 0.5±0.0 e | 0.7±0.1 d | 1.2±0.1 c | 3.6±0.1 b | 4.6±0.0 a | 0.6±0.0 e |
| ***β*-(1,3)-glucanase (U/kg)** | 1.6±0.2c | 2.2±0.2bc | 2.4±0.4bc | 6.2±0.3a | 5.4±0.6a | 3.1±0.3b |
| ***β*-(1,3),(1,4)-glucanase (U/kg)** | 1.8±0.6a | 1.9±0.2a | 2.3±0.3a | 2.3±0.4a | 1.8±0.2a | 2.0±0.5a |
| **Total Phenolic Compounds (mg GAE/g)** | 7.3±0.1 a,b | 6.8±0.1 c | 7.0±0.1 c | 7.5±0.2 a | 7.2±0.0 b | 7.4±0.2 a,b |
| **Condensed Tannins (% CE)** | 0.14±0.00 a | 0.06±0.00 f | 0.07±0.00 e | 0.12±0.00 c | 0.09±0.00 d | 0.13±0.00 b |
| **Total Malting Loss (%)** | 24.2±0.5 b | 24.9±0.2 b | 26.9±0.2 a | 18.7±0.1 c | 17.9±0.0 c | 14.1±0.1 d |

Values are mean±StDev. Treatments within a line having a letter in common are not statistically different according to Tukey's HSD test ($p<0{\cdot}05$). $H_2O$ (16 h in distilled water), NaOH (16 h in 0.2% NaOH), $Ca(OH)_2$ (16 h in 0.1% $Ca(OH)_2$), C+B (16 h in a *B. subtilis* culture diluted with distilled water to $10^8$ cells/mL), C (16 h in distilled water containing *B. subtilis* cells at $10^8$ cells/mL), and B (16 h in the cell-free supernatant obtained after centrifuging a culture diluted as in C+B).

High percentages of inhibition were obtained with biocontrol treatments were compared to dilute alkalines and distilled water steeping (≈99.99% for C+B and B treatments). All seven have been inhibited by *B. subtilis* S499 to differing degrees, as follows: (a) *F. oxysporum* (++), (b) *F. solani* (+), (c) *A. niger* (+++), (d) *A. versicolor* (+), (e) *Penicillium expansum* (+), (f) *Penicillium chrysogenum* (+++), *(g) penicillium sp*. (+++).

The effect of a *B. subtilis* population on malt enzyme activities is presented in Figure 3.

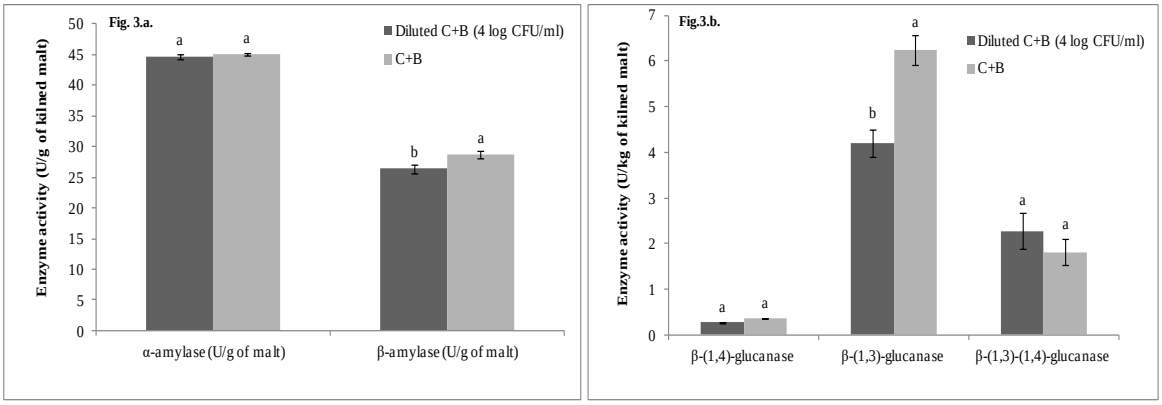


**Figure 3. Effect of *B. subtilis* dilution on kilned malt *α*-amylase, *β*-amylase and *β*-glucanase activities.**

Values are mean±StDev. Treatments having a letter in common are not statistically different according to Tukey's honestly significant difference test ($p<0{\cdot}05$). C+B : steeping 16 h in a *B. subtilis* culture diluted with distilled water to $10^8$ cells/mL; Diluted C+B (4 log CFU/mL): steeping 16 h in a *B. subtilis* culture diluted with distilled water to $10^4$ cells/mL

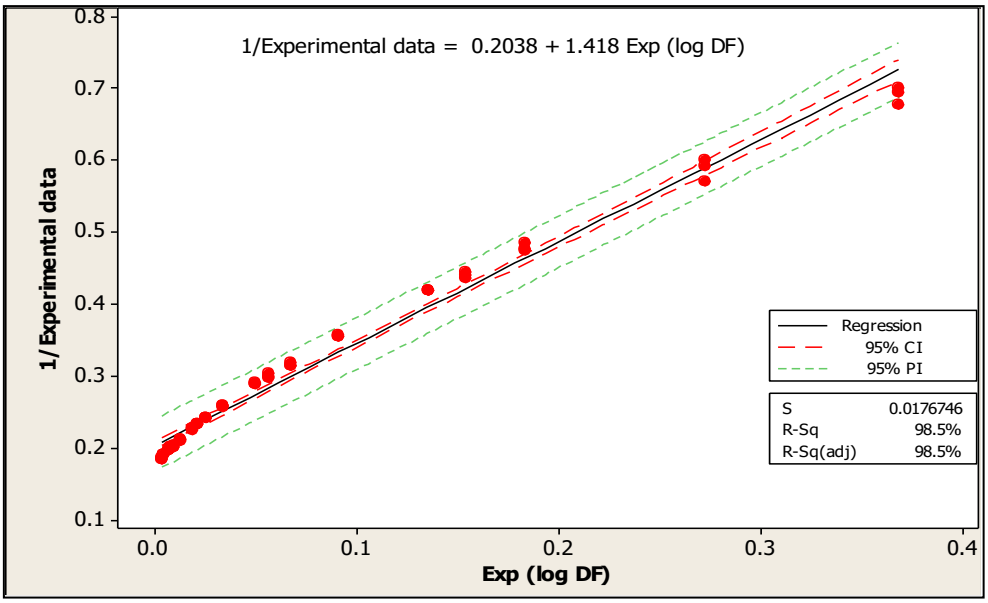


**Figure 4. Regression line of 1/experimental values of total fungal count versus exp(log DF) for the first iteration ($\gamma$=1)**

The effect of a *B. subtilis* population in the steeping liquor on mould development has been modelled as described in the materials and methods. The result of the first approximation is presented in Figure 4 and according to the result obtained in Figure 4, Eq.3. can be written as follows:

$y^{-1} = 0.2038 + 1.418 \exp(x)$ Eq. 4

From Eq. 4., approximate values of $\alpha$ and $\beta$ have been calculated ($\alpha \approx 0.6166$ and $\beta \approx 4.907$).

The final model equation obtained using Minitab 16 software was:

TFC (log CFU) = 4.67/(0.79 + 5.10 exp(0.74 log DF)) Eq. 5

Where $\alpha = 0.793518$, $\beta = 5.89016$ and $\gamma = 0.739028$. The experimental and predicted data scatterplot is presented in Figure 6 and the RMSE of the fit (for the steeping treatment C+B) was 0.23 log CFU. Sorghum kilned malt properties of biocontrol treatments as compared with those obtained after dilute alkaline steeping (NaOH and $Ca(OH)_2$) − malts obtained after steeping in distilled water as the control – are presented in Table 1.

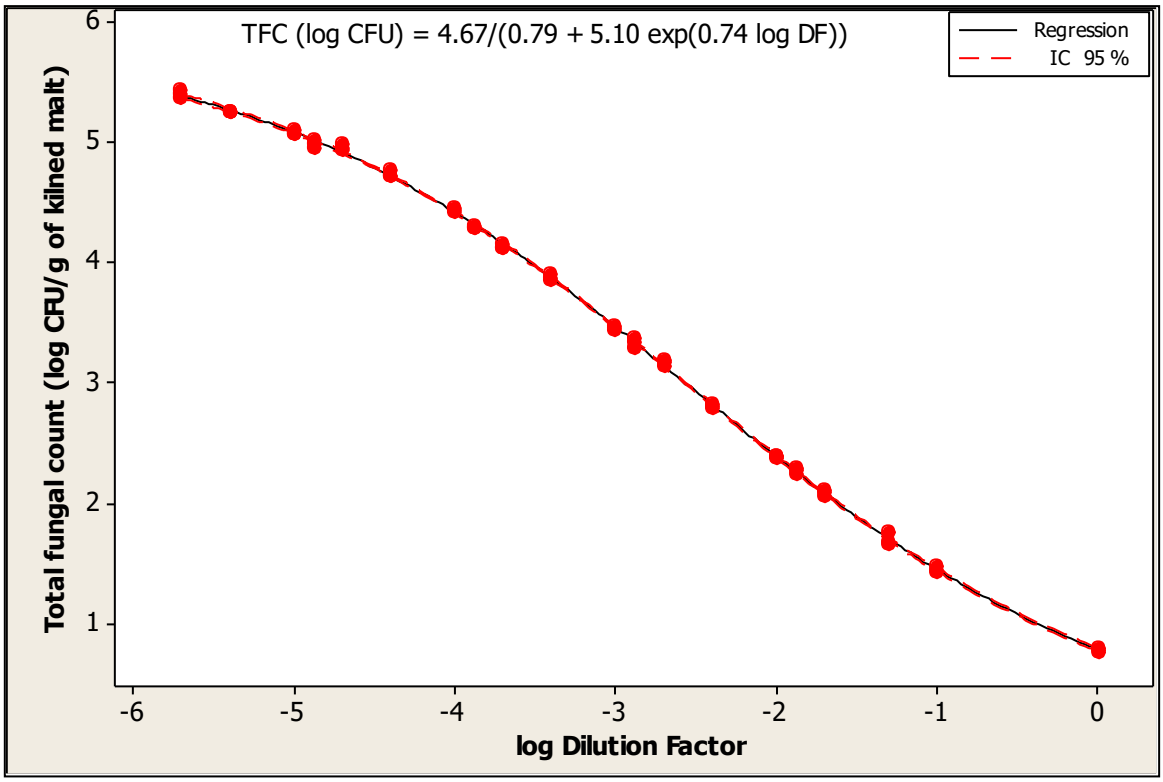


**Figure 5. Kilned malt total fungal count as affected by the logarithm of *B. subtilis* culture dilution factor (final model)**

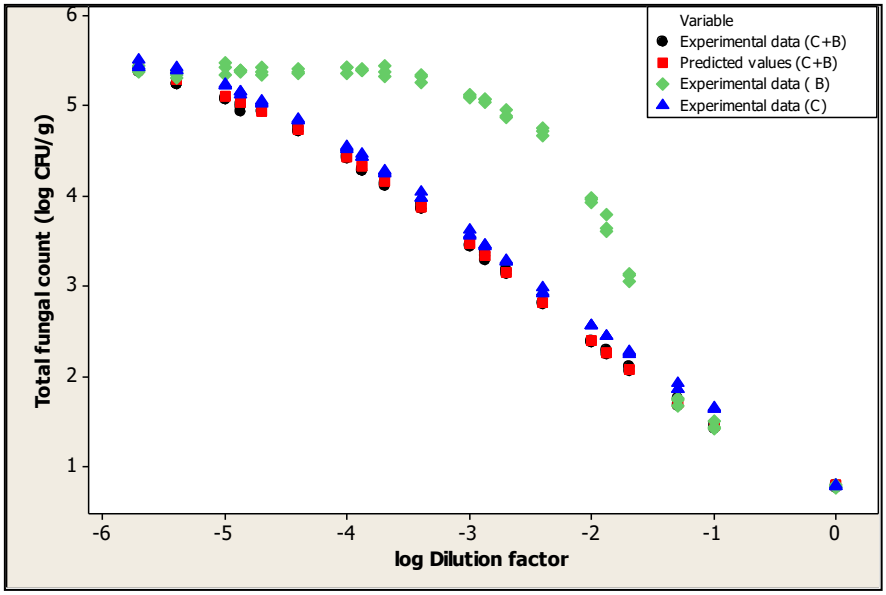


**Figure 6. Kilned malt total fungal count scatterplot (experimental data for steeping in C+B, C and B) and C+B predicted values**

### *2.4. Discussion*

The inhibitory effect of *B. subtilis* on mould growth is well known and is often attributed to the presence of lipopeptides produced by *B. subtilis* species (Romero *et al.*, 2007; Touré *et al.*, 2004). Indeed, as shown in the results section, the *B. subtilis* strain used is capable of producing significant amounts of lipopeptides and inhibiting 7 moulds isolated from red sorghum grain. This inhibition (*in*

*vitro*) is to differing degrees and dependent on the strain involved. However, during malting, the situation is far from reduced to simple mould inhibition by *B. subtilis*, but must still take into account the effect of the use of large amounts of microorganisms on grain metabolism. Indeed, it is known that plant-microbial ecosystem interactions are complex and the molecular dialogue that occurs between microorganisms and plants has never been fully elucidated (Camphnhoui *et al.*, 1998). Our results (Figure 6) show that the inhibition is not exclusively the effect of lipopeptide production. Indeed, when the culture is diluted to a dilution factor (DF) of 1/10000, for example, the lipopeptide concentration is extremely low and not sufficient to exert an effect on mould growth; but when both *B. subtilis* cells and cell-free supernatant or when *B. subtilis* washed cells are used in the steeping liquor, mould growth is still reduced due to the presence of *B. subtilis* cells. In other words, the increase in total fungal count is not proportional to the dilution of *B. subtilis* culture since the lipopeptide levels are above the minimal inhibitory dilution (minID) which corresponds − on Figure 6 – to the region at the beginning of dilution (high *B. subtilis* population and high lipopeptide concentrations). Examining the minID, phase one is an area where the loss of efficacy of the culture is proportional to the dilution. This area represents the range of dilution where the inhibitory effect is simply due to the concentration of *B. subtilis*. During the second phase, when *B. subtilis* cell-free supernatant is used alone as the steeping liquor, an increase in dilution is accompanied by a sudden jump in the total fungal count (Figure 6: experimental data (B)). A third phase starts from the maximal inhibitory dilution (MaxID) – at which, the dilution of *B. subtilis* culture no longer affects the development of the total fungal count; this range of dilution is simply inefficacious. The advantage of such a model is to determine, based on the initial fungal load, the required dilution range to obtain certain levels of reduction in the development of the total fungal count. The inflection point (log DF = -2.51 and TFC = 2.94) of the function represented by the Eq. 5 can be obtained by solving the following equation:

$$d^2y/dx^2=\{[66.5152\times e^{1.48x}]-[10.3033\times e^{0.74x}]\}\times[0.79+5.10\times e^{0.74x}]^{-3} \quad \text{Eq. 6}$$

Where y and x represent, respectively, the TFC and the log DF.

So, when we consider an infinitesimal variation of log DF (x) around the inflection point of the function represented by the Eq. 4 which corresponds to an infinitesimal variation in total fungal count (y), and we apply the principle of the right triangle, one obtains the slope of the line (y = -1.0935 x + 0.200) whose intersections with the asymptotes ($y_0 = \alpha$ and $y_\infty = \beta$) represent approximately the minID (log DF ≈-0.540) and the MaxID (log DF ≈ -5.223), respectively. When *B. subtilis* is used as a starter, malts obtained have low levels of fungal contamination, but the NaOH steeping treatment has not presented significant differences from biocontrol steeping treatments C and B. The inhibitory effect of NaOH on mould growth has indeed been previously demonstrated (Lefyedi and Taylor, 2006). Thus, the advantage of using a biocontrol should not simply be reduced by decreasing the growth of mould. As shown in Table 1− although soaking in alkaline solutions has given the best $\alpha$- and $\beta$-amylase

activities, total polyphenol compounds and condensed tannins reduction − the use of the biocontrol allows malts with high *β*-glucanase activities and low malting losses to be obtained. It is known that the hydrolysis of *β*-glucans and the reduction on malting loss are major concerns during the malting and brewing of sorghum (Etokakpan, 1992; Ogbonna, 2011). Table 1 also shows that the residual phenolics represented 84.3% after NaOH and 86.6% after $Ca(OH)_2$, and residual condensed tannins represented 37.0 and 40.7% respectively. Total phenolic compounds were unaffected and condensed tannins only slightly affected in the presence of the cell-free supernatant conditioned by *B. subtilis* S499, and the presence of bacterial cells (C+B, C) had no significant effect on the level of phenolics compared with steeping in distilled water ($H_2O$). According to the Tukey's HSD test, the level of condensed tannins was significantly lower in the presence of washed cells (C) than in the presence of a conditioned medium with or without cells (C+B, B).

The question remains, why does the use of *B. subtilis* not give good levels of *α*- and *β*-amylase activities? Several hypotheses can be made in relation to the effect of lipopeptides on malt enzyme activities and various interactions between the large bacterial population used in steeping liquor and grain germination. Biosurfactants produced by *B. subtilis* have antioxidant properties (Yalçın *et al.*, 2010). In fact, it has been shown that amylose becomes less susceptible to enzymatic hydrolysis through the formation of complexes with surfactants and many surfactants can also interact with proteins and alter their secondary and tertiary structures (Rodríguez *et al.*, 2006). The high levels of polyphenols and condensed tannins can also affect enzyme activities. The grain steep-out moisture in the biocontrol treatments is lower than those of grains obtained after soaking in dilute alkaline solutions, thus confirming the results of (Dewar *et al.*, 1997), according to which the grain moisture influences the malt diastatic power. Another mechanism involves the fact that when the steeping liquor is not aerated, the microbial ecosystem can compete with steeped grain for available oxygen (Lewis and Bamforth, 2006), which may have a significant effect on seed germination.

Malt obtained with the biocontrol presented enzyme activities higher than those obtained after steeping in distilled water. Therefore, the results of Figure 3 show that the dilution of the culture exerted an effect on the various enzymatic activities, thus confirming the effect of the microbial ecosystem on malt properties (Camphnhoui *et al.*, 1998; Laitila, 2007).

### *2.5. Conclusion*

*B. subtilis* can be used to control mould growth. The effect of *B. subtilis* dilution on total fungal growth can be modelled using a sigmoidal-like function. When *B.-subtilis*-based treatments are used during red malting sorghum – grain steeping conducted without aeration − mould growth is strongly inhibited compared with dilute alkaline steeping (NaOH and $Ca(OH)_2$), while malt *α*- and *β*-amylase activities are lower than those obtained after steeping in alkaline solutions. The use of the biocontrol

also leads to the production of malts with high levels of phenolic compounds. Compared with steeping in dilute alkaline, the total malting losses obtained with the biocontrol treatment remained at lower levels. A *B. subtilis* starter used during red sorghum malting affected both the total fungal development and the grain physiology (the level of hydrolytic key enzymes). It is important to study the interactions between *B. subtilis* and seed germination to take advantage of the beneficial effects of the *Bacillus-subtilis*-based biocontrol.

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# Chapter III. Optimizing red sorghum malt quality when *Bacillus subtilis* is used during steeping to control mould growth

## *Présentation du chapitre*

Les résultats du chapitre 2 nous ont montré que lorsque *B. subtilis* S499 est utilisé comme biocontrôle des moisissures lors du maltage du sorgho rouge, la qualité des malts obtenus est globalement inférieure à celle de ceux obtenus avec les traitements chimiques. Ces malts ont par contre des faibles niveaux de contamination fongique, des freintes relativement faibles et des activités $\beta$-glucanasiques élevées. Quelques hypothèses ont été émises pour justifier l'infériorité du biocontrôle par rapport aux traitements chimiques en ce qui concerne les activités amylasiques des malts, à savoir :

1. Une possible compétition en oxygène entre la population de *B. subtilis* S499 utilisée et les grains maltés.
2. La faible humidité obtenue avec le biocontrôle comparée aux traitements alcalins.
3. La possibilité d'inhibition de l'hydrolyse de l'amidon par les lipopeptides produits par *B. subtilis* S499.
4. La présence en quantité relativement élevée des polyphénols dans les malts obtenus avec les biocontrôles comparés aux malts obtenus avec les traitements alcalins.
5. Toutes les interactions, complexes par nature, entre les grains maltés et la souche utilisée.

Dans le chapitre 3 nous avons essayé d'améliorer les propriétés des malts obtenus avec le biocontrôle en nous basant sur des approches bien connues de l'effet des conditions de maltage sur les propriétés du malt.

## *Abstract*

Previous work having shown that *Bacillus-subtilis*-S499-based biocontrol treatments applied without aeration at the steeping stage of red sorghum malting offered good mould reduction but yielded malts with low levels of key hydrolytic enzymes, we attempted to raise these levels by aerating the steeping liquor, varying the steeping time (from 8 to 40 h) and temperature (from 25 to 35°C), and combining a biocontrol treatment with prior steeping in 0.2% NaOH. Aeration proved particularly important whenever *B. subtilis* cells were present in the steep liquor. The optimal temperatures for $\alpha$- and $\beta$-amylase were 30 and 25°C, respectively. By increasing the steeping time it was possible to improve the $\alpha$-amylase activity, but the $\beta$-amylase activity peaked sharply between 16 and 20 h, depending on the steeping medium. A good compromise was steeping in biocontrol medium for 14 to 16 h at 30°C. Combination steeping treatments (0.2% NaOH for 8 h followed by biocontrol for 8 h) yielded malts of a quality approaching that afforded by dilute alkaline treatment.

Keywords: red sorghum, biocontrol, mould reduction, malt quality, amylases, *Bacillus subtilis*

## *3.1. Introduction*

When *B. subtilis* S499 is used as starters during sorghum malting, the key hydrolytic activities ($\alpha$- and $\beta$-amylases) are low compared with the dilute alkaline steeping-based treatments (see chapter 2). In this work, based on well-known approaches such as the effect of aeration, temperature and soaking time on red sorghum malt properties, we tried to improve the properties of red sorghum malt by using *B. subtilis* as starters to control mould growth. We persevered with this treatment, despite its inferiority to chemical treatment, because it leads to malts with high $\beta$-glucanase activity. Improvement of this activity is very interesting to the brewing industry as it avoids all the problems associated with the hydrolysis of sorghum $\beta$-glucans, which affects wort lautering, beer filtration and stability. One strategy studied in this work is the combination of a chemical treatment (0.2% NaOH) and the biocontrol in order to improve red sorghum malt properties.

## *3.2. Material and Methods*

### 3.2.1. *B. subtilis* S499 cultures and preparation of biocontrol treatment fractions

*B. subtilis* S499 strain was obtained from the Walloon Center of Industrial Biology (CWBI) and grown on Luria Broth (LB) agar at 37°C for 24 h. An inoculating loopful was transferred to 100 ml Landy broth optimized for *B. subtilis* S499 lipopeptide production and incubated for 16 h. Finally, 10 ml was transferred to 350 ml optimized Landy broth and incubated at 30°C (under rotary shaking at 130 rpm) for 72 h. After cell counting under a microscope in a Burker cell, the culture was diluted with distilled water to $10^8$ cells $ml^{-1}$. This suspension (henceforth called "C+B" for "cells plus broth") was either used as such or centrifuged at 10,000×g to yield two fractions: a supernatant, which was centrifuged two more times at 10,000×g to eliminate as many cells as possible (the resulting fraction is henceforth called "B"), and a cell pellet, which was subjected to two rounds of rinsing in distilled water and centrifuging and finally resuspended in distilled water so as to obtain a concentration of $10^8$ cells $ml^{-1}$.

### 3.2.2. Total fungal and total mesophilic aerobe counts

Fungal populations and the total mesophilic aerobe count were evaluated after kilning as in Badau (2006).

### 3.2.3. Malting

Red sorghum was obtained from the D.R. Congo. After sorting and cleaning, 150 g sorghum grains was placed in a perforated stainless steel cylinder. The cylinder was immersed to about 3/4 of its height in a JULABO HP35 water bath containing 18 L steeping liquor (the amount of liquid in the cylinder was about 300 ml). When aeration was required, compressed air (0.7 bars) was pumped continuously into the bath from below (at about 15 L $min^{-1}$) and the steeping liquor was recirculated

through the cylinder from above. After steeping, the grains were rinsed in distilled water and allowed to germinate at 30°C for 72 h in a plastic mini greenhouse, with the grains in the upper portion and water underneath to saturate the air. The green malts obtained were kilned at 40°C for 48 h and rootlets were hand removed by gentle brushing.

#### 3.2.4. Steeping treatments

All biocontrol treatments were applied at the steeping step. The grains were steeped at 25°C, 30°C, or 35°C in either C+B, C, or B for a time ranging from 0 to 40 h. Two controls were included: soaking in distilled water and in 0.2% NaOH. Combination treatments were also tested. These involved steeping in 0.2% NaOH for 2 to 14 h, rinsing the grains, and then subjecting them for 14 to 2 h to a biocontrol treatment (total steeping time: 16 h).

#### 3.2.5. Sorghum malt characteristics

The percentage of chitted grains after steeping was calculated as follows: 20 g grain was taken at random after steeping and divided into lots of approximately 5 g. The chitted grains of three randomly selected lots were counted, and the percentage of chitted grains was calculated as the average of the three measurements. All enzymatic assays were performed on extracts of malt flour obtained by grinding 20 g kilned malt in an IKA mill followed by sieving (mesh size: 0.5 mm). $\alpha$- and $\beta$-amylase activities were extracted and assayed using Megazyme methods (Ceralpha Method: K-CERA 08/05 and Betamyl-3 Method: K-BETA 10/10 respectively). $\beta$-Glucanase extraction was carried out for 15 min at 30°C in a centrifugation tube containing 0.5 g malt flour and 8 ml extraction buffer (50 mM Na-acetate, pH 4.8), with vigorous vortexing every 5 min. The mixture was then centrifuged at 1000xg for 10 min and the supernatant collected. The assay was performed at 40°C in a reaction mixture containing 0.5 mL extract and 0.5 mL of 2% carboxymethyl cellulose as substrate. The incubation time was 5 min and the reaction was stopped by immersing the tubes in boiling water. The amount of glucose released was then determined by the method of Nelson-Somogyi (Primarini and Yoshiyuki, 2000) and the $\beta$-glucanase activity was expressed in µmoles of glucose released per minute per kg of kilned malt. Total phenolics were assayed using the method optimized by Georgé *et al.* (2005) without eliminating water-soluble compounds, and results are expressed in mg gallic acid equivalents per gram of dried malt (mg GAE $g^{-1}$). Condensed tannins were estimated by the modified vanillin/HCl method of Price *et al.* (1978) and results are expressed in percent catechin equivalents (% CE). EBC methods were used for the following characteristics: EBC method 3.2. for raw sorghum moisture content, EBC method 3.4, 2004 for the thousand corn weight (TCW), EBC method 3.5.2 for germination capacity (GC), and EBC method 3.6.3 for germination energy (GE) (Analytica EBC, 2004). Soluble nitrogen was determined after mashing and filtration as described in EBC method 4.5.1 (Analytica EBC, 2004).

The nitrogen content was determined in the wort before and after 2 hours of boiling and the soluble nitrogen/total nitrogen ratio (S/N) was calculated.

#### 3.2.6. Rehydration curves

After sorting and cleaning, the sorghum grains were soaked in 0.2% NaOH or supernatant B for 40 h. After 0.5, 1, 2, 4, 6, 8, 12, 14, 16, 20, 24, 36, and 40 h, the wet weight ($w_w$) was measured in triplicate. The dry weight ($w_d$) was determined by heating the grains in an oven at 105°C until the weight remained unchanged. The dry-basis (d.b.) steep-out moisture content was calculated by subtracting $w_d$ from $w_w$ and dividing by $w_d$. Peleg's model (Peleg, 1988) was used to draw the rehydration curves. This model can be written as: $1/M_t-M_0 = k_2 + k_1t^{-1}$, where t is the rehydration time, $M_0$ is the initial moisture content (d.b.), and $M_t$ is the moisture content (d.b.) at time t. The parameters $k_1$ (Peleg's rate constant) and $k_2$ (Peleg's capacity constant) are determined by plotting $1/M_t-M_0$ versus $t^{-1}$ and fitting a straight line to the data. From these parameter values it is possible to deduce an initial sorption rate ($\mu_s$ = 1/k1) and a final (equilibrium) moisture content ($\omega_0=\mu_0+1/k_2$).

#### 3.2.7. Lipopeptide assays

Surfactins, iturins, and fengycins were assayed by reversed-phase high-performance liquid chromatography hyphenated to diode array detection electrospray ionization mass spectrometry (RP-HPLC/(ESI+)DAD-MS) as described by Nihorimbere *et al.* (2011).

#### 3.2.8. Statistical analyses

XLSTAT 2011.4.01 software was used for statistical analyses. The Student-Neuman-Keuls (SNK) test was used to determine the significance of differences between results obtained under different conditions. Three-way ANOVA was applied to the $\alpha$- and $\beta$-amylase data obtained with different steeping treatments after different steeping times and at different temperatures. The goodness of fit of Peleg's model to the rehydration data was checked by estimating the mean relative percentage deviation modulus (E). Minitab 15 software was used to produce graphs and to perform the 3-way ANOVA.

### *3.3. Results*

#### 3.3.1. Red sorghum characteristics

The characteristics of the red sorghum used here were as follows. Moisture content (w.b.): 11.14%±0.19; thousand grain weight: 26.20 g ±0.12; germination capacity: 96.33% ± 0.29; germinative energy: 95.53% ± 0.50; initial fungal plate count: 4.85±0.14 log cfu $g^{-1}$.

### 3.3.2. Effect of aeration

As our previous study had been conducted without aeration (chaper 1), we first compared the effects of aeration on our three biocontrol treatments (C+B, C, and B) and our two controls ($H_2O$, NaOH). As shown in Table 2, aeration was found to have a slight, albeit positive effect on the wet-basis (w.b.) steep-out moisture. The presence of the culture broth also seemed to increase, very slightly, the steep-out moisture (C+B and B versus C). On the other hand, aeration clearly stimulated germination, as indicated by increases in the number of chitted grains at the end of the steeping step. The most spectacular increases occurred in the presence of *B. subtilis* cells: 28- and 78-fold for treatments C and C+B, respectively, as compared to about 2-fold in distilled water and 5-fold in dilute alkaline solution. Also noteworthy is the high percentage of chitted grains observed after all three biocontrol treatments, as compared to either $H_2O$ or NaOH. This last treatment would appear to have a germination-delaying effect (NaOH vs. $H_2O$, NA and A).

**Table 2. Effect of aeration on steep-out moisture and percentage of chitted grains**

| Treatment | Steep-out moisture content (%, w.b.) | | % of chitted grains | |
|---|---|---|---|---|
| | **NA** | **A** | **NA** | **A** |
| **$H_20$** | $30.7\pm0.2^{f}$ | $32.6\pm0.3^{e}$ | $25.5\pm0.5^{f}$ | $59.7\pm3.8^{d}$ |
| **NaOH** | $42.1\pm0.1^{b}$ | $43.8\pm0.5^{a}$ | $9.3\pm1.5^{h}$ | $45.7\pm3.5^{e}$ |
| **C+B** | $36.5\pm0.1^{d}$ | $37.9\pm0.2^{c}$ | $1.0\pm1.0^{i}$ | $78.0\pm1.0^{b}$ |
| **C** | $35.6\pm0.1^{d}$ | $36.8\pm0.2^{d}$ | $3.3\pm1.2^{i}$ | $85.0\pm1.5^{a}$ |
| **B** | $36.6\pm0.0^{d}$ | $38.6\pm0.1^{c}$ | $16.0\pm2.0^{f}$ | $74.3\pm4.2^{c}$ |

For each characteristic measured, data having a letter in common are not statistically different according to the SNK test ($p<0.05$). NA: Not aerated during steeping, A: aerated during steeping.

Table 3 shows the effect of aeration on the post-kilning fungal count. It appeared to enhance, slightly, the antifungal efficacy of all three biocontrol treatments (C+B, B, and C, A vs. NA) while reducing that of 0.2% NaOH. The biocontrol treatments were always more effective than dilute alkaline treatment. In terms of their fungus-reducing efficacy, the biocontrol treatments (with or without aeration) ranked as follows: B+C > B > C.

Table 3 also shows the total malting loss, total phenolics, and condensed tannins after the various treatments. In the absence of aeration, the total malting loss was 46 to 48% lower after biocontrol treatment than after NaOH treatment. Aeration increased the total malting loss recorded after the biocontrol treatments (by 21%, 11%, and 24% for C+B, C, and B respectively) without affecting the loss after NaOH treatment, but the loss remained 34 to 39% lower after biocontrol treatment than after NaOH treatment. Aeration had no effect on total phenolics or condensed tannins, whose levels were lower after steeping in NaOH than after any biocontrol treatment.

**Table 3. Effect of aeration on total fungal count, total malting loss, total phenolics, and condensed tannins**

| Treatment | Total fungal count (logCFU/g) | | Total malting loss (%) | | Total phenolics (mg GAE $g^{-1}$) | | Condensed tannins (% CE) | |
|---|---|---|---|---|---|---|---|---|
| | NA | A | NA | A | NA | A | NA | A |
| **$H_20$** | 4.81±0.02$^{a}$ | 4.76±0.04$^{a}$ | 21.1±1.1$^{b}$ | 25.0±0.5$^{a}$ | 7.9±0.2$^{ab}$ | 8.0±0.3$^{a}$ | 0.15±0.01$^{a}$ | 0.16±0.01$^{a}$ |
| **NaOH** | 2.87±0.07$^{c}$ | 3.16±0.03$^{b}$ | 26.0±0.5$^{a}$ | 25.7±0.6$^{a}$ | 5.9±0.1$^{e}$ | 6.0±0.1$^{e}$ | 0.06±0.01$^{c}$ | 0.06±0.01$^{c}$ |
| **C+B** | 1.63±0.06$^{f}$ | 1.40±0.07$^{g}$ | 13.5±1.2$^{e}$ | 16.3±0.6$^{cd}$ | 7.5±0.1$^{c}$ | 7.4±0.2$^{cd}$ | 0.12±0.02$^{a}$ | 0.12±0.02$^{a}$ |
| **C** | 2.11±0.08$^{d}$ | 1.87±0.04$^{e}$ | 14.1±1.1$^{de}$ | 15.7±1.1$^{cde}$ | 7.2±0.0$^{d}$ | 7.2±0.1$^{d}$ | 0.09±0.01$^{b}$ | 0.09±0.01$^{b}$ |
| **B** | 1.81±0.03$^{e}$ | 1.61±0.06$^{f}$ | 13.7±2.1$^{e}$ | 17.0±1.0$^{c}$ | 7.7±0.1$^{bc}$ | 7.5±0.1$^{c}$ | 0.12±0.02$^{a}$ | 0.12±0.02$^{a}$ |

For each characteristic measured, data having a letter in common are not statistically different according to the SNK test ($p<0.05$). NA: Not aerated during steeping, A: aerated during steeping.

Table 4 shows the effect of aeration on three key hydrolytic enzymes. In the absence of aeration, as observed previously (Chapter 2), the *α*- and *β*-amylase levels achieved were lower after biocontrol treatment than after NaOH treatment, but higher than after steeping in water alone. Aeration improved these levels whatever the steeping conditions, but in all cases but one (*β*-amylase, treatment B), it had a greater effect when the grains were soaked in water (+64% for *α*-amylase, +67% for *β*-amylase) or subjected to a biocontrol treatment (*α*-amylase: +95%, +82%, and +42%, for C+B, C, and B respectively; *β*-amylase: +38% and +23% for C+B and C, respectively) than when they were steeped in 0.2% NaOH (*α*-amylase: +11%; *β*-amylase:+17% ). Aeration also enhanced the level of *β*-glucanase activity under all steeping conditions. Interestingly, the highest levels of this enzyme activity were reached in the presence of *B. subtilis* cells (C+B and C, A and NA).

Given the considerable boosting effect of aeration on these enzyme activities, especially during biocontrol treatment, all subsequent experiments were performed with aeration.

**Table 4. Effect of aeration on enzyme activities**

| Treatment | *α*-amylase (U/g malt) | | *β*-amylase (U/g malt) | | *β*-glucanase (U/kg malt) | |
|---|---|---|---|---|---|---|
| | NA | A | NA | A | NA | A |
| **$H_20$** | 45.0±0.7$^{i}$ | 73.6±2.6$^{g}$ | 22.9±1.1$^{h}$ | 38.3±1.5$^{f}$ | 0.6±0.0$^{g}$ | 0.9±0.0$^{f}$ |
| **NaOH** | 262.5±1.8$^{b}$ | 291.8±2.7$^{a}$ | 50.6±2.8$^{b}$ | 59.5±1.7$^{a}$ | 1.8±0.2$^{e}$ | 3.1±0.2$^{d}$ |
| **C+B** | 69.6±1.0$^{h}$ | 135.8±1.9$^{d}$ | 32.0±2.2$^{g}$ | 44.1±1.2$^{d}$ | 3.9±0.1$^{c}$ | 4.6±0.1$^{b}$ |
| **C** | 80.5±3.8$^{f}$ | 147.3±1.7$^{c}$ | 40.4±1.7$^{ef}$ | 49.6±0.7$^{c}$ | 4.8±0.2$^{b}$ | 5.8±0.1$^{a}$ |
| **B** | 67.4±0.7$^{h}$ | 96.1±2.0$^{e}$ | 38.9±1.7$^{ef}$ | 42.2±0.6$^{de}$ | 1.1±0.1$^{f}$ | 1.6±0.1$^{e}$ |

For each characteristic measured, data having a letter in common are not statistically different according to the SNK test ($p<0.05$). NA: Not aerated during steeping, A: aerated during steeping.

### 3.3.3. Effects of steeping time and temperature on *α*- and *β*-amylase activities

We next examined whether the diastatic power of malts obtained after biocontrol treatment might be improved by extending the steeping time or changing the steeping temperature. Figure 7. A and B show how these factors affected the *α*- and *β*-amylase activities. 3-way ANOVA applied to these data

showed significant effects of steeping treatment, time, and temperature, and their interactions (Tables 5 and 6). At each temperature and for each amylase, the peak activity recorded was highest after steeping in 0.2% NaOH, but at 35°C, the *β*-amylase activity declined faster after peaking in the NaOH-treated samples than in the broth-containing biocontrol samples (C+B and B). In all treatment media the optimal temperature for *α*-amylase activity was 30°C (Figure 7.A.). When treated at this temperature, samples steeped in NaOH showed a sharp rise in *α*-amylase between 8 and 16 h, followed by a broad peak (highest value recorded: 286.3±1.0 U $g^{-1}$, at 24 h) and a final decline. Interestingly, the biocontrol treatments containing *B. subtilis* cells at high concentration led to higher *α*-amylase activities than steeping in conditioned broth alone (Figure 7.A, C+B and C vs. B).

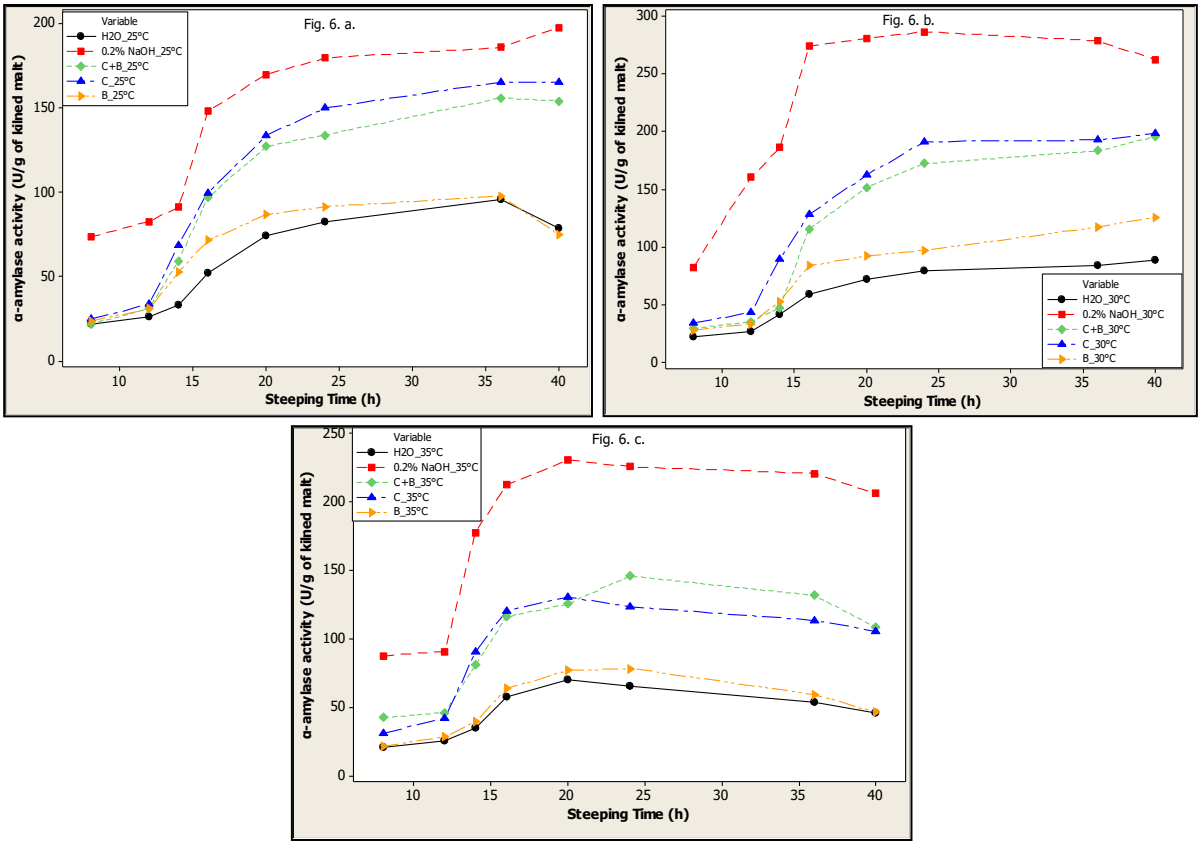


**Figure 7. A. Effects of steeping time, temperature, and steeping liquor composition on *α*-amylase activities in kilned malts [A1, A2, A3: *α*-amylase activities respectively for 25°C, 30°C and 35°C for 25°C, 30°C and 35°C]**

In the case of these cell-containing treatments, the *α*-amylase activity rose quickly for 24 h and then more slowly until the end of the experiment. The increase beyond 16 h (time corresponding to Table 2-4) was 31% for treatment C+B, 27% for treatment C, and 11% for treatment B. Extending the steeping time is thus one way to increase the *α*-amylase activity of a red sorghum malt obtained after biocontrol treatment at 30°C.

A different picture was obtained for *β*-amylase activity, which peaked between 12 and 20 h, and declined - often sharply - thereafter. The peak level obtained after NaOH treatment was about the

same at 25°C and 30°C (respectively 56.3 U $g^{-1}$ and 55.3 U $g^{-1}$), but markedly lower at 35°C (41.5 U $g^{-1}$). The peak levels recorded after biocontrol treatment were less sensitive to temperature, although 35°C appeared generally less favorable. At 30°C (the optimum for $\alpha$-amylase activity), the $\beta$-amylase peak was higher after steeping in cell-containing medium (41.6 U $g^{-1}$for C+B and 42.0 U $g^{-1}$ for C) than after treatment in the conditioned broth alone (26 U $g^{-1}$).

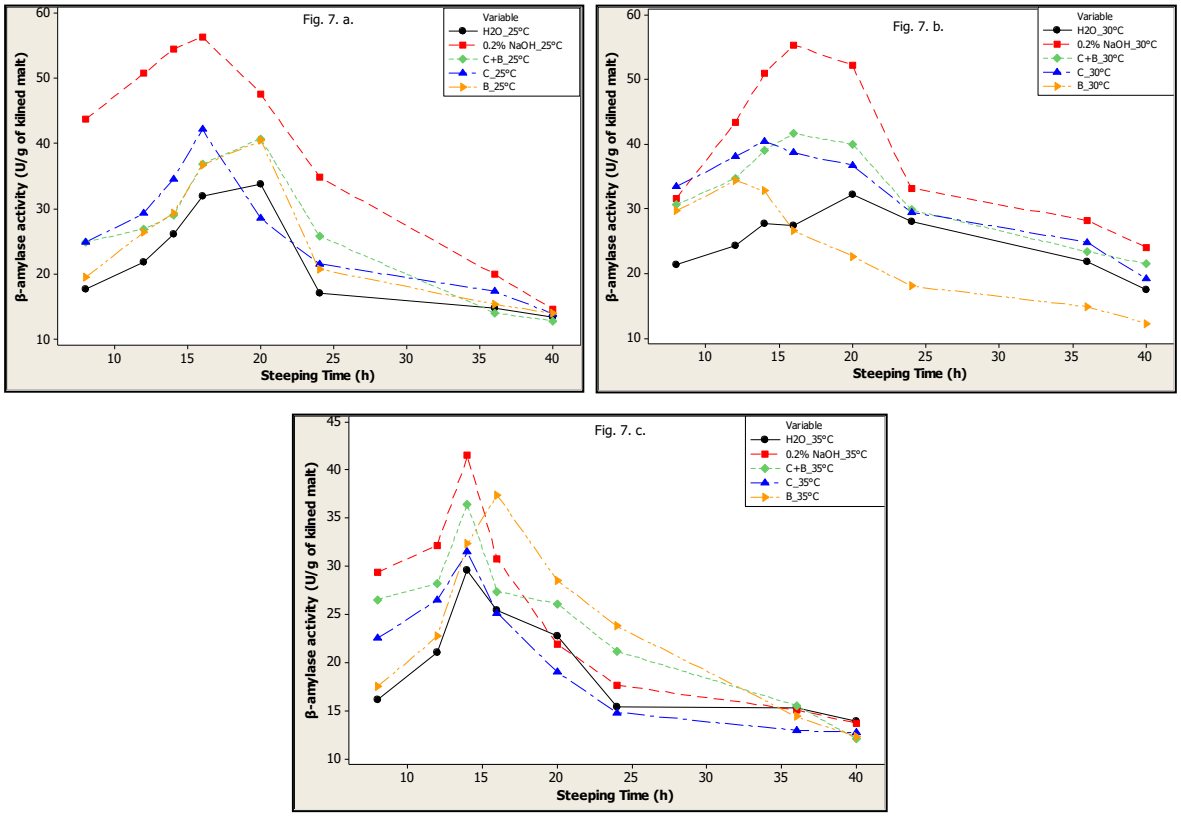


**Figure 7. B. Effects of steeping time, temperature, and steeping liquor composition on beta-amylase activities in kilned malts [B1, B2, B3: abeta-amylase activities respectively for 25°C, 30°C and 35°C]**

**Table 5. Results of 3-way ANOVA applied to the data of Figure 7.A. (for $\alpha$-amylase vs. steeping treatment, steeping temperature, and steeping time) using the general linear model**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| **Steeping treatments [STT]** | 4 | 732130 | 183033 | 5490.35 | 0.0000 |
| **Steeping temperature [ST] (°C)** | 2 | 62491 | 31246 | 937.26 | 0.0000 |
| **Steeping time [SD] (h)** | 7 | 580027 | 82861 | 2485.55 | 0.0000 |
| **STT*ST** | 8 | 56745 | 7093 | 212.77 | 0.0000 |
| **STT*SD** | 28 | 78634 | 2808 | 84.24 | 0.0000 |
| **ST*SD** | 14 | 42028 | 3002 | 90.05 | 0.0000 |
| **STT*ST*SD** | 56 | 26116 | 466 | 13.99 | 0.0000 |
| **Error** | 240 | 8001 | 33 | | |
| **Total** | 359 | 1586173 | | | |
| **S = 5.77383** | **R-Sq = 99.50%** | | | **R-Sq(adj) = 99.25%** | |

**Table 6. Results of 3-way ANOVA applied to the data of Figure 7.B. (for *β*-amylase vs steeping treatment, steeping temperature and steeping time) using general linear model.**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| **Steeping treatments [STT]** | 4 | 6947.21 | 1736.80 | 745.42 | 0.0000 |
| **Steeping temperature [ST] (°C)** | 2 | 4021.97 | 2010.98 | 863.09 | 0.0000 |
| **Steeping time [SD] (h)** | 7 | 19896.86 | 2842.41 | 1219.93 | 0.0000 |
| **STT*ST** | 8 | 2391.97 | 299.00 | 128.33 | 0.0000 |
| **STT*SD** | 28 | 2200.84 | 78.60 | 33.73 | 0.0000 |
| **ST*SD** | 14 | 1558.82 | 111.34 | 47.79 | 0.0000 |
| **STT*ST*SD** | 56 | 2589.96 | 46.25 | 19.85 | 0.0000 |
| **Error** | 240 | 559.19 | 2.33 | | |
| **Total** | 359 | 40166.82 | | | |
| **S = 1.52642** | **R-Sq = 98.61%** | | **R-Sq(adj) = 97.92%** | | |

### 3.3.4. Enzyme levels in relation to grain hydration

Dewar *et al.* (1997) have attributed the increased DP observed after dilute NaOH treatment to increased water uptake by the grains during steeping, likely due to opening-up of the pericarp cell walls by the dilute alkaline solution. To investigate this matter, we measured the moisture content of the sorghum grains over a 40-h period of steeping under various conditions. Under all conditions tested, the dry-basis (d.b.) moisture content was found to increase over the entire period, being higher in the NaOH-treated samples than in the biocontrol samples. To get a really good fit with Peleg's model (Pelg, 1988), it was necessary to limit the time period considered to 16 h (data and model curves: Figure 9; parameter values presented in Table 7). We attribute this to the changes accompanying grain germination, as attested by the appearance of chitted grains around this time. As shown by others (Sopade and Obekpa, 1990; Sopade *et al.*, 1994; Abu-Ghannam and McKenna, 1997; Akpinar, 2006; Gowen *et al.*, 2007; Kashiri *et al.*, 2010), the initial sorption rate μs, as calculated from the model fitted to the 16-h data, was found to increase with the temperature according to the Arrhenius equation (not shown). This was not true of the value obtained after fitting to the 40-h data (not shown). The model fitted to the 16-h data markedly underestimated the moisture at 40 h, by 4.1 to 8.3% when the grains were steeped in 0.2% NaOH and by 5.4 to 13.7% when they were steeped in *B. subtilis* culture broth. This suggests that the equilibrium moisture values (ωo) calculated on the basis of this model should be considered with caution. There appeared no absolute correlation between moisture content and the *α*- and *β*-amylase activities presented in the previous section. On the one hand, it is true that both the moisture content and the amylase activities were highest after steeping in 0.2% NaOH. It is also true that the *α*-amylase activity rose concomitantly with the moisture content over the first 20 hours. Yet both enzyme activities were lower at 35 than at 30°C, and *β*-amylase reached its highest levels at 25°C (both steeping liquors).

**Table 7. Red sorghum rehydration - kinetic parameters (Peleg's model)**

| Treatment* | T (°C) | $k_1$ | $k_2$ | $\mu_s$ | $\omega_0$ | E |
|---|---|---|---|---|---|---|
| **16 h steeping in 0.2% NaOH** | **25°C** | 2.92 | 1.59 | 0.34 | 0.75 | 0.55 |
| | **30°C** | 2.53 | 1.31 | 0.40 | 0.88 | 0.43 |
| | **35°C** | 2.07 | 1.17 | 0.48 | 0.97 | 0.32 |
| **16 h steeping in B** | **25°C** | 8.14 | 1.89 | 0.12 | 0.65 | 1.10 |
| | **30°C** | 7.17 | 1.73 | 0.14 | 0.70 | 1.42 |
| | **35°C** | 5.89 | 1.66 | 0.17 | 0.72 | 0.83 |

### 3.3.5. Combining steeping in 0.2% NaOH with a biocontrol treatment

We then examined the possibility of combining NaOH treatment with a biocontrol treatment, so as possibly to cumulate the advantages of both. In an experiment where the total steeping time was set at 16 h, steeping in 0.2% NaOH for 2 to 14 h was followed by 14 to 2 hours of resteeping under conditions C+B or C. Figure 8 shows that as the time spent in the dilute alkaline solution increased, the fungal count was found to rise, the percentage of chitted grains to decrease, and the moisture content to rise before stabilizing at 8 h. To maximize the moisture content while minimizing the mould count increase and germination lag, 8 h in NaOH followed by 8 h biocontrol treatment (C+B or C) was chosen for the next experiment. As shown in Table 9, the characteristics of the malts obtained are encouraging. As compared to the corresponding 16-h biocontrol treatments (see Tables 6 and 7, tests with aeration), both combination treatments offered higher $\alpha$-amylase activities and lower levels of total phenolics and tannins. $\beta$-Amylase activity was improved only by the combination treatment with C+B. These improvements were associated with an increased steep-out moisture (to a level only 3% lower than for the 16-h NaOH treatment). Although the combination treatments gave rise to somewhat higher malting losses and total fungal counts than the corresponding16-h biocontrol treatments, these characteristics remained more favorable than after the 16-h NaOH treatment. There was no significant difference between treatments as regards the soluble-to-total nitrogen content after 2 h of boiling. Both tested combination treatments thus offer slightly better mould control than a 16-h NaOH treatment, while yielding malts of approaching quality.

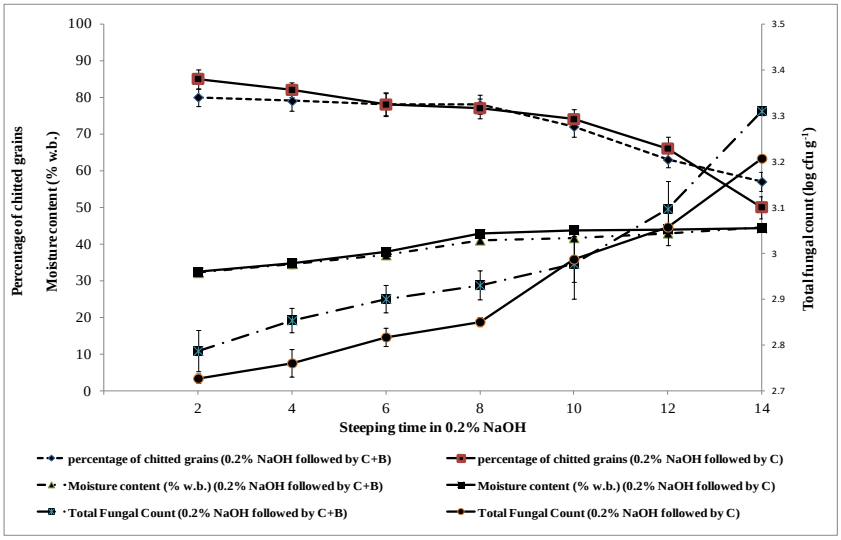


**Figure 8. Steep-out moisture, percentage of chitted grains, and total fungal count as affected by residence time in 0.2% NaOH prior to rinsing and transfer to C+B or C (total steeping time: 16 h)**

### 3.3.6. Lipopeptides and total mesophilic aerobes

We next examined whether lipopeptide production by *B. subtilis* S499 and/or competition between moulds and bacteria might be responsible for the biocontrol effects observed here. Table 8 shows the total mesophilic aerobe counts and lipopeptide (surfactin, iturin, and fengycin) levels observed after 16 hours of C+B, C, or B treatment, and Figure 10 and 11 show their variations in the course of each treatment. As expected of extracellular compounds in a culture medium optimized for their production, all three lipopeptide families were found at high levels in the presence of conditioned broth (C+B and B). Although these levels decreased somewhat in the course of the experiment, they remained high at the end of the 16-h period. In contrast, all three families were undetectable for the first 8 hours of treatment with rinsed cells, reaching low levels by the end of the experiment. As expected, the total mesophilic aerobe count was highest in the cell-based treatments. It was lower in the case of treatment B (centrifuged broth) than after NaOH treatment.

**Table 8. Lipopeptides and mesophilic aerobes in steeping liquor**

| Treatment | Surfactins ($\mu g\ ml^{-1}$) | Iturins ($\mu g\ ml^{-1}$) | Fengycins ($\mu g\ ml^{-1}$) | Total mesophilic aerobe count ($log\ cfu\ ml^{-1}$) |
|---|---|---|---|---|
| C+B | 332.9 | 100.0 | 249.8 | 10.02 |
| C | 26.6 | 11.2 | 18.8 | 10.64 |
| B | 368.4 | 89.6 | 176.7 | 4.01 |
| 0.2% NaOH | - | - | - | 6.0 |

**Table 9. Characteristics of malts obtained with combined steeping treatments**

| Treatment | Steep-out moisture (%,w.b.) | Total fungal count (log cfu $g^{-1}$) | Total malting loss (%) | $\alpha$-amylase (U $g^{-1}$) | $\beta$-amylase (U $g^{-1}$) | $\beta$-glucanase (U $kg^{-1}$) | Total phenolics (GAE $g^{-1}$) | Condensed tannins (%CE) | Soluble nitrogen/total nitrogen (%) | Soluble nitrogen/total nitrogen after 2h boiling (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| **NaOH, C+B** | $^{*}$40.8±0.9$^{b}$ | 1.65±0.04$^{c}$ | 20.1±0.9$^{b}$ | $^{*}$224.9±4.0$^{c}$ | $^{*}$46.5±0.7$^{c}$ | $^{*}$4.3±0.1$^{b}$ | $^{*}$6.6±0.3$^{b}$ | *0.08±0.01$^{b}$ | 35.9±1.0$^{b}$ | 32.7±0.7$^{a}$ |
| **NaOH, C** | $^{*}$40.9±0.9$^{b}$ | 2.05±0.03$^{b}$ | 21.1±0.4$^{b}$ | $^{*}$254.5±4.4$^{b}$ | $^{*}$49.2±1.6$^{b}$ | $^{*}$6.0±0.0$^{a}$ | $^{*}$6.0±0.2$^{a}$ | 0.08±0.01$^{b}$ | 38.5±0.9$^{b}$ | 33.8±1.0$^{a}$ |
| **NaOH** | 43.8±0.5$^{a}$ | 3.16±0.03$^{a}$ | 25.7±0.6$^{a}$ | 291.8±4.9$^{a}$ | 59.5±1.7$^{a}$ | 3.1±0.2$^{c}$ | 6.0±0.1$^{a}$ | 0.06±0.01$^{a}$ | 41.4±0.2$^{a}$ | 33.2±1.5$^{a}$ |

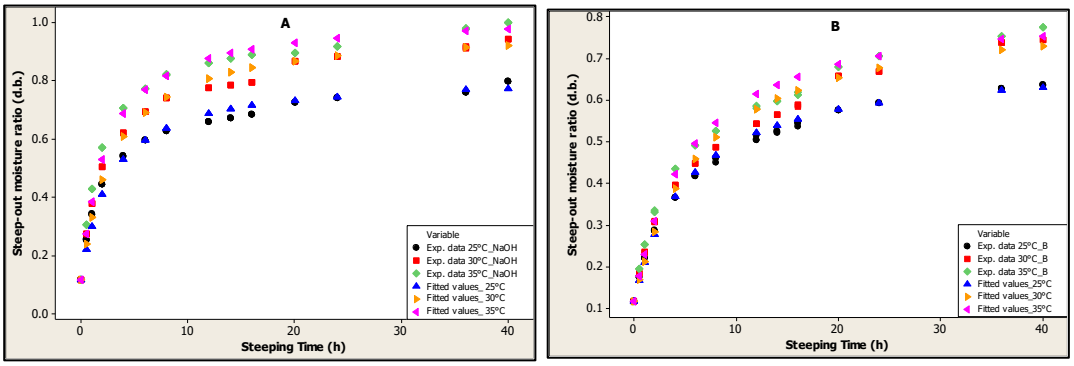


**Figure 9. Sorghum grain hydration over a 16-h steeping period (A) in 0.2% NaOH or (B) under biocontrol conditions (B : conditioned *B. subtilis* culture broth)**

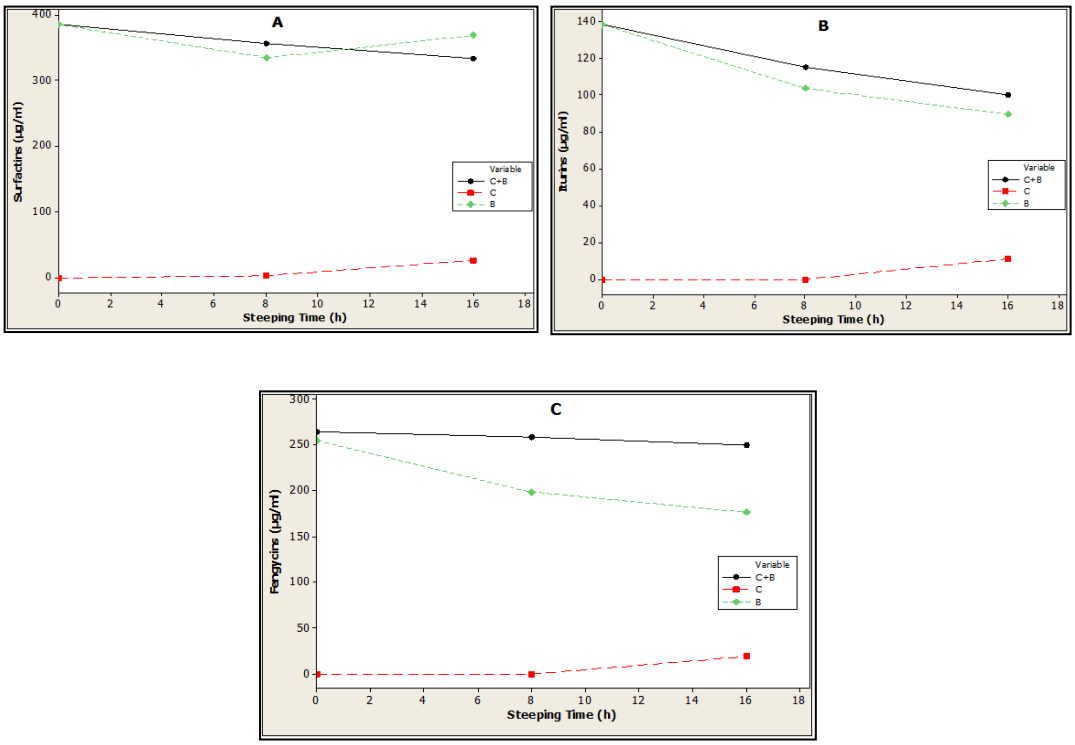


**Figure 10. Variation of surfactins (A), iturins (B), and fengycins (C) levels in the course of different 16-h biocontrol steeping treatments (C : *B. subtilis* cells in water; B : conditioned *B. subtilis* culture broth ; (B+C : cells in their culture broth)**

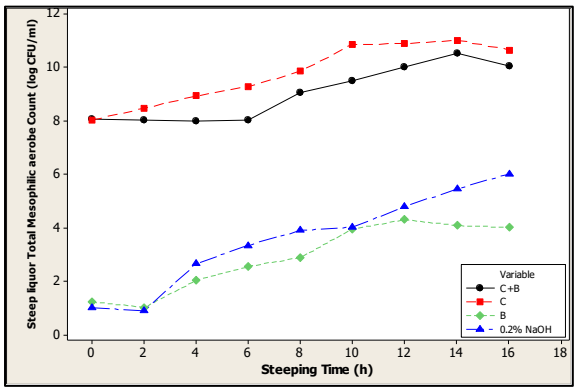


**Figure 11. Variation of the total mesophilic aerobe count (log cfu ml$^{-1}$ of steeping liquor) in the course of different 16-h biocontrol steeping treatments (B+C: cells in their culture broth, C: *B. subtilis* cells in water; B: conditioned *B. subtilis* culture broth, and in 0.2% NaOH)**

### *3.4. Discussion*

We show here that during sorghum malting, the use of steeping treatments based on *B. subtilis* S499 can greatly reduce the post-kilning level of fungal contamination. All of the biocontrol treatments tested (with and without aeration, with and without cells or conditioned broth, combined or not with NaOH treatment) reduced the total fungal count from an initial 4.85 log cfu $g^{-1}$ to below the limit of 3 log cfu $g^{-1}$ recommended by AFRNOR (Ballogou *et al.*, 2011). The results of Lefyedi and Taylor (2007) offer a comparison: these authors reduced the total fungal count in malt from an initial $2.6\times10^3$ cfu $g^{-1}$ down to $1.3\times10^2$ cfu $g^{-1}$ with a half-and-half mixture of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* at $10^7$ cells $ml^{-1}$. All of the biocontrol treatments tested here appeared to limit the post-kilning mould count more effectively than NaOH treatment.

The mechanism(s) underlying mould control by *Bacillus-subtilis*-based steeping treatments are as yet unclear. On the one hand, lipopeptides produced by *Bacillus subtilis* are known to inhibit the growth of plant pathogens such as *Fusarium oxysporum* and *Rhizoctonia solani* (Nihorimbere *et al.*, 2011; Qi-qin *et al.*, 2006). Another possible mechanism is competition between moulds and the biocontrol agent during steeping. Yet our results cannot readily be explained by either of these mechanisms, as strong biocontrol effects were observed after both B treatment (low mesophilic aerobe count) and C treatment (low lipopeptide levels after 16 h of steeping). Perhaps bacterial compounds other than lipopeptides exert antifungal effects. Or perhaps both mechanisms intervene, since the strongest effects were observed with cells and broth together. This matter deserves further investigation.

We confirm here, as shown by others (see Introduction), the DP-enhancing power of steeping in 0.2% NaOH during sorghum malting. With biocontrol treatments alone, we failed to approach the $\alpha$- and $\beta$-amylase levels recorded for dilute alkaline treatment. Interestingly, however, the recorded $\beta$-glucanase activity was between 1.5 and 2.7 times as high after steeping with *B. subtilis* cells (alone or with their broth) as after dilute alkaline treatment. The reason for this enhanced activity is unclear. The effect was not observed with conditioned culture broth alone, which seems to rule out penetration of a secreted *B. subtilis* enzyme into the grains. A high $\beta$-glucanase activity could be an advantage in situations where sorghum malt is used for brewing (Etokakpan, 1992). Cell wall $\beta$-glucan hydrolysis and solubilization during malting and brewing are two important steps of the process, because they affect the viscosity of the mash and the stability of the beer. Yet preliminary results (unpublished) fail to show any significant change in $\beta$-glucan levels according to the steeping treatment.

Several measures taken here to raise the enzyme activities achieved after biocontrol treatment were successful. Aeration is clearly of the essence, since all measured enzyme levels were found to increase with aeration, whatever the steeping liquor. The effect was particularly pronounced for $\alpha$-amylase, when *B. subtilis* cells were added to the steeping mixture. This suggests that the bacterial cells

compete strongly with the grains for the available oxygen during steeping without aeration. This is further suggested by the marked increases in the percentage of chitted grains observed upon aeration, most pronounced when *B. subtilis* cells are present. Alternatively or additionally, the mixing and bubbling that accompany aeration might favor evacuation of $CO_2$, known to inhibit the synthesis of certain enzymes, especially $\alpha$-amylase (Weith and Klaushofer, 1963). It would be interesting to test different types and levels of aeration to see if enzyme levels after biocontrol treatment might be improved further.

Regarding the steeping time, Dewar *et al.* (1997) report a significant increase in DP with the steeping time, whereas Novéllie (1962) reports no effect. Our results suggest that increasing the steeping time is a two-edged sword. On the one hand, at 25 or 30°C, the $\alpha$-amylase level can be increased somewhat by extending the steeping time beyond 16 h. On the other hand, the $\beta$-amylase level in the kilned malt shows a rather sharp peak, the timing of which shifts according to the steeping conditions. One must thus be careful not to extend the steeping time beyond the peak. In the case of treatment C+B or C carried out at 30°C, this means limiting the treatment to about 16 h.

Changing the temperature affects the $\alpha$- and $\beta$-amylase activities differently. The former was highest at 30°C and the latter, at 25°C. Overall, the cell-containing biocontrol treatments yielded higher activities than treatment with supernatant alone, with the exception of $\beta$-amylase at 35°C. A good compromise would appear to be treatment C+B or C at 30°C with a 14- to 16-h steeping time (see Figure 7. A and B).

The relationship between water uptake and individual enzyme levels is unclear. A higher moisture content might explain the superiority of NaOH treatment (in terms of $\alpha$- and $\beta$-amylase activities) over biocontrol or $H_2O$ treatment, but it can explain neither the temperature-related variations in enzyme levels presented here nor the superiority of 0.1% $Ca(OH)_2$ treatment over 0.2% NaOH treatment observed in chapter 1. If necessary, the moisture content can be corrected by spraying the grains during germination, but limitations to endosperm modification caused by suboptimal moisture are hard to correct later in the germination process (Palmer, 2006).

We highlight here a limitation of the use of Peleg's equation to model grain hydration. This model appears to offer a good description up to the time the grains show signs of germination, but less so thereafter. This is not really surprising, as Peleg's model is intended for modeling diffusion into a homogeneous medium. It takes into account neither the grains' internal structure (water may have successive barriers to pass, as shown by Holmbero and Hámáláinen (1997)) nor the physiological changes that occur when a plantlet emerges and begins to take up water and nutrients actively.

By combining dilute alkaline treatment with a biocontrol treatment, we have obtained malts whose quality approaches that of malts produced from NaOH-steeped grains, the most noteworthy improvement being at the level of the recorded enzyme activities. Disappointingly, we observed no synergy between the mould-reducing effects of NaOH and biocontrol treatment. In fact, the total fungal count was not quite so effectively reduced with the combined treatment as with the 16-h biocontrol treatments, but it remained at an acceptably low level.

Our results raise several questions worth answering. It would certainly be important to identify any compounds that contribute to the antifungal effects of steeping treatments based on *B. subtilis* S499 (see above). Another question is: how do the tested biocontrol treatments induce grain chitting? Some *Bacillus* species such as *B. pumilus* and *B. licheniformis* can produce gibberellic acid *(*Gutiérrez-Mañero *et al.*, 2001*)*, which can induce barley germination and affect both *α*-amylase synthesis and the activation of preformed *β*-amylase in barley. It would also be useful to understand in depth the relationship between grain chitting during steeping and the development of enzyme activities. With such knowledge gained from biocontrol experiments, it might be possible to devise new biocontrol strategies or to produce bioadditives with which to modulate the effects of dilute alkaline treatment.

### *3.5. Conclusions*

It is possible to limit mould development during red sorghum malting by means of *Bacillus-subtilis*-S499-based biocontrol treatments applied at the steeping stage. Aeration of the biocontrol steeping medium improves both the fungal-count reduction and the levels of hydrolytic enzymes in the kilned malts. Steeping in 0.2% NaOH for 8 h followed by an 8-h biocontrol treatment yields malts approaching the quality of those obtained after steeping for 16 h in dilute alkaline solution, with better mould control.

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# Chapter IV. Modelling the *α*-and *β*-amylase activities during red sorghum malting when *Bacillus subtilis*-S499-biocontrol based steeping treatment is used

## *Présentation du chapitre*

Les résultats du chapitre 3 nous ont montré qu'un trempage dans 0.2% NaOH suivi d'un retrempage dans le biocontrôle permettait d'obtenir des malts ayant des propriétés acceptables mais que les maxima pour les activités *α*- et *β*-amylases n'étaient pas atteints dans les mêmes conditions. Lorsque l'on ne connait pas de façon exacte comment varient ces deux activités amylasiques, décider de l'arrêt du maltage devient difficile. Un seul modèle a été proposé pour le suivi de l'activité *α*-amylase lors du maltage du sorgho par Egwim and Adenomon (2009). La fonction polynomiale proposée, lorsqu'elle est appliquée, donne des $R^2$ acceptables mais la décomposition de la somme des carrés des écarts montre l'existence des inadéquations significatives. Dans ce travail, nous basant sur les lois individuelles de distribution des nuages des points, des modèles ont été proposés pour les phases de synthèse de ces deux enzymes clés, à savoir l'*α*- amylase et la *β*-amylase. Les modèles proposés (modèle de Weibull à 4 paramètres pour les phases d'induction de ces 2 enzymes : *α*- et *β*-amylases) et le modèle linéaire généralisé avec liaison logarithmique pour la phase de répression de la synthèse de la *β*-amylase ont l'avantage de montrer l'effet des conditions de maltage sur les activités amylasiques.


## *Abstract*

In this work the suitability of the Weibull 4 Parameters Model (W-4-PM) to predict sorghum malt *α*-amylase activity during the enzyme induction stage of red sorghum germination has been compared with those of 2nd Order Polynomial Model (2nd O.P.M.) and General Linear Model (GLM). Results obtained showed that the W-4-PM could be used to predict *α*-amylase activity with significant goodness of fit when compared to the 2nd O.P.M. and to the GLM. The effects of steeping and germination conditions on the *β*-amylase activity had been described using 2 functions: a W-4-PM combined with a General Linear Model with Logarithm Link (GLMLL) with significant goodness. Steeping conditions (combined use of NaOH and *B. subtilis* S499) affects the synthesis capacity of grain: when *B. subtilis* culture used in the steeping step is diluted, *lnα* increases, suggesting a loss of treatment efficacy for both *α*- and *β*-amylase activities. When the *B. subtilis* culture used as starter is diluted, the treatment efficacy to develop *α*-amylase activity was lost. The germination temperature affects the amylase synthesis rate during the induction phase: the germination temperature increase is accompanied by an increase of *α*-amylase development rate and a decrease of the *β*-amylase development rate. During the repression phase of *β*-amylase synthesis, the effect of malting conditions was found to taper.


Keywords: *B. subtilis* biocontrol, amylase, Weibull 4-parameter model, red sorghum malting

## *4.1. Introduction*

The main purpose of the malting step is to favour the production of enzymes that will render the grain constituents (starch, proteins) more digestible. When *B. subtilis* is used during the steeping step, malt $\alpha$- and $\beta$-amylase activities are significantly improved compared to steeping in distilled water (see chapter 2). It can be useful, when one wants to optimize the malting process, to have a good model of their development in the course of germination. During the germination step of the malting process, malt amylase activities, reache a maximum, and then finally drop. The maltster, whose mission is to guide these transformations, must make a number of decisions, which must be the result of a compromise which is not always easy. One of the concerns of the maltster is to know exactly when the malting must be stopped. However, conditions for achieving the target values of the enzyme activities, residual grain reserves, etc. are not the same. It is therefore imperative to precisely determine the factors governing the evolution of interesting malt properties in order to allow a factual decision. In this work, the $\alpha$- and $\beta$-amylase activities are modelled and the effect of soaking and germination conditions on these two enzyme activities is presented.

For $\alpha$-amylase we have examined the performance of three models: the 2$^{nd}$ order polynomial model (2$^{nd}$ OPM) (the only model proposed to date for $\alpha$-amylase activity during sorghum malting by Egwim and Adenomon (2009)), the Weibull 4-parameter model (W-4-PM) chosen according to the individual distribution of experimental data using Minitab 16 software, and the general linear model (GLM) constructed using stepwise regression. The time course of the development of $\beta$-amylase activity during red sorghum germination is modelled using a Weibull 4-parameter Model (W-4-PM) (for the induction phase of $\beta$-amylase synthesis) and a General Linear Model with Logarithm Link (GLMLL) (for the repression phase of $\beta$-amylase synthesis).

## *4.2. Materials and methods*

### 4.1.2. Choice of variables

Malt enzymes activities during the sorghum malting process may be affected by several factors including the following: the nature of the steeping liquor (Ezeogu and Okolo, 1996; Dewar and Orovan, 1997; Beta *et al.*, 2000), the steeping temperature and time, aeration during steeping (Dewar *et al.*, 1997), the final warm water steep and air rest cycles (Ezeogu and Okolo, 1994), the steep-out moisture (Dewar *et al.*, 1997), the use of microbial starters: such as lactic acid bacteria and yeasts (Lefyedi and Taylor, 2007), germination temperature *(*Taylor and Robbins, 1993; Taylor *et al.*, 2006*)* and germination time (De Clerck, 1962, Bekele *et al.*, 2012).

Therefore in this study, the following steeping conditions were set: aeration, temperature, time, and

nature of the steeping solution for the initial 8 h steeping. The difference between steeping treatments was made by varying the dilution of the *B. subtilis* S499 culture (ln *BSP*: natural logarithm of the *B. subtilis* S499 population) used as the biocontrol during the last 8 h of steeping. For the germination conditions, two factors were manipulated: the germination temperature (*GT*) and the germination time (*GD*).

#### 4.1.3. Red sorghum malting

*B. subtilis* strain S499 was obtained from the Walloon Centre of Industrial Biology (CWBI) and grown on the Luria broth agar at 37°C for 24 h. An inoculating loopful was transferred to 100 ml of Landy broth optimized for *B. subtilis* S499 lipopeptide production, and incubated for 16 h. Finally, 10 ml was transferred to 350 ml of optimized Landy broth and incubated at 30°C (with rotary shaking at 130 rpm) for 72 h. The culture (containing approximately $10^{11}$ cells/ml) was diluted with distilled water to $10^8$ and $10^4$ cells/ml and used as the steep liquors during the biocontrol step of the steeping process (Bwanganga *et al.*, 2012).

The red sorghum cultivar used was obtained from the D.R. Congo and has been described previously in chapters 2 and 3. It contains: 3.8% ± 0.3 fat content, 2.9% ± 0.1 $\beta$ -glucans, 73.9% ± 1.9 total starch (as assayed by the Megazyme K-TSTA 07/11 method), 27.6% ± 1.4 amylose (assayed by the Megazyme K-AMYL 07/11 method), 8.1 mg Gallic Acid Equivalents per gram of dried malt (assayed using the method optimized by Georgé *et al.* (2005) without eliminating water-soluble compounds) and 0.18% Cathechin Equivalent (assayed using the modified vanillin method of Price *et al.* (1978)).

Sorghum malts were obtained by manual sorting, aerated steeping at 30°C for 8 h in 0.2% NaOH followed by 8 h resteeping in the biocontrol steep liquor (distilled water containing $10^8$, $10^4$ *B. subtilis* S499 cells (respectively for B1 and B2), distilled water alone being used as a control (C)); germination in the dark (to exclude photosynthesis) at the indicated temperature (25, 30, or 35°C) for the indicated time (6, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168 and 180 h), and kilning for 48 h at 40°C. The total malting loss (*TML*) was calculated as follows: $(M_{1000RG}-M_{1000MG})/(M_{1000RG})$; where $M_{1000RG}$ and $M_{1000MG}$ were respectively the mass (g) of 1000 raw grains and 1000 malted grains dried to a constant weight.

#### 4.1.4. Sorghum malts $\alpha$-amylase assays

Alpha-amylase assays were performed on extracts of sorghum malt flour obtained by grinding kilned malt in an IKA mill followed by sieving (mesh size: 0.5 mm). The activity was extracted and assayed using the Megazyme method (Ceralpha Method K-CERA 08/05): sodium maleate (100 mM, pH 6.0) plus $CaCl_2$ (5 mM) and sodium azide (0.02%) as the extraction buffer, azurine cross-linked amylose as

the substrate, incubation at 40°C for exactly 10 min and 2% (w/v) Trizma base as the stopping solution. The absorbance was read at 590 nm against the reaction blank.

#### 4.1.5. Sorghum malt *β*-amylase assays

Beta-amylase activities were extracted and assayed using Megazyme methods Betamyl-3 (K-BETA3 10/10). Sorghum *β*-amylase was extracted using 0.05 M Tris-HCl buffer plus 1 mM EDTA, dilution was made in 0.1 M MES buffer plus 1 mM EDTA, 1.0 mg/ml of BSA and 0.02% w/v sodium azide. The assay was performed at 40°C for exactly 10 min in a reaction mixture of p-nitrophenyl-*β*-D-maltotrioside (PNP*β*-G3) plus *β*-glucosidase (50 U) and stabilizers as the substrate. The reaction was stopped by 1% (w/v) Trizma base (approx. pH 8.5) and the absorbance was read at 400 nm against distilled water.

#### 4.1.6. Modelling the *α*-amylase activity

The first function used to represent the *α*-amylase activity data collected for the above-mentioned steeping and germination conditions was the 2nd order polynomial model as proposed by Egwim and Adenomon *(9)*.

$$AA=a+b(GD)+c(GD)^2 \qquad \text{Eq. 7}$$

where *AA* is the *α*-amylase activity. Parameters a, b, and c were fitted by stepwise regression performed with Minitab 16 software.

The second model was a classical general linear model obtained after stepwise regression of *α*-amylase experimental values using ln *BSP*, *GT* and *GD* with their 1st and 2nd order interaction.

The third model tested was the W-4 PM

$$AA = AA_0 + (AA_\omega - AA_0) \exp(-\alpha \times GD^\beta) \qquad \text{Eq. 8}$$

where $AA_0$ is the *α*-amylase activity at the start of germination and *AA*ω is the value towards which the activity tends during the induction phase of the *α*-amylase synthesis.

Eq. 7 can be written as: $\ln[\ln((AA_\omega - AA_0)/(AA - AA_0))] = \ln\alpha + \beta \ln GD$ Eq. 9

This is the equation of a straight line with slope (*β*) and x-intercept -(ln *α*)/*β*. The parameters *α* and *β* were obtained by plotting experimental data according to Eq. 9 and for each case the smallest and the highest value were first considered respectively as $AA_0$ and *AA*ω. The true values of $AA_0$ and *AA*ω were obtained using the Gauss-Newton algorithm and a convergence tolerance of 0.00001, after fixing the values of *α* and *β* equal to those obtained with experimental data (straight line of Eq. 9) using Minitab software. $AA_\# = [((AA_\omega - AA_0)/(AA - AA_0))]$ is dimensionless and tends to one at the maximum

activity. The value of $\beta$ is the expression of the speed at which the maximum activity is achieved [$\beta = \partial\ln(\ln AA_{\#})/\partial\ln GD$], and -(ln $\alpha$)/$\beta$ is the starting point, i.e., the advantage offered by the steeping treatment or the expression of the capacity of the treatment to improve $\alpha$-amylase synthesis. Minitab 16 software was used for statistical analyses: analysis of variance, goodness of fit, stepwise regression, general linear model, fitted line plot and scatterplot.

#### 4.1.7. Modelling the $\beta$-amylase

The $\beta$-amylase activity (*BA*) has been modelled as a function of steeping treatment (C, B1 and B2: the natural logarithm of the *B. subtilis* population was used as a factor), the germination time (*GD*) and the germination temperature (*GT*).

Two models have been chosen among those proposed by Minitab 16 software using quality tools for the identification of the individual distribution of experimental data:

1. A W-4-PM:

$$BA = BA_0 + (BA\omega - BA_0)\times\exp(-\alpha\times GD^{\beta}) \quad \text{Eq. 10}$$

for the induction phase of the synthesis of $\beta$-amylase activity, i.e., before obtaining the maximum for this activity.

2. A GLMLL:

$$BA = \exp(u+vGD) \quad \text{Eq. 11}$$

for the repression phase of the synthesis of $\beta$-amylase activity, i.e., after obtaining the maximum for this activity.

From the Eq. 12 we obtain:

$$(BA-BA_0)/(BA_{\omega}-BA_0) = \exp(-\alpha\times GD^{\beta}) \quad \text{Eq. 12}$$

Eq. 14 can also be written as:

$$\ln[\ln((BA_{\omega}-BA_0)/(BA-BA_0))] = \ln\alpha+\beta\ln GD \quad \text{Eq. 13}$$

Where $BA_0$ and is the initial $\beta$-amylase activity and $BA\omega$ the maximal value towards which the $\beta$-amylase activity tends during the induction phase of the $\beta$-amylase synthesis.

Eq. 15 was obtained by plotting experimental data and is a line with slope is ($\beta$) and x-intercept -(ln $\alpha$)/$\beta$. The variable $BA_{\#} = [((BA_{\omega}-BA_0)/(BA-BA_0))]$ is dimensionless and measures the effort remaining prior to achieving the maximum activity. $\beta$ is the expression of the speed at which the maximum activity is achieved [$\beta = \partial\ln(\ln BA\#)/\partial\ln GD$], and -(ln $\alpha$)/$\beta$ is the starting point, i.e., the advantage offered by the treatment or the expression of the capacity of the treatment to improve $\beta$-amylase synthesis. W-4 PM was obtained using the Non Linear function of Minitab 16 software. $BA_0$ and $BA\omega$ used in the final model were obtained using Minitab 16 software: a maximum of 200 iterations was performed using the Gauss-Newton algorithm with a convergence tolerance of 0.00001 on the experimental data, and fixing the values of $\alpha$ and $\beta$ equal to those obtained with experimental data.

For the regression phase, ln*BA* (experimental data) was plotted versus germination time (*GD*) to obtain a straight line as follows:

$$\ln BA = u + vGD \quad \text{Eq. 14}$$

u and v were obtained using experimental data. "v" is the expression of the *β*-amylase repression speed ($v=\partial \ln BA/\partial GD$) and –(u/v) the time after which ln *BA* = 0 during the repression phase of the *β*-amylase synthesis.

### *4.3. Results*

#### 4.3.1. Modelling *α*-amylase activity during germination

Three-way ANOVA was applied to the experimental data obtained with different steeping treatments after different germination times and temperatures; and results are presented in Table 10. All of the main effects and their 1st and 2nd order interactions were significant ($p<0.05$).

**Table 10. Three-way Analysis of Variance for *α*-amylase activity**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| *GT* | 2 | 77733 | 38866 | 19755.61 | 0.000 |
| ln *BSP* | 2 | 88332 | 44166 | 22449.49 | 0.000 |
| *GD* | 9 | 2075955 | 230662 | 117244.42 | 0.000 |
| *GT**ln *BSP* | 4 | 1359 | 340 | 172.75 | 0.000 |
| *GT*GD* | 18 | 183965 | 10220 | 5194.92 | 0.000 |
| ln *BSP*GD* | 18 | 48361 | 2687 | 1365.64 | 0.000 |
| *GT**ln *BSP*GD* | 36 | 19158 | 532 | 270.49 | 0.000 |
| Error | 180 | 354 | 2 | | |
| Total | 269 | 2495216 | | | |
| S = 1.40263 | R-Sq = 99.99% | | R-Sq(adj) = 99.98% | | |

*GT*: germination temperature, *BSP*: *B. subtilis* population, *GD*: germination time.

The kinetic parameters of the 2nd OPM and W-4-PM obtained with the experimental data are presented in Table 11. The equation of the GLM obtained using Minitab software after stepwise regression was as follow:

$$AA = -9.97 + 0.107\, GT*GD + 0.174 \ln BSP*GD - 0.00455\, GT*\ln BSP*GD - 0.00967 GD^2 \quad \text{Eq. 15}$$

The regression analysis of the 3 models is presented as supplementary data in Table 17-20. Predicted and experimental data scatterplots are presented in Figure 12.

**Table 11. Kinetic parameters of W-4-PM and 2nd OPM**

| Germination Temperature (°C) | Steeping Treatment | W-4-PM | | | 2nd OPM | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ln $\alpha$ | $\beta$ | Lack of fit (p-value) | a | b | c | R-sq (%) | R-sq(adj) | Lack of fit (p-value) |
| **25** | C | 14.4032972 | -3.40443 | 0.696 | 23.3 | -0.342 | 0.0229 | 96.4 | 96.2 | 0.000 |
| | B1 | 12.7068479 | -3.32415 | 0.507 | -21.2 | 3.72 | -0.00810 | 93.6 | 93.2 | 0.000 |
| | B2 | 13.8054602 | -3.39737 | 0.600 | 4.3 | 1.20 | 0.0123 | 94.4 | 94.0 | 0.000 |
| **30** | C | 14.2209757 | -3.53351 | 0.423 | -2.2 | 1.63 | 0.00939 | 93.8 | 93.4 | 0.000 |
| | B1 | 12.7938593 | -3.5137 | 0.713 | -36.5 | 5.55 | -0.0239 | 94.4 | 94.0 | 0.000 |
| | B2 | 13.3374748 | -3.45218 | 0.417 | -20.9 | 3.40 | -0.00485 | 93.5 | 93.0 | 0.000 |
| **35** | C | 14.0778748 | -3.4764 | 0.000 | -45.3 | 6.22 | -0.0370 | 87.3 | 86.3 | 0.000 |
| | B1 | 12.6115378 | -3.79624 | 0.125 | -52.6 | 8.74 | -0.0589 | 94.8 | 94.4 | 0.000 |
| | B2 | 12.9715405 | -3.6857 | 0.591 | -53.3 | 7.57 | -0.0471 | 91.7 | 91.1 | 0.000 |

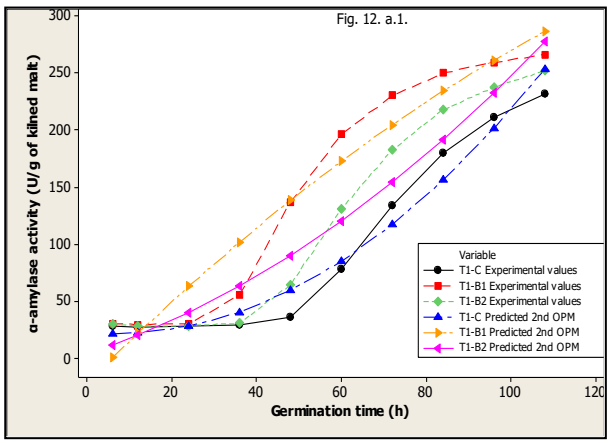
Fig. 12. a.1.
α-amylase activity (U/g of kilned malt)
Germination time (h)
Variable
T1-C Experimental values
T1-B1 Experimental values
T1-B2 Experimental values
T1-C Predicted 2nd OPM
T1-B1 Predicted 2nd OPM
T1-B2 Predicted 2nd OPM

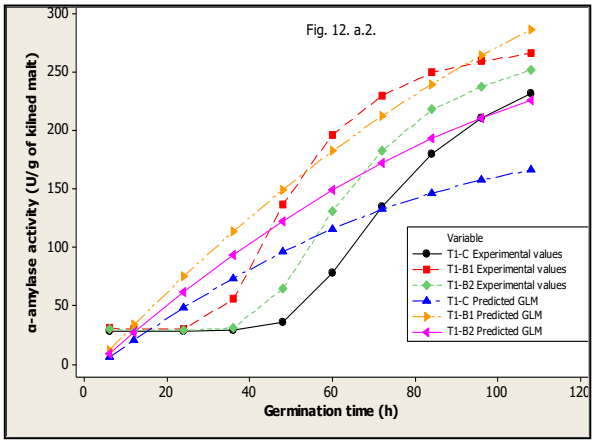
Fig. 12. a.2.
α-amylase activity (U/g of kilned malt)
Germination time (h)
Variable
T1-C Experimental values
T1-B1 Experimental values
T1-B2 Experimental values
T1-C Predicted GLM
T1-B1 Predicted GLM
T1-B2 Predicted GLM

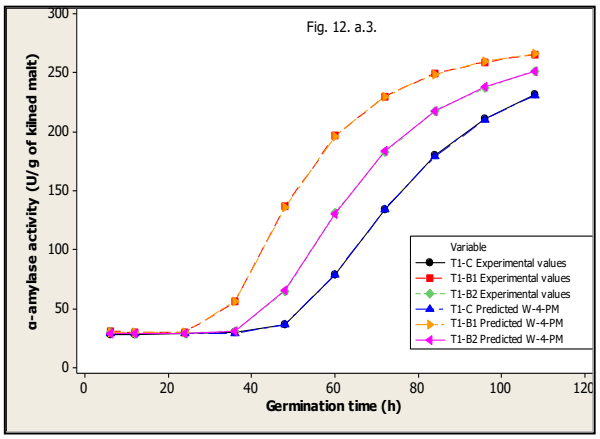
Fig. 12. a.3.
α-amylase activity (U/g of kilned malt)
Germination time (h)
Variable
T1-C Experimental values
T1-B1 Experimental values
T1-B2 Experimental values
T1-C Predicted W-4-PM
T1-B1 Predicted W-4-PM
T1-B2 Predicted W-4-PM

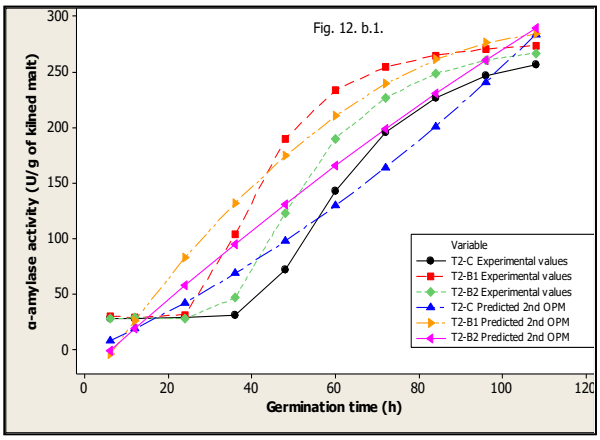
Fig. 12. b.1.
α-amylase activity (U/g of kilned malt)
Germination time (h)
Variable
T2-C Experimental values
T2-B1 Experimental values
T2-B2 Experimental values
T2-C Predicted 2nd OPM
T2-B1 Predicted 2nd OPM
T2-B2 Predicted 2nd OPM

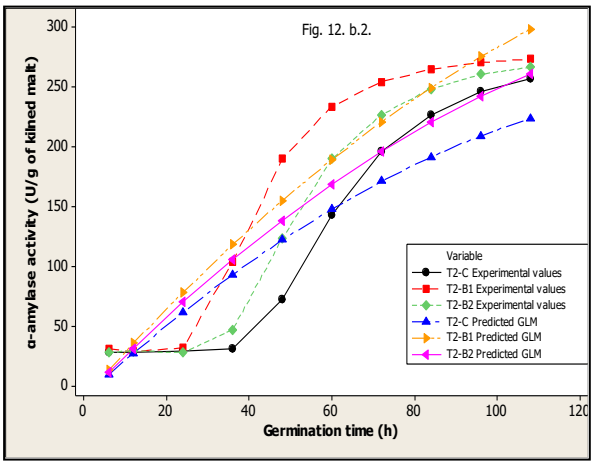
Fig. 12. b.2.
α-amylase activity (U/g of kilned malt)
Germination time (h)
Variable
T2-C Experimental values
T2-B1 Experimental values
T2-B2 Experimental values
T2-C Predicted GLM
T2-B1 Predicted GLM
T2-B2 Predicted GLM

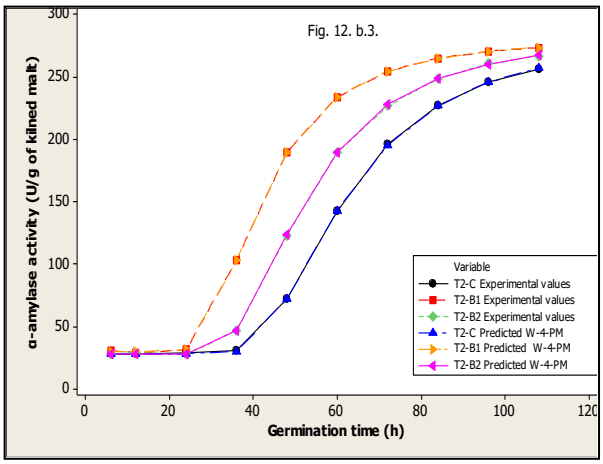
Fig. 12. b.3.
α-amylase activity (U/g of kilned malt)
Germination time (h)
Variable
T2-C Experimental values
T2-B1 Experimental values
T2-B2 Experimental values
T2-C Predicted W-4-PM
T2-B1 Predicted W-4-PM
T2-B2 Predicted W-4-PM

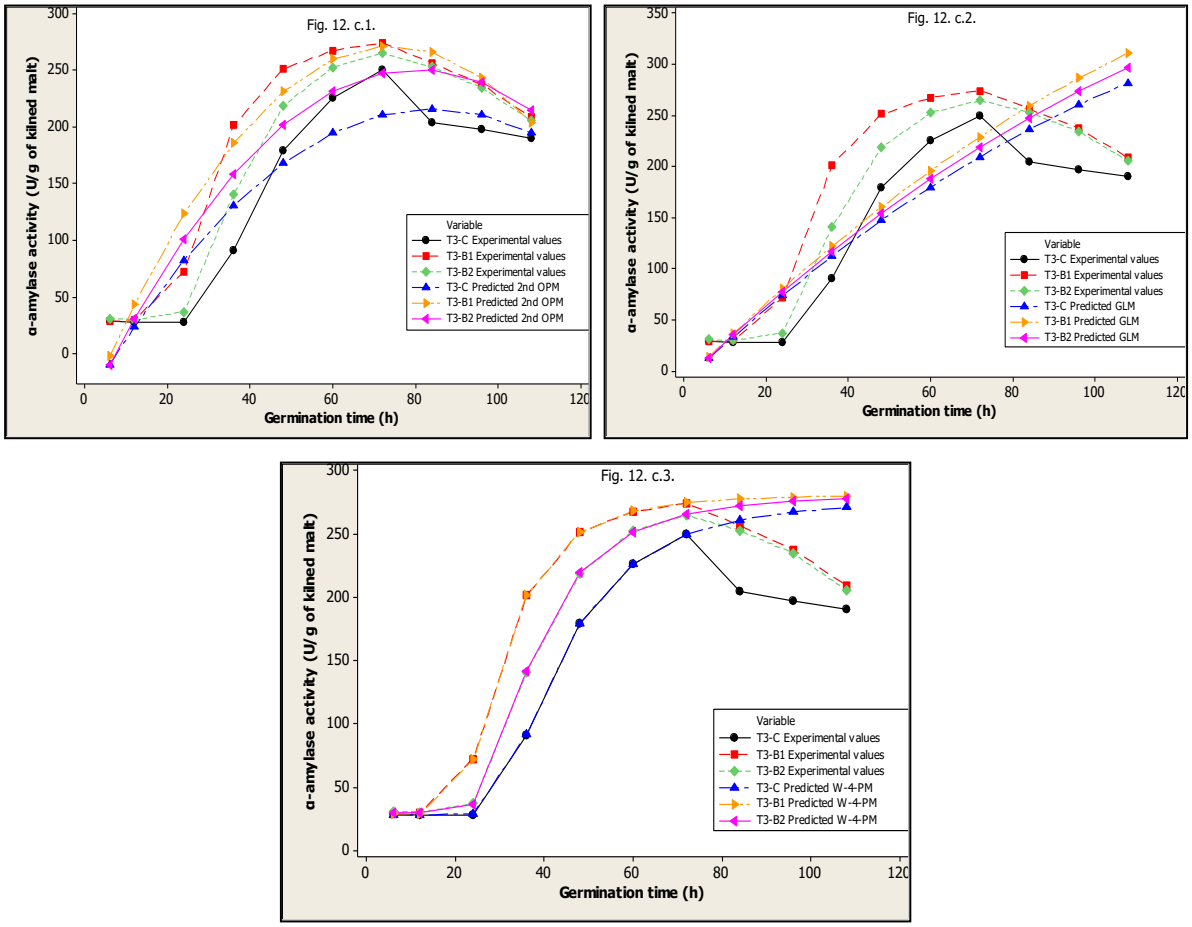


**Figure 12. Experimental and 2nd OPM, GLM and W-4-PM predicted data (at different temperatures: T1=25°C, T2=30°C and T3=35°C for different steeping treatments: C, B1 and B2)**

❖ ***The effect of germination temperature and steeping treatment on α-amylase activity***

The question to be asked is: how does each of the malting factors affect $\alpha$-amylase synthesis?

To answer this question, two approximations were made according to the results obtained with the experimental data (Table 11):

1. Parameter $\beta$ varies very little with steeping treatments for a given temperature so that it can be considered as a constant regardless of the steeping treatment. This parameter is a function of germination temperature and the effect of germination temperature on $\beta$ is shown in Figure 13.
2. $ln\ \alpha$ varies minimally for a given treatment regardless of the temperature so that it can be considered as a constant in this study. The effect of steeping treatment on this parameter is presented in Figure 14.

So, from Eq. 9 to be combined with the regression equations of Figure 13 and 14 we obtained the following model:

$$\ln(\ln AA_{\#}) = 14.20 - 0.08306 \ln(BSP) - (0.02775\, GT + 2.677) \ln GD \qquad \text{Eq. 16}$$

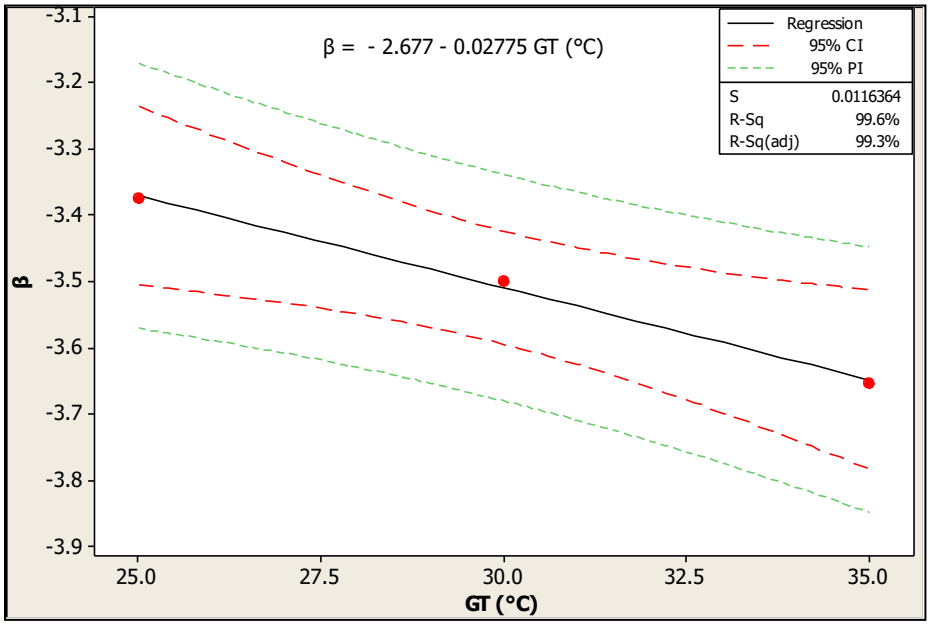


**Figure 13. Effect of germination temperature on the rate of *α*-amylase synthesis**

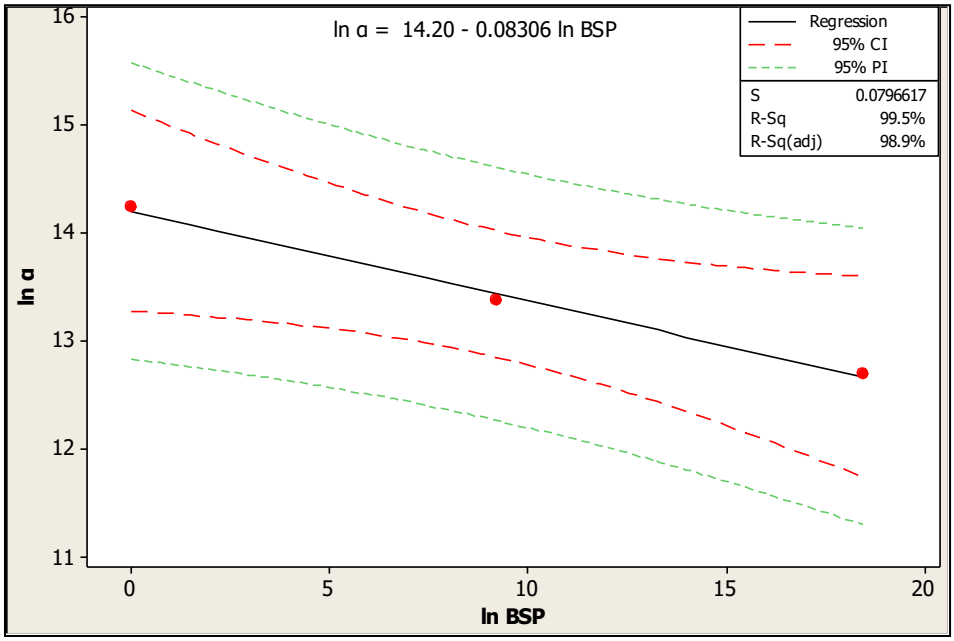


**Figure 14. Effect of steeping treatment on the capacity of the *α*-amylase synthesis**

❖ ***Goodness of fit***

The goodness of fit of all 3 models obtained using the decomposition of the residual error is presented as supplemental data Table 17-20, for the 2nd OPM, W-4-PM and GLM. The mean square error [MSE = $n^{-1}$(SSE)] and its root (RMSE) are presented in Table 12. SSE (sum of square error) = ∑(experimental data-predicted data)$^2$.

**Table 12. Goodness of fit for *α*-amylase activity**

| | GLM | 2ndOPM | W-4-PM |
|---|---|---|---|
| **MSE** | 1568.49096 | 570.669896 | 0.90030675 |
| **RMSE** | 39.6041786 | 23.8886981 | 0.94884496 |

When we consider only the values of R-sq (adj) obtained: 86.3 - 96.2% for the 2nd OPM and 82.8% for the GLM one may be tempted to consider these two models as good fits. However, the decomposition of the residual error associated to the fits (Table 17-20) and the RMSE (Table 12) clearly showed that neither the 2nd OPM nor the GLM gave good fits (the lack of fit being significant for these two models). From this point of view, the W-4-PM can be considered as a good fit: RMSE = 0.95 and for all fits obtained, except for the steeping in control and germination at 35°C, where the lack of fit was not significant.

### 4.3.2. Modelling *β*-amylase activity during germination

Three-way analysis of variance (ANOVA) was performed using experimental data and results are presented in Table 21. All factors [steeping treatment (*ST*), *GT* and *GD*] and their 1st and 2nd order interactions effects are significant at $\alpha < 0.05\%$.

**Table 13. Three-way ANOVA for *β*-amylase activity**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| *ST* | 2 | 9159,12 | 4579,56 | 4922,69 | 0,000 |
| *GT* | 2 | 3216,64 | 1608,32 | 1728,82 | 0,000 |
| *GD* | 15 | 57842,77 | 3856,18 | 4145,12 | 0,000 |
| *ST*GT* | 4 | 487,18 | 121,79 | 130,92 | 0,000 |
| *ST*GD* | 30 | 5197,47 | 173,25 | 186,23 | 0,000 |
| *GT*GD* | 30 | 3146,04 | 104,87 | 112,73 | 0,000 |
| *ST*GT*GD* | 60 | 1159,17 | 19,32 | 20,77 | 0,000 |
| Error | 288 | 267,93 | 0,93 | | |
| Total | 431 | 80476,32 | | | |
| | S = 0,964519 | R-Sq = 99,67% | R-Sq(adj) = 99,50% | | |

Kinetic parameters of W-4-PM and those of the GLMLL were calculated as describe in the materials and methods section and the results are presented in Table 22.

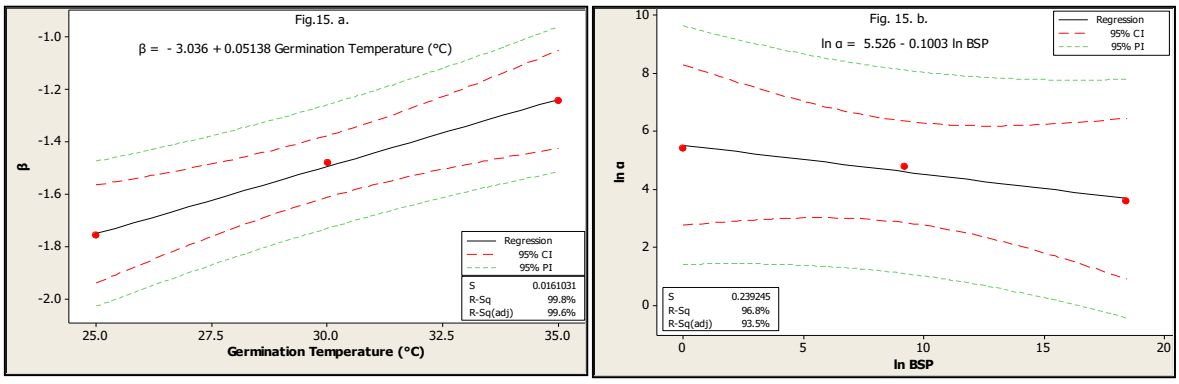


**Figure 15. Effect of Germination Temperature (°C) on *α* and *β***

Two approximations can be made to the values presented in Table 22 for the W-4-PM:

1. $\beta$ does not depend on steeping treatment and is a function of temperature, values of a given temperature can be regarded as constant;
2. $\alpha$ does not depend on temperature, and is therefore a function of the steeping treatment, values for a given treatment can be considered constant.

This determines, for the W-4-PM, the relationship between the rate parameter $\beta$ and the germination temperature and the relationship between the capacity parameter ($\ln\alpha$) and the steeping treatment presented in Figure 15 (a and b). The final W-4-PM has been obtained by using the relations obtained with Figure 15 (a and b) and the Eq. 15 and can be written as follows:

$$\ln[\ln(BA\#)] = 5.526 - 0.1003 \ln BSP - (3.036 - 0.05138 \times GT) \ln GD \qquad \text{Eq. 17}$$

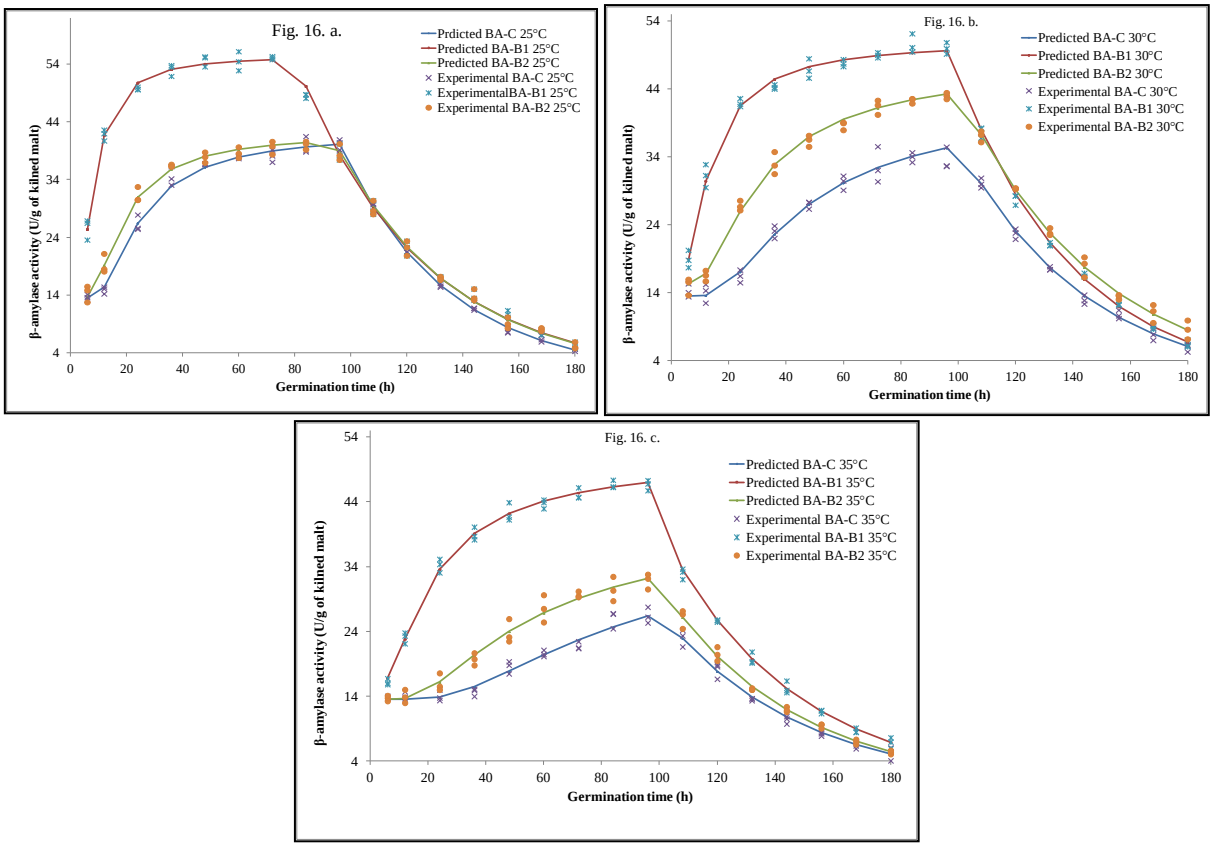


**Figure 16. Predicted and experimental data for $\beta$-amylase activity**

**Table 14. Experimental kinetic parameters**

| Steeping treatments | Weibull 4-Parameter Model | | | | | | General Linear Model with Logarithm Link | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 25°C | | 30°C | | 35°C | | 25°C | | 30°C | | 35°C | |
| | *α* | *β* | *α* | *β* | *α* | *β* | u | v | u | v | u | v |
| **C** | 226.855 | -1.7815 | 227.787 | -1.478 | 228.785 | -1.2405 | 6.1988 | -0.0261 | 5.8003 | -0.0222 | 5.4004 | -0.021 |
| **B1** | 34.951 | -1.762 | 35.8966 | -1.4887 | 36.8081 | -1.2512 | 5.8228 | -0.0227 | 6.2400 | -0.0241 | 5.8886 | -0.022 |
| **B2** | 119.5117 | -1.7296 | 122.6649 | -1.476467 | 121.3947 | -1.239867 | 5.8915 | -0.0232 | 5.8415 | -0.0206 | 5.6302 | -0.0219 |

With values obtained in Table 22 for the GLMLL, the repression phase can be considered as not greatly affected by *ST* and *GT*, so one function can approximately be used to describe this germination phase:

$BA = \exp[ (5.857\pm0.19) - (0.023\pm0.001)GD]$ Eq. 18

The intercept between Eq. 17 and Eq. 18 corresponds approximately to the maximal value of *β*-amylase activity.

The root mean square error (RMSE) of the fit was 0.93 U/g and was calculated as follows:

$RMSE = [(1/n)\sum(\text{Predicted value-Experimental value})^2]^{1/2}$ Eq. 19

The residual analysis of these combined models (residual error vs fitted values) is presented in Figure 17. The random pattern of the residual error indicates that the model provides a decent fit to the data. Predicted and experimental data scatterplots are presented in Figure 16.

Parallel to the monitoring of *β*-amylase activity, the evolution of the residual starches content (*RS*) for a single treatment B1 was determined in order to elucidate what happens in the endosperm and the total malting loss (*TML*) - which is a major concern during malting sorghum (Nout and Davies, 1982; Okon and Uwaifo, 1985) - to get an idea of the seed growth (living part of the grain). The results for these two parameters are presented as supplemental data in Figure 18.

These results show that the residual starch content decreases when the total malting loss increases and this increase is elevated when the germination temperature is high.

It can be shown that beyond 48 h of germination, a convex asymptotic model fits well with the *TML*. So the total malting loss function can be written as:

$TML = \pi-\rho\times\exp(\sigma GD)$ Eq. 20

and hence as:

$\ln[1/(\pi-TML)] = \ln\rho+\sigma GD$ Eq. 21

The parameters $\pi$, $\rho$ and $\sigma$ can thus be obtained by tracing the straight line of Eq. 21 assuming $\pi$=100%. The results obtained using Minitab 16 software are presented in Table 23.

**Table 15. Total Malting Loss as affected by germination time and temperature**

| | | | | | |
|---|---|---|---|---|---|
| ***1st model regression analysis: TML* (25°C) = 100 - 101.465 exp(-0.00355203 *GD*)** | | | | | |
| **Source** | DF | SS | MS | F | P |
| **Error** | 16 | 17.7695 | 1.11059 | | |
| **Lack of fit** | 4 | 1.6670 | 0.41676 | 0.31 | 0.865 |
| **Pure Error** | 12 | 16.1024 | 1.34187 | | |
| ***2nd model regression analysis: TML*(30°C) = 100 - 99.0872 exp(-0.00375068 *GD*)** | | | | | |
| **Source** | DF | SS | MS | F | P |
| **Error** | 16 | 16.3518 | 1.02199 | | |
| **Lack of fit** | 4 | 2.3095 | 0.57737 | 0.49 | 0.741 |
| **Pure Error** | 12 | 14.0424 | 1.17020 | | |
| ***3rd model regression analysis: TML*-35°C = 100 - 102.67 exp(-0.00488772 *GD*)** | | | | | |
| **Source** | DF | SS | MS | F | P |
| **Error** | 16 | 19.0813 | 1.19258 | | |
| **Lack of fit** | 4 | 1.7553 | 0.43882 | 0.30 | 0.870 |
| **Pure Error** | 12 | 17.3260 | 1.44384 | | |

As shown in the model equations (Table 23), when the approximation was made of equal lnρ for all temperatures, a non-linear dependence between σ and germination temperature can be shown.

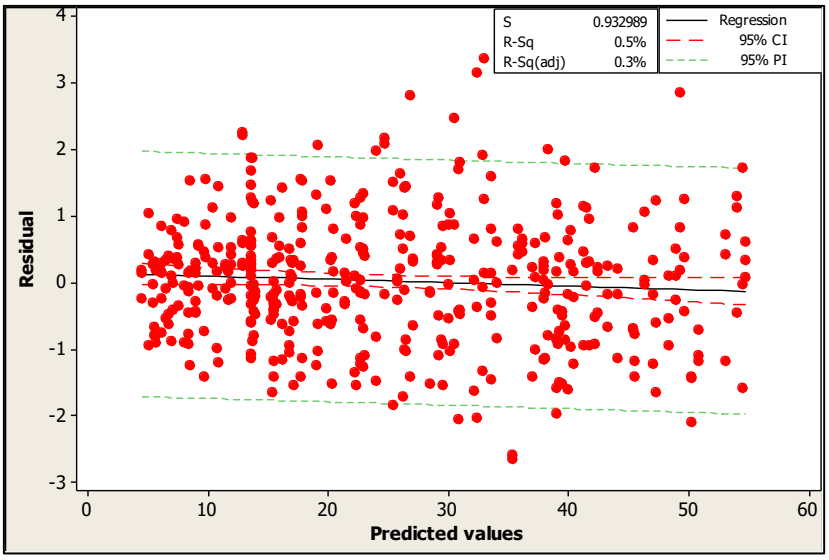


**Figure 17. Residual vs fitted values**

**Table 16. General Linear Model Stepwise (forward+backward) regression for *α*-amylase activity**

| Step | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Constant | 11.028 | 9.802 | 7.672 | -9.971 | -22.705 | -22.705 | -202.3 | -216.5 |
| *GT***GD* | 0.0813 | 0.0702 | 0.0720 | 0.1067 | 0.0592 | 0.0458 | -0.034 | -0.034 |
| T-Value | 30.29 | 24.43 | 24.71 | 12.95 | 4.14 | 5.06 | -2.07 | -2.09 |
| P-Value | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.040 | 0.037 |
| ln*BSP***GD* | | 0.0385 | 0.1055 | 0.1744 | 0.0799 | 0.0363 | 0.0363 | 0.0159 |
| T-Value | | 7.38 | 4.25 | 6.11 | 2.19 | 7.39 | 7.82 | 1.81 |
| P-Value | | 0.000 | 0.000 | 0.000 | 0.029 | 0.000 | 0.000 | 0.071 |
| *GT**ln*BSP***GD* | | | -0.00229 | -0.00455 | -0.00145 | | | |
| T-Value | | | -2.75 | -4.79 | -1.21 | | | |
| P-Value | | | 0.006 | 0.000 | 0.229 | | | |
| *GD*^2 | | | | -0.0097 | -0.0150 | -0.0150 | -0.0150 | -0.0150 |
| T-Value | | | | -4.47 | -6.03 | -6.03 | -6.38 | -6.46 |
| P-Value | | | | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| *GD* | | | | | 2.08 | 2.48 | 4.87 | 5.05 |
| T-Value | | | | | 4.01 | 6.23 | 8.66 | 9.04 |
| P-Value | | | | | 0.000 | 0.000 | 0.000 | 0.000 |
| *GT* | | | | | | | 6.0 | 6.0 |
| T-Value | | | | | | | 5.72 | 5.79 |
| P-Value | | | | | | | 0.000 | 0.000 |
| Ln*BSP* | | | | | | | | 1.54 |
| T-Value | | | | | | | | 2.74 |
| P-Value | | | | | | | | 0.007 |
| S | 45.9 | 41.9 | 41.4 | 40.0 | 38.9 | 38.9 | 36.8 | 36.3 |
| R-Sq | 77.40 | 81.23 | 81.75 | 83.03 | 84.00 | 83.92 | 85.69 | 86.09 |
| R-Sq(adj) | 77.31 | 81.09 | 81.54 | 82.77 | 83.70 | 83.67 | 85.42 | 85.77 |
| Mallows Cp | 159.0 | 89.0 | 81.2 | 59.1 | 42.8 | 42.4 | 11.1 | 5.6 |

**Table 17. 2nd OPM regression analysis [Germination temperature = 25°C] for *α*-amylase activity**

***AA* (for T1, C) = 23.3 - 0.342 *GD* + 0.0229 *GD*²**

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | 23.285 | 8.114 | 2.87 | 0.008 |
| *GD* | -0.3424 | 0.3474 | -0.99 | 0.333 |
| *GD*^2 | 0.022885 | 0.003018 | 7.58 | 0.000 |

S = 15.7074 R-Sq = 96.4% R-Sq(adj) = 96.2%

Analysis of Variance

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 2 | 179331 | 89665 | 363.43 | 0.000 |
| Residual Error | 27 | 6661 | 247 | | |
| Lack of Fit | 7 | 6647 | 950 | 1319.73 | 0.000 |
| Pure Error | 20 | 14 | 1 | | |
| Total | 29 | 185992 | | | |

***AA* (for T1, B1) = - 21.2 + 3.72 *GD* - 0.00810 *GD*²**

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | -21.19 | 13.43 | -1.58 | 0.126 |
| *GD* | 3.7164 | 0.5752 | 6.46 | 0.000 |
| *GD*^2 | -0.008097 | 0.004998 | -1.62 | 0.117 |

S = 26.0080 R-Sq = 93.6% R-Sq(adj) = 93.2%

Analysis of Variance

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 2 | 269210 | 134605 | 199.00 | 0.000 |
| Residual Error | 27 | 18263 | 676 | | |
| Lack of Fit | 7 | 18248 | 2607 | 3343.61 | 0.000 |
| Pure Error | 20 | 16 | 1 | | |
| Total | 29 | 287473 | | | |

***AA* (for T1, B2) = 4.3 + 1.20 *GD* + 0.0123 *GD*²**

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | 4.35 | 11.54 | 0.38 | 0.709 |
| *GD* | 1.2018 | 0.4939 | 2.43 | 0.022 |
| *GD*^2 | 0.012288 | 0.004291 | 2.86 | 0.008 |

S = 22.3315 R-Sq = 94.4% R-Sq(adj) = 94.0%

Analysis of Variance

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 2 | 227745 | 113872 | 228.34 | 0.000 |
| Residual Error | 27 | 13465 | 499 | | |
| Lack of Fit | 7 | 13447 | 1921 | 2155.67 | 0.000 |
| Pure Error | 20 | 18 | 1 | | |
| Total | 29 | 241210 | | | |

**Table 18. 2nd OPM regression analysis [Germination temperature = 30°C] for *α*-amylase activity**

**AA (for T2, C) = - 2.2 + 1.63 *GD* + 0.00939 $GD^2$**

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | -2.19 | 12.62 | -0.17 | 0.863 |
| *GD* | 1.6345 | 0.5404 | 3.02 | 0.005 |
| *GD*^2 | 0.009394 | 0.004695 | 2.00 | 0.056 |

S = 24.4324 R-Sq = 93.8% R-Sq(adj) = 93.4%

Analysis of Variance

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 2 | 245571 | 122786 | 205.69 | 0.000 |
| Residual Error | 27 | 16117 | 597 | | |
| Lack of Fit | 7 | 16097 | 2300 | 2249.21 | 0.000 |
| Pure Error | 20 | 20 | 1 | | |
| Total | 29 | 261689 | | | |

**AA (for T2, B1) = - 36.5 + 5.55 *GD* - 0.0239 $GD^2$**

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | -36.47 | 13.16 | -2.77 | 0.010 |
| *GD* | 5.5480 | 0.5633 | 9.85 | 0.000 |
| *GD*^2 | -0.023936 | 0.004894 | -4.89 | 0.000 |

S = 25.4700 R-Sq = 94.4% R-Sq(adj) = 94.0%

Analysis of Variance

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 2 | 295351 | 147676 | 227.64 | 0.000 |
| Residual Error | 27 | 17515 | 649 | | |
| Lack of Fit | 7 | 17489 | 2498 | 1867.98 | 0.000 |
| Pure Error | 20 | 27 | 1 | | |
| Total | 29 | 312866 | | | |

**AA (for T2, B2) = - 20.9 + 3.40 *GD* - 0.00485 $GD^2$**

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | -20.93 | 13.82 | -1.51 | 0.142 |
| *GD* | 3.3982 | 0.5918 | 5.74 | 0.000 |
| *GD*^2 | -0.004849 | 0.005142 | -0.94 | 0.354 |

S = 26.7602 R-Sq = 93.5% R-Sq(adj) = 93.0%

Analysis of Variance

| | | | | | |
|---|---|---|---|---|---|
| Regression | 2 | 276520 | 138260 | 193.07 | 0.000 |
| Residual Error | 27 | 19335 | 716 | | |
| Lack of Fit | 7 | 19312 | 2759 | 2401.37 | 0.000 |
| Pure Error | 20 | 23 | 1 | | |
| Total | 29 | 295855 | | | |

**Table 19. 2nd OPM regression analysis [Germination temperature = 35°C] for *α*-amylase activity**

***AA* (for T3, C) = - 45.3 + 6.22 *GD* - 0.0370 $GD^2$**

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | -45.34 | 16.34 | -2.77 | 0.010 |
| *GD* | 6.2175 | 0.6998 | 8.89 | 0.000 |
| *GD*^2 | -0.036977 | 0.006080 | -6.08 | 0.000 |

S = 31.6398 R-Sq = 87.3% R-Sq(adj) = 86.3%

Analysis of Variance

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 2 | 184974 | 92487 | 92.39 | 0.000 |
| Residual Error | 27 | 27029 | 1001 | | |
| Lack of Fit | 7 | 26947 | 3850 | 940.58 | 0.000 |
| Pure Error | 20 | 82 | 4 | | |
| Total | 29 | 212003 | | | |

***AA* (for T3, B1) = - 52.6 + 8.74 *GD* - 0.0589 $GD^2$**

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | -52.57 | 11.72 | -4.49 | 0.000 |
| *GD* | 8.7384 | 0.5017 | 17.42 | 0.000 |
| *GD*^2 | -0.058856 | 0.004359 | -13.50 | 0.000 |

S = 22.6853 R-Sq = 94.8% R-Sq(adj) = 94.4%

Analysis of Variance

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 2 | 253300 | 126650 | 246.10 | 0.000 |
| Residual Error | 27 | 13895 | 515 | | |
| Lack of Fit | 7 | 13838 | 1977 | 693.65 | 0.000 |
| Pure Error | 20 | 57 | 3 | | |
| Total | 29 | 267194 | | | |

***AA* (for T3, B2) = - 53.3 + 7.57 *GD* - 0.0471 $GD^2$**

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | -53.28 | 14.67 | -3.63 | 0.001 |
| *GD* | 7.5719 | 0.6283 | 12.05 | 0.000 |
| *GD*^2 | -0.047090 | 0.005459 | -8.63 | 0.000 |

S = 28.4078 R-Sq = 91.7% R-Sq(adj) = 91.1%

Analysis of Variance

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 2 | 241721 | 120860 | 149.76 | 0.000 |
| Residual Error | 27 | 21789 | 807 | | |
| Lack of Fit | 7 | 21692 | 3099 | 637.03 | 0.000 |
| Pure Error | 20 | 97 | 5 | | |
| Total | 29 | 263510 | | | |

**Table 20. W-4-PM regression analysis [Germination temperature = 25°C] for $\alpha$-amylase activity**

$$AA \text{ (for T1, C)} = 28.8 + 251.8 * \exp(-1.831*10^{6} * GD^{-3.40443})$$

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Error | 22 | 62.1085 | 2.82311 | | |
| Lack of fit | 6 | 12.0526 | 2.00877 | 0.64 | 0.696 |
| Pure Error | 16 | 50.0559 | 3.12850 | | |

$$AA \text{ (for T1, B1)} = 29.8 + 250.5 * \exp(-333540 * GD^{-3.32415})$$

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Residual Error | 22 | 84.0109 | 3.81868 | | |
| Lack of fit | 6 | 21.5233 | 3.58722 | 0.92 | 0.507 |
| Pure Error | 16 | 62.4876 | 3.90548 | | |

$$AA \text{ (for T1, B2)} = 29.1 + 251.6 * \exp(-997895 * GD^{-3.39737})$$

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Residual Error | 22 | 64.8472 | 2.94760 | | |
| Lack of fit | 6 | 14.6137 | 2.43562 | 0.78 | 0.600 |
| Pure Error | 16 | 50.2335 | 3.13959 | | |

**Table 21. W-4-PM regression analysis [Germination temperature = 30°C] for $\alpha$-amylase activity**

$$AA \text{ (for T2, C)} = 28.2 + 252.8 * \exp(-1520390 * GD^{-3.53351})$$

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Residual Error | 22 | 48.9772 | 2.22624 | | |
| Lack of fit | 6 | 13.9662 | 2.32770 | 1.06 | 0.423 |
| Pure Error | 16 | 35.0110 | 2.18819 | | |

$$AA \text{ (for T2, B1)} = 29.8 + 250.0 * \exp(-361059 * GD^{-3.5137})$$

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Residual Error | 22 | 27.8758 | 1.26708 | | |
| Lack of fit | 6 | 5.2512 | 0.87519 | 0.62 | 0.713 |
| Pure Error | 16 | 22.6246 | 1.41404 | | |

$$AA \text{ (for T2, B2)} = 28.1 + 253.3 * \exp(-617336 * GD^{-3.45218})$$

| Source | DF | S | MS | F | P |
|---|---|---|---|---|---|
| Residual Error | 22 | 187.990 | 8.54500 | | |
| Lack of fit | 6 | 54.066 | 9.01096 | 1.08 | 0.417 |
| Pure Error | 16 | 133.924 | 8.37026 | | |

**Table 22. W-4-PM regression analysis [Germination temperature = 35°C] for $\alpha$-amylase activity**

**$AA$ (for T3, C) = 28.1 + 250.3 * exp (-353576 * $GD^{-3.4764}$)**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Residual Error | 13 | 748.140 | 57.549 | | |
| Lack of fit | 3 | 719.937 | 239.979 | 85.09 | 0.000 |
| Pure Error | 10 | 28.203 | 2.820 | | |

**$AA$ (for T3, B1) = 28.8 + 252.6 * exp (-307238 * $GD^{-3.79624}$)**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Residual Error | 13 | 55.5943 | 4.27648 | | |
| Lack of fit | 3 | 23.4807 | 7.82691 | 2.44 | 0.125 |
| Pure Error | 10 | 32.1135 | 3.21135 | | |

**$AA$ (for T3, B2) = 29.7 + 251.2 * exp (-446978 * $GD^{-3.68857}$)**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Residual Error | 13 | 48.1187 | 3.70144 | | |
| Lack of fit | 3 | 8.0191 | 2.67303 | 0.67 | 0.591 |
| Pure Error | 10 | 40.0996 | 4.00996 | | |

**Table 23. GLM regression analysis for $\alpha$-amylase activity**

The regression equation is

**$AA$ = - 9.97 + 0.107 $GT$*$GD$ + 0.174 ln$BSP$*$GD$ - 0.00455 $GT$*ln$BSP$*$GD$ - 0.00967 $GD^2$**

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | -9.971 | 6.060 | -1.65 | 0.101 |
| *GT*GD* | 0.106678 | 0.008237 | 12.95 | 0.000 |
| ln*BSP*GD* | 0.17443 | 0.02853 | 6.11 | 0.000 |
| *GT**ln*BSP*GD* | -0.0045463 | 0.0009484 | -4.79 | 0.000 |
| *GD*^2 | -0.009667 | 0.002161 | -4.47 | 0.000 |

S = 39.9741  R-Sq = 83.0%  R-Sq(adj) = 82.8%

Analysis of Variance

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 4 | 2071765 | 517941 | 324.13 | 0.000 |
| Residual Error | 265 | 423451 | 1598 | | |
| Lack of Fit | 85 | 423097 | 4978 | 2530.10 | 0.000 |
| Pure Error | 180 | 354 | 2 | | |
| Total | 269 | 2495216 | | | |

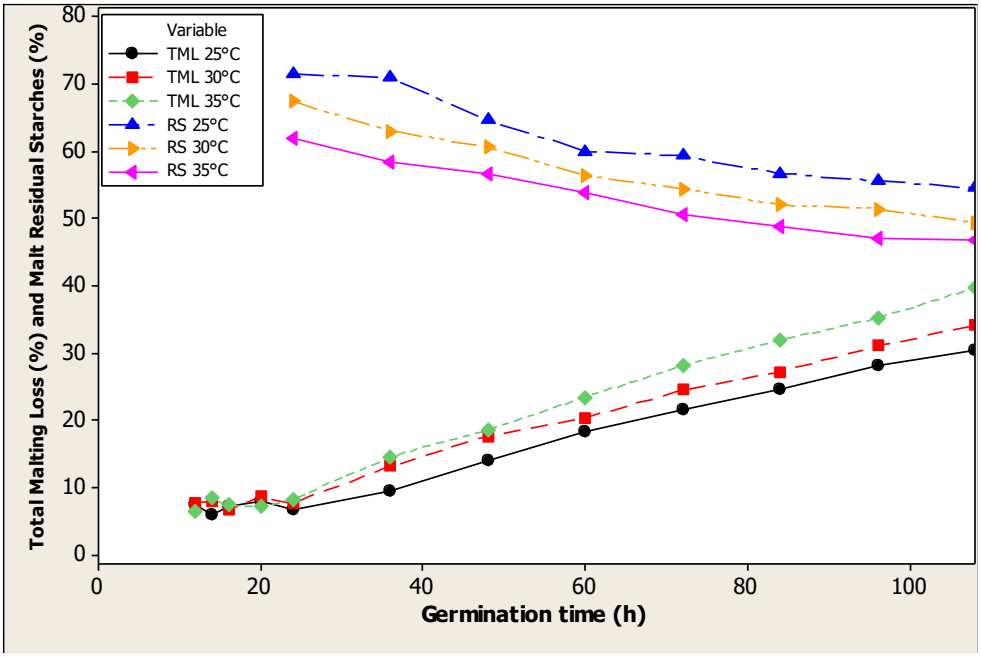


**Figure 18. Residual starches and Total Malting Loss as affected by germination time and temperature for grains steeped in B1**

### *4.4. Discussion*

Seed germination is well documented and it is known that sorghum germination is under hormonal control (Pagano *et al.*, 1997). In addition, when germination is well advanced, the seed in contact with the external environment can synthesize ABA, known to be involved in stress responses to changing environmental conditions (León and Sheen, 2003). The role of carbohydrates in the regulation of plant hormone action has been extensively discussed (Koch, 1996; Sheen *et al.*, 1999; Ohto *et al.*, 2001; Koch, 2004). The steeping and germination conditions can improve, to a greater or lesser extent, the release of absorbable simple sugars and induce ABA synthesis and/or activation. The presence of excess glucose during seedling development, for example, induces growth arrest and differentiation, which some authors attribute to the biosynthesis of the ABA and ABA signalling (León and Sheen, 2003). The ABA is important in the blockage of germination by reducing the permeability of membranes and its action is highly modulated by the concentration of glucose (León and Sheen, 2003). Thus it is clear that when using a model to predict enzyme activity, steeping and germination conditions have to be taken into account.

The effects of steeping conditions on amylase activities during sorghum malting are well known. Steeping conditions are known to be able to affect grain moisture (Dewar *et al.*, 1997), phenolic content (Beta *et al.*, 2000), cell walls degradation (Dewar and Orovan, 1997), protein matrix hydrolysis (Hamaker, 1987) and $\alpha$-amylase activity (Dewar and Orovan, 1997; Beta *et al.*, 2000, Dewar *et al.*, 1997). It is also known that during germination, amylase activities rise, reache a maximum, and then finally drop. Everything that happens after the peak (maximum $\alpha$-amylase activity) - despite the effect of germination conditions - is strongly related to the underlying

phenomenon, the growth of the seedling. The 2nd OPM or the GLM can be used in modelling *α*-amylase activity during the red sorghum germination step. When such models are employed, R-square, Chi-square, F-test and/or the root mean square error (RMSE) are often used to evaluate the goodness of fit. The basis of these statistics is the sum of square total (SST) (deviation from the average) and the sum of square error (deviation from the model's predicted values). Egwim and Adenomon (2009) obtained an R-sq value of between 67 to 90% using the 2nd OPM to model *α*-amylase activity during sorghum malting and suggested that the model could be used to predict future values. It is known that when a lack of fit exists, standard deviations for regression coefficients are overestimated, and this gives rise to confidence intervals that are too large (Martínez *et al.*, 2000). This can be seen in Tables 12, 14-20, and in such a situation the acceptable values of R-sq and the R-sq (adj) do not guarantee the goodness of the fit. The advantage of such models is they cover the entire process and therefore can offer an idea of the germination time corresponding to the maximum for the enzyme activity. We obtain the R-sq (adj) of between 86.3 to 96.0% using the 2nd OPM but the corresponding p-values for the lack of fit were less than 0.05 (Table 12). The same observation was made with the GLM which gave an R-sq (adj) = 82.82% but the lack of fit was significant. From this point of view, W-4-P M presented significant goodness, the lack of all fits was not significant except for the model obtained from steeping treatment C, followed by germination at 35°C (see Table 11).

During the malting process the effect of conditions created by the maltster must be evaluated correctly to be sure whether or not malting conditions have to be improved, as during germination, on the one hand there are a series of reactions that take place in the non-living part of the grain (endosperm), which can be controlled by the maltster, and on the other hand all reactions taking place in the living part of the grain are highly regulated (Lewis and Bamforth, 2006). The sensitivity of enzyme synthesizing cells varies, so that the aleurone layer cells are not affected by the levels of the sugars while in the embryo; the repression of enzyme synthesis is effective (Perata *et al.*, 1997). In sorghum, it has been reported that the synthesis of enzymes during germination is mainly achieved in the scutellum (Aisien, 1982; Aisien and Palmer, 1983) and therefore more sensitive to repression by sugars. The conditions affecting the occurrence of these two phases are strongly influenced by the conditions of malting (effect of malting conditions on the hydrolysis of the endosperm reserve, etc.). From this point of view, the W-4-PM clearly highlighted the effect of steeping and germination conditions on *α*-amylase activity during malting. The ST affected the grain capacity to develop *α*-amylase activity ($\partial \ln \alpha / \partial \ln BSP = 0.08306$) and the germination temperature affected the rate of the *α*-amylase synthesis ($\partial \beta / \partial GT = -0.02775$) as shown in Figure 13 and 14. It is therefore understandable that the value of $AA\omega$ estimated in this study is only a potential value. This model is cut by the curve of the repression phase of the *α*-amylase synthesis (see Figure 12.c. experimental values). The true maximum is found at the intersection of the two models with two different bases: that is to say the junction of the synthesis phase and that of the repression phase. It explains the peak obtained early

(around 72 h germination) with a germination temperature of 35°C (Figure 12.c.). Knowledge of this phenomenon is crucial. Indeed, when comparing two steeping treatments, one tends to fix conditions for germination. However, the steeping treatment and germination temperature have an effect over time after which this maximum is reached. Thus two treatments should not be compared on this basis. This is one of the advantages of the W-4-PM; which takes into account the effect of steeping conditions on the one hand, and that of germination conditions on the other hand.

The results obtained for $\beta$-amylase activity show that, the W-4-PM combined with the GLMLL can be used to predict the $\beta$-amylase activity during red sorghum germination. When these models are used, a significant goodness of fit is obtained (RMSE = 0.93 and random pattern of the residual error). The germination temperature affects the synthesis rate of $\beta$-amylase activity. Taylor and Robbins (1993) suggested a low temperature as optimal to obtain high $\beta$-amylase activities while Agu and Palmer (1997) suggested a sorghum malting temperature of 30°C produces high levels of $\alpha$- and $\beta$-amylase activities. The W-4-PM shows clearly the effect of germination temperature on the synthesis rate (Figure 15) of $\beta$-amylase activity. Contrary to $\alpha$-amylase synthesis, it can be seen shown that *GT* affects negatively the rate of the $\beta$-amylase synthesis. The results agree with those of Taylor and Robbins (1993) which clearly show that at 24°C the synthesis rate of $\beta$-amylase synthesis is higher than at 28 or 32°C. When this model is applied to values obtained by Taylor and Robbins (1993) the relationship between the rate of synthesis of $\beta$-amylase and the germination temperature can be clearly observed. The germination temperature increase is accompanied by an increase in the model parameter $\beta$ and means the minimization of the dimensionless variable *BA#*, or better, the maximization of $\beta$-amylase activity. The increase in $\beta$-amylase activity associated with the decrease in germination temperature is in our opinion due to the suggestion by Kaplan and Guy (2005) and Kaplan *et al.* (2004). Indeed, upon exposure to low temperatures, the hydrolysis of starch is accompanied by the synthesis of $\beta$-amylase and hence the release of maltose and maltotriose associated with cold stress.

The W-4-PM shows that, the steeping conditions affect the $\beta$-amylase synthesis capacity of sorghum. Similar observations regarding the effect of steeping conditions on $\beta$-amylase activity have been made by many researchers (reviewed by Taylor *et al.*, 2006). This model also allows the comparison of soaking treatments. Indeed, the use of *B. subtilis* induces an increase in $\beta$-amylase activity, which is characterized by the low value obtained for $ln\alpha$ (Table 22). When the culture is diluted, $ln\alpha$ increases, suggesting a loss of treatment efficacy. These models can be used to calculate the germination time after which the maximum value of $\beta$-amylase is reached. Moreover, if the measure is not made at this particular time, but during the phase of repression, the measured activity will be less than the maximum. When employing treatments aimed at improving the final quality of malts, it should be appreciated that if the malting goes beyond a certain limit before the goals are achieved, control of all the transformations which took place in the grain will be gradually taken up by the seed. A second

highly-regulated phase, which closely resembles the same laws as the phase manipulated by the maltster, begins; the residual starches decrease and the subsequent release of sugars activates abscissic acid and affects *β*-amylase synthesis. For the red sorghum used in this study, the *β*-amylase activity reaches its maximum before the *α*-amylase activity (not shown) suggesting that the effect of released sugar (expressed by the decrease in residual starches content) is not equivalent for these two enzyme activities. Indeed, this would possibly be due to the *β*-amylase synthesis occuring mainly in the scutellum (Aisen and Palmer, 1983) and is therefore more sensitive to inhibition due to the concentration of simple sugars (Perata *et al.*, 1997).

### *4.5. Conclusions*

The *α*-amylase activity time course during the first germination phase, characterized by the induction of the *α*-amylase synthesis, can be suitably modelled using the W-4-PM with significant goodness of fit (all models obtained haven't presented significant lack of fit except the fit obtained with the control (C) when the germination temperature was 35°C). The advantage of such a model is to highlight the effect of steeping and germination conditions. The W-4-PM hasn't been used to model the entire germination step as the second phase of this process is highly regulated and should be approached differently. This limitation of the model isn't a problem when the objective is the monitoring of the malting process. In fact, despite the importance of *α*-amylase during malting, what is sought is not always the maximum of this activity, but a compromise between a range of characteristics: the other enzymatic activity levels (*β*-amylase, *α*-glucosidase, limit dextrinase, *β*-glucanase, endo- and exo-peptidases, etc.), the reduction of the Total Malting Loss, the achievement of good grain modification level, reduced phenolic compounds content, etc. The *β*-amylase activity can be modelled using two different functions taking into account the specific phases of germination: the induction phase and the repression phase of *β*-amylase synthesis. The steeping treatment affects the capacity of *β*-amylase synthesis while the temperature affects the rate of *β*-amylase synthesis during the induction phase. During the repression phase, the effect of treatment tapers, while the temperature exerts more or less of an effect. The residual starch decreases during germination which then stabilizes and the total malting loss increases and then becomes an exponential rate.

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# Chapter V. Effect of the use of dilute alkaline prior to *Bacillus subtilis*-based biocontrol steeping and germination conditions on red sorghum malt *β*-glucanase activities and residual *β*-glucans

## *Présentation du chapitre*

Un autre problème devant mériter une attention particulière lors du maltage du sorgho – surtout lorsque les malts sont destinés à une utilisation brassicole – est l'hydrolyse des *β*-glucanes. Dans les chapitres 2 et 3 nous avons trouvé que les activités *β*-glucanasiques des malts obtenus avec le biocontrôle étaient supérieures à celles des traitements chimiques. Dans une étude préliminaire, nous avons constaté que, malgré leurs activités *β*-glucanasiques élevées, les teneurs en *β*-glucanes résiduels des malts obtenus avec les biocontrôles restaient supérieures à celles des malts obtenus après trempage dans 0.2% NaOH – du moins pour les malts obtenus dans les mêmes conditions de maltage. Dans ce chapitre 5, nous avons essayé d'élucider ce phénomène qui est une préoccupation des malteurs du sorgho et pour lequel les avis des chercheurs travaillant sur le maltage et le brassage du sorgho sont parfois très divergents. La stratégie utilisée dans cette étude est celle proposée dans le chapitre 3, à savoir la combinaison du trempage alcalin et du biocontrôle pour permettre de tirer profit de l'effet du NaOH sur la disponibilisation des *β*-glucanes et celui du biocontrôle sur la synthèse des *β*-glucanases. L'utilisation des contrôles (trempage dans l'eau distillée et trempage dans le biocontrôle non combiné au traitement chimique) a permis la mise en évidence des effets des traitements ; et les substrats utilisés - pour les mesures aussi bien des *β*-glucanes résiduels que des activités *β*-glucanases – ont permis l'étude de la spécificité des enzymes synthétisées sur les *β*-glucanes des malts de sorgho.


## *Abstract*

Malting is the ideal stage to deal with *β*-glucans. Their hydrolysis is very important as the diffusion of both hormones and hydrolytic enzymes in the endosperm of germinated grain depend on it. A high malt *β*-glucanase activity is not a guarantee of an extensive hydrolysis of *β*-glucans. When *Bacillus subtilis* is used to control mould growth, red sorghum malt *β*-glucanase activity (measured using carboxymethylcellulose as the substrate) was improved without significantly affecting the hydrolysis of malt *β*-glucans. Thus, in order to reduce the residual *β*-glucans content, soaking in 0.2% NaOH was combined with a biocontrol. Soaking in 0.2% NaOH is recognized as capable of improving grain hydration by opening-up the endosperm cell walls. The combined use of 0.2% NaOH with *Bacillus subtilis*-based biocontrol treatments during red sorghum malting leads to malt with increased *β*-glucanase activity and a significant reduction of residual *β*-glucans when compared to the steeping 16 h in the biocontrol without prior steeping in 0.2% NaOH. *β*-Glucanase activity increases with increased germination temperature and time while, inversely, the residual *β*-glucans content of the

malts decreases. Indeed, while the level of *β*-glucanase was not greatly different between the malts obtained after steeping in distilled water and those obtained after 8 h steeping in 0.2% NaOH followed by 8 h resteeping in distilled water (treatment NaOH+$H_2O$), their residual *β*-glucans levels differ significantly. *Bacillus subtilis*-based treatment leads to malt with improved *β*-(1-3)- and *β*-(1-4)-glucanase activities without significantly improved malt *β*-(1-3),(1-4)-glucanase activity. While malts obtained after 84 h germination weren't significantly different in terms of malt *β*-(1-3),(1-4)-glucanase activities for all steeping treatments, the use of 0.2% NaOH steeping prior to resteeping led to malts with improved *β*-glucans content. Combining the steeping in dilute alkaline and biocontrol enables taking advantage of the effect of the dilute alkaline on residual *β*-glucans content, due probably to the opening-up the cell walls and the improvement of water uptake, and that of the biocontrol (improvement of *β*-glucanase synthesis).

Key words: Dilute alkaline, *Bacillus subtilis* biocontrol, *β*-glucans and *β*-glucanase, red sorghum malting

### *5.1. Introduction*

Seed germination is a very complex process which continues to be a mystery (Nonogaki *et al.*, 2010). Indeed, during the imbibition phase of grain germ development, a range of reactions from simple to the more complex take place including: hydration of polymers, solubilization of simple molecules, activation and / or deactivation of hormones, activation of preformed enzymes, induction and / or repression of enzyme synthesis, hydrolysis of molecules, all metabolic reactions, cellular reorganization and various interconnections. The sum of current knowledge on germination is far from primitive; however, it is nonetheless clear that this phenomenon is far from fully understood (Nonogaki *et al.*, 2010). Raw grain tannin levels, starch gelatinization, endosperm cell wall hydrolysis and *β*-amylase activity in malt are major unresolved problem areas in sorghum malting and brewing (reviewed by Taylor *et al.*, 2006). The *β*-(1,3)-(1,4)-D-glucan – a polymer of *β*-D-glucose with *β*-(1,3) and *β*-(1,4)-glucosidic bonds − is one of the major constituents of the envelope of grasses (Asian *et al.*, 2007). *β*-glucans are present in the endosperm and the aleurone layer of cereals and account for up to 70% of the walls in the starchy endosperm of barley (Fincher, 1975). There are several structural variants of *β*-glucans in cereals (see Hrmova and Fincher, 2009 for details).

Endo-*β*-(1,3)-glucanases (EC 3.2.1.39.), endo-*β*-(1,3)-(1,4)-D-glucan-4-glucanhydrolases (EC 3.2.1.73) are enzymes which hydrolyse *β*-glucans in cereals (Hrmova and Fincher, 2009). Bacterial endo-*β*-(1,3)/(1,3)-(1,4)-D-glucanases (non-specific) (EC 3.2.1.6), endo-*β*-(1,3)-D-glucanases (EC 3.2.1.39) and endo-*β*-(1,3)-(1,4)-D-glucanases (lichenase) (EC 3.2.1.73) can also degrade *β*-D-glucans (Hrmova and Fincher, 2009).

Despite their prebiotic properties, *β*-glucans are disadvantageous during the brewing process when present at high levels as they reduce the wort filtration rate and negatively affect the extraction yield, the fermentation process and the stability of the beer (Lewis and Bamforth, 2006). According to Lewis and Bamforth (2006), malting is the ideal step for eliminating *β*-glucans.

Glennie (1984) and Palmer (1989), cited by Etokakpan and Palmer (1990), results show that the cell wall endosperms of barley are extensively degraded during malting, whilst those of sorghum are not. Contrary to these results Ogu *et al.* (2004) and Ogbonna and Egunwu (1994) reported an extensive (1-3),(1-4)-*β*-D-glucans hydrolysis during sorghum malting. This apparent contradiction has been clearly reviewed by Taylor *et al.* (2006). According to Etokakpan and Palmer (1990), malting sorghum developed mainly endo-*β*-(1,3)-D-glucanases while malting barley developed endo-*β*-(1,3)-(1,4)-D-glucanases.

Previous work has shown that when *Bacillus subtilis* cells are used in the steep liquor to control mould growth, the level of *β*-glucanase was improved in kilned malt (Bwanganga *et al.*, 2012), while unfortunately the malt residual *β*-D-glucans content was not. It is known that the hydrolysis of *β*-glucans is important for the diffusion of enzymes in the malted grain during germination (Bruggeman *et al.*, 2001); this hydrolysis has a great effect on the degree of grain modification. Factors affecting *β*-glucans hydrolysis include: grain moisture, *β*-glucans availability, pH and temperature (see Lewis and Bamforth, 2006). It has been shown that the use of dilute NaOH probably causes the endosperm cell wall to open up, resulting in a subsequent increase in water uptake (Dewar and Orovan, 1997). Other questions to be addressed are: (1) how does the use of *Bacillus subtilis* affect the main red sorghum malt *β*-glucanase activities, namely: *β*-(1,3)-, *β*-(1,4)- and *β*-(1,3)-(1,4)-D-glucanases; and (2) what is the nature of the *β*-glucans residual fraction ? This is crucial due to the fact that if the developed malt *β*-glucanases aren't specific to the grain residual *β*-glucans, their subsequent hydrolysis will not be possible.

Hence, in this study, we have sought to further investigate the combined treatment strategy focusing on the malt residual *β*-glucans and *β*-glucanase during the germination process. The aim of this study was to decrease red sorghum *β*-glucans by combining steeping in dilute alkaline and steeping in the *Bacillus subtilis* biocontrol, and by studying the effect of germination conditions (germination temperature (GT) and germination duration (GD)) on malt *β*-glucanase activities and residual *β*-glucans content.

### *5.2. Materials and Methods*

*Bacillus subtilis* strain S499 was obtained from the Walloon Center of Industrial Biology (CWBI) and grown on Luria Broth agar at 37 °C for 24 h. An inoculating loopful was transferred to 100 mL Landy

broth optimized for *B. subtilis* S499 lipopeptide production and incubated for 16 h. Finally, 10 mL was transferred to 350 mL optimized Landy broth and incubated at 30 °C (with rotary shaking at 130 rpm) for 72 h. The culture was diluted with distilled water to $10^8$ cells/mL in order to obtain the steep liquor used during the biocontrol step (Bwanganga *et al.*, 2012; Bwanganga *et al.*, 2013).

Sorghum malts were obtained as in chapters 3 and 4 by manual sorting and aerated steeping at 30 °C. In order to evaluate the effect of the use of dilute alkaline prior to the biocontrol (use of *Bacillus subtilis* starters) on the synthesis of *β*-glucanase (carboxymethylcellulose (CMC) being used as the substrate) and the hydrolysis of red sorghum grain *β*-glucans (barley mixed *β*-glucans being used as the substrate), two steeping treatments (ST) were applied: (1) treatment (NaOH+B): steeping for 8 h in dilute alkaline (0.2% NaOH) followed by resteeping for 8 h in distilled water containing $10^8$ *Bacillus subtilis* cells/mL, and (2) treatment (NaOH+$H_2O$): steeping for 8 h in dilute alkaline (0.2% NaOH) followed by resteeping in distilled water. Steeping for 16 h in distilled water (henceforth called $H_2O$) and steeping for 16 h in distilled water containing $10^8$ *Bacillus subtilis* cells/mL (henceforth called B) were used as the controls. Steeped grains were germinated in the dark at 25, 30, or 35 °C for 6, 12, 24, 36, 48, 60, 72, 84, 96 or 108 h, and kilned for 48 h at 40 °C.

In order to evaluate the specificity of the developed *β*-glucanase and the nature of the grain residual *β*-glucans, 84 h-germinated malts were obtained for all treatments. Red sorghum malt residual *β*-glucans and *β*-glucanase activities were then assayed as described below (3 methods for *β*-glucanase activities and 2 methods for the residual *β*-glucans).

According to Etokakpan and Palmer (1990), sorghum malt *β*-glucanase activity consists of 3 different enzymes, namely: *β*-(1,3)-, *β*-(1,4)- and *β*-(1,3)-(1,4)-D-glucanases. These 3 enzyme activities were assayed as follows:

**Method 1: (**see Bwanganga *et al.*, 2012): *β*-(1-4)-glucanase activity was assayed by grinding kilned malt in an IKA mill which was then sieved (mesh size: 0.5 mm), followed by *β*-glucanase extraction for 15 min at 30 °C in a centrifugation tube containing 0.5 g sorghum malt flour and 8 mL extraction buffer (50 mM Na-acetate, pH 4.8), with vigorous vortexing every 5 min. The mixture was then centrifuged at 1000xg for 10 min and the supernatant collected. The assay was performed at 40 °C in a reaction mixture containing 0.5 mL extract and 0.5 mL of 2% carboxymethylcellulose (CMC) as the substrate. The incubation time was 5 min and the reaction was stopped by immersing the tubes in boiling water. The amount of glucose released was then determined by the method of Nelson-Somogyi (Primarini and Yoshiyuki, 2000) and the *β*-glucanase activity was expressed in µmoles of glucose released per minute per kg of kilned malt.

**Method 2:** *β*-(1-3),(1-4)-glucanase activity of the malts was then assayed using the Megazyme S-ABG100 03/11 method and Azo-Barley glucan − a chemically modified (to increase solubility), dye-labelled barley *β*-glucan (1% w/v) with 0.02% sodium azide used as the substrate.

**Method 3:** approximately 0.5 g of sorghum malt flour was used, to which 5 mL of Na-acetate buffer (2 M, pH 5.0) was added and the mixture (in a centrifugation tube) was vigorously vortexed. The enzyme was allowed to extract for 15 min at room temperature with occasional mixing. The tubes were centrifuged at 1000xg and the Megazyme assay procedure for cereal flours and malt endo-(1,3)-*β*-glucanase activity was then used.

Methods 2 and 3 were only used for malt obtained after 84 h germination.

Sorghum *β*-glucans were assayed using 2 methods: method 1 for the mixed-linkage *β*-glucans and method 2 for *β*-(1,3)-glucans.

**Method 1** Megazyme mixed-linkage *β*-glucans assay procedure (McCLEARY method): approximately 0.5 g of sorghum raw grain (or malt) flour was treated with 1 mL of 50% ethanol. 5 mL of sodium phosphate buffer (20 mM, pH 6.5) was added and the mixture was incubated in a boiling water bath. The tubes were equilibrated at 40 °C and 0.2 mL lichenase enzyme (10 U) was added, and then further incubated for 60 min at 40 °C. Na-acetate buffer (50 mM, pH 4.0) was added and the tubes centrifuged for 10 min, after which aliquots (0.1 mL) were removed and treated with *β*-glucosidase (0.2 U), for a further 10 min. The mixture was incubated with GOPOD reagent for 20 min at 40 °C and the absorbance read at 510 nm against a reagent blank.

**Method 2** (for malts obtained after 84 h germination): approximately 0.5 g of sorghum raw grain (or malt) flour was treated with 1 mL of 50% ethanol. 5 mL of sodium phosphate buffer (20mM, pH 6.5) was added and the mixture was incubated in a boiling water bath. A 0.1 mL aliquot (in duplicate) was transferred to the bottom of a glass test tube. 0.1 mL of exo-(1,3)-*β*-glucanase (20 U/mL) plus *β*-glucosidase (4 U/mL) in sodium acetate buffer (20 mM, pH 5.0) was added to each tube. The tubes were vortexed and incubated at 40 °C for 60 min. The mixture was incubated with GOPOD reagent for 20 min at 40 °C and the absorbance read at 510 nm against a reagent blank.

### *5.3. Statistical analysis*

Two-way ANOVA was performed to study the effects of steeping treatments (B or C) and germination conditions (*GT* and *GD*) on red sorghum malt *β*-glucanase activity (assayed using method 1) and residual *β*-glucans (assayed using method 1). Tukey's Honestly Significant Difference test was used to compare malt obtained after 84 h germination at 25, 30 or 35 °C in 3 steeping conditions (treatments

B, C and $H_2O$) in terms of their *β*-(1,3)-, *β*-(1,4)- and *β*-(1,3)-(1,4)-D-glucanase activities and residual *β*-(1,3)- and *β*-(1,3)-(1,4)-D-glucans content.

## *5.4. Results*

### 5.4.1. Red sorghum raw grain characteristics

The red sorghum cultivar used was obtained from the D.R. Congo and has been described previously (Bwanganga *et al.*, 2012; Bwanganga *et al.*, 2013); it contained: 3.8% ± 0.3 fat content; 29.4 g/kg ± 1.0 *β*-glucans (assayed by the Megazyme K-BGLU 04/06 method, 2006); 73.9% ± 1.9 total starch (as assayed by the Megazyme K-TSTA 07/11 method) and 27.6% ± 1.4 amylose (assayed by the Megazyme K-AMYL 07/11 method).

### 5.4.2. Effect of steeping and germination conditions on red sorghum residuals *β*-glucans (assayed using method 1) and *β*-glucanase activity (assayed using method 1)

Sorghum malt residual *β*-glucans and *β*-glucanase activity assayed using method 1 for both residual *β*-glucans and *β*-glucanase activity are presented in Figure 19 (a, b and c). Figure 19 (a, b and c) shows that during red sorghum germination, the malt residual *β*-glucans content decreases while the malt *β*-glucanase activity increases. Three-way ANOVA applied to the data of Figure 19 (presented in Table 24) shows that all factors affect significantly the residual *β*-glucans level, and except the interaction *ST*GT*GD* which had no significant effect on kilned malt *β*-glucanase activity (assayed using method 1), all factors exerted also a significant effect on kilned malt *β*-glucanase activity.

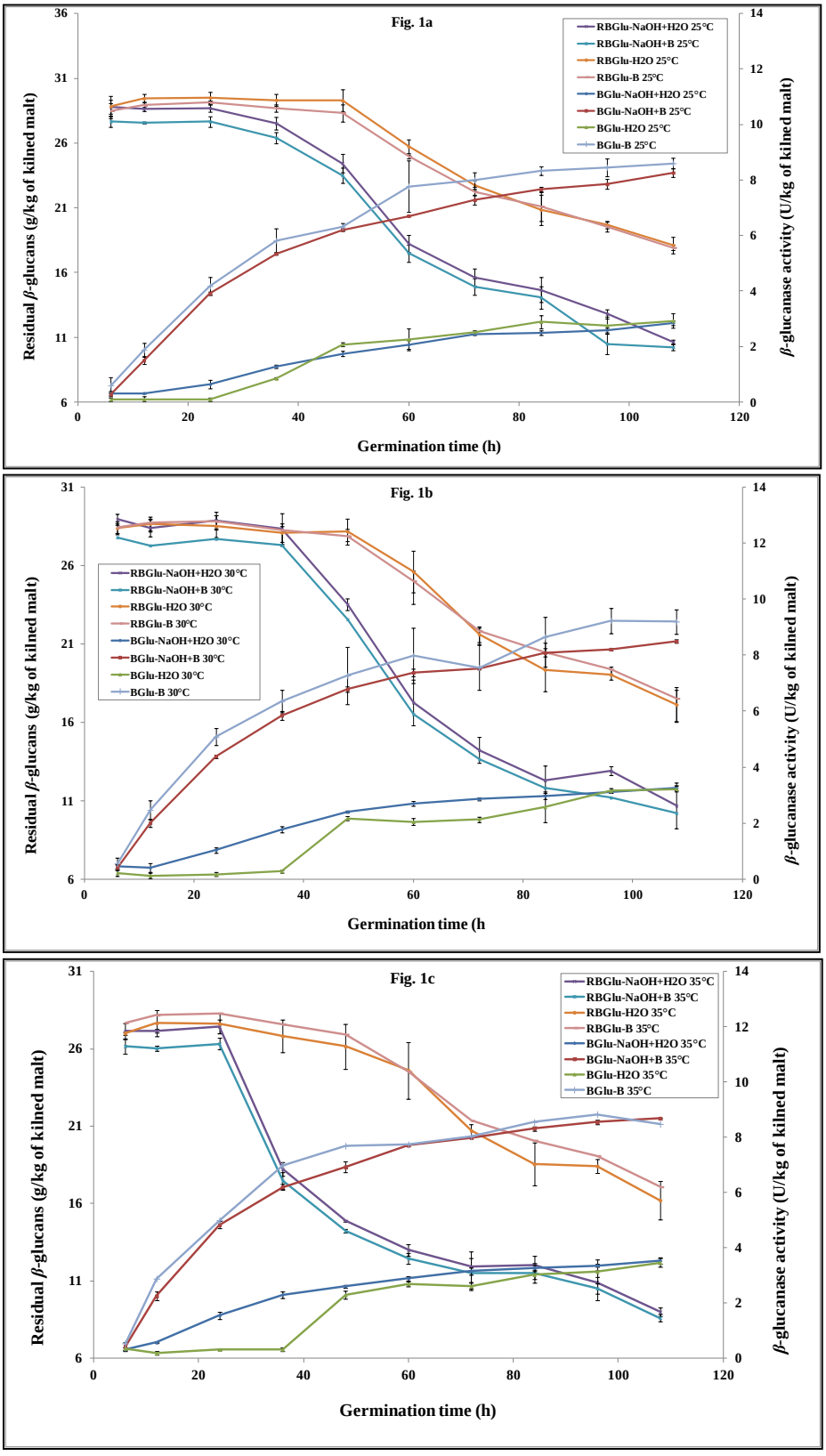


**Figure 19. *β*-glucanase activity (U/kg of kilned malt) and malt residual *β*-glucans (g/kg of kilned malt) (Figure 19a, 19b and 19c respectively for germination temperatures of 25, 30 and 35°C)**

**Table 24. Three-way ANOVA for residual *β*-glucans and *β*-glucanase activity assayed using methods 1**

| Source | DF | *β*-glucanase activity (U/kg of kilned malt) | | | | Residual *β*-glucans (g/kg of kilned malt) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | SS | MS | F | P | SS | MS | F | P |
| ST | 3 | 1571.665 | 523.888 | 4153.42 | 0.000 | 2601.59 | 867.20 | 1830.57 | 0.000 |
| GT | 2 | 15.404 | 7.702 | 61.06 | 0.000 | 433.70 | 216.85 | 457.75 | 0.000 |
| GD | 9 | 1268.126 | 140.903 | 1117.09 | 0.000 | 10777.17 | 1197.46 | 2527.73 | 0.000 |
| ST*GT | 6 | 3.324 | 0.554 | 4.39 | 0.000 | 121.70 | 20.28 | 42.81 | 0.000 |
| ST*GD | 27 | 268.852 | 9.957 | 78.94 | 0.000 | 957.28 | 35.45 | 74.84 | 0.000 |
| GT*GD | 18 | 3.881 | 0.216 | 1.71 | 0.038 | 232.49 | 12.92 | 27.26 | 0.000 |
| ST*GT*GD | 54 | 8.891 | 0.165 | 1.31 | 0.092 | 176.09 | 3.26 | 6.88 | 0.000 |
| Error | 240 | 30.272 | 0.126 | | | 113.70 | 0.47 | | |
| Total | 359 | 3170.415 | | | | 15413.70 | | | |
| | | S = 0.355154 | | | | S = 0.688280 | | | |
| | | R-sq = 99.05 % | | | | R-sq = 99.26 % | | | |
| | | R-sq (adj)= 98.57 % | | | | R-sq (adj) = 98.90 % | | | |

When the soaking is performed in distilled water (treatment $H_2O$), the percentage of *β*-glucans content reduction is too low − the highest values being between 38.4 and 44.9% obtained after 108 h of germination at 35°C. The highest value of the *β*-glucanase activity (9.2±0.5 U/kg of kilned malt) was obtained with steeping treatment B after 96 h germination at 30°C. The lowest residual *β*-glucans content (8.6±0.2 g/ kg of kilned malt) was obtained with steeping treatment "NaOH+B" after 108 h germination at 35°C. The use of *Bacillus subtilis* biocontrol leads to malts with high *β*-glucanase activities (high *β*-glucanase activities obtained with the steeping treatments B and "NaOH+B" when compared to "$H_2O$" and "NaOH+$H_2O$" (see Figures 19, 21 and 22)] while steeping in 0.2% NaOH prior to resteeping leads to malts with low residual *β*-glucans contents (low *β*-glucans contents obtained with the steeping treatments NaOH+$H_2O$ and NaOH+B). This can be seen when considering for example the 84 h-germinated malts at 30°C (84 h being the time from which the *β*-glucanase activity does not increase substantially) and comparing different treatments based on their contents *β*-glucans and their *β*-glucanase activities. Unlike the treatment B which has a very high *β*-glucanase activity but leads malts with high residual *β*-glucans contents, the steeping treatment NaOH+$H_2O$ shows a percentage of *β*-glucans reduction (58.2%) close to that of the steeping treatment NaOH + B (59.8%), whereas these treatments are significantly different in terms of their beta-glucanase activities. This means that an increased malt *β*-glucanase activity does not necessarily induce a reduction in residual *β*-glucans content.

The regression lines of the grain residual *β*-glucans content (assayed by method 1) vs the *β*-glucanase activity for each steeping treatment are presented in Figure 20 (R-sq (adj) ≈55.7−86%).

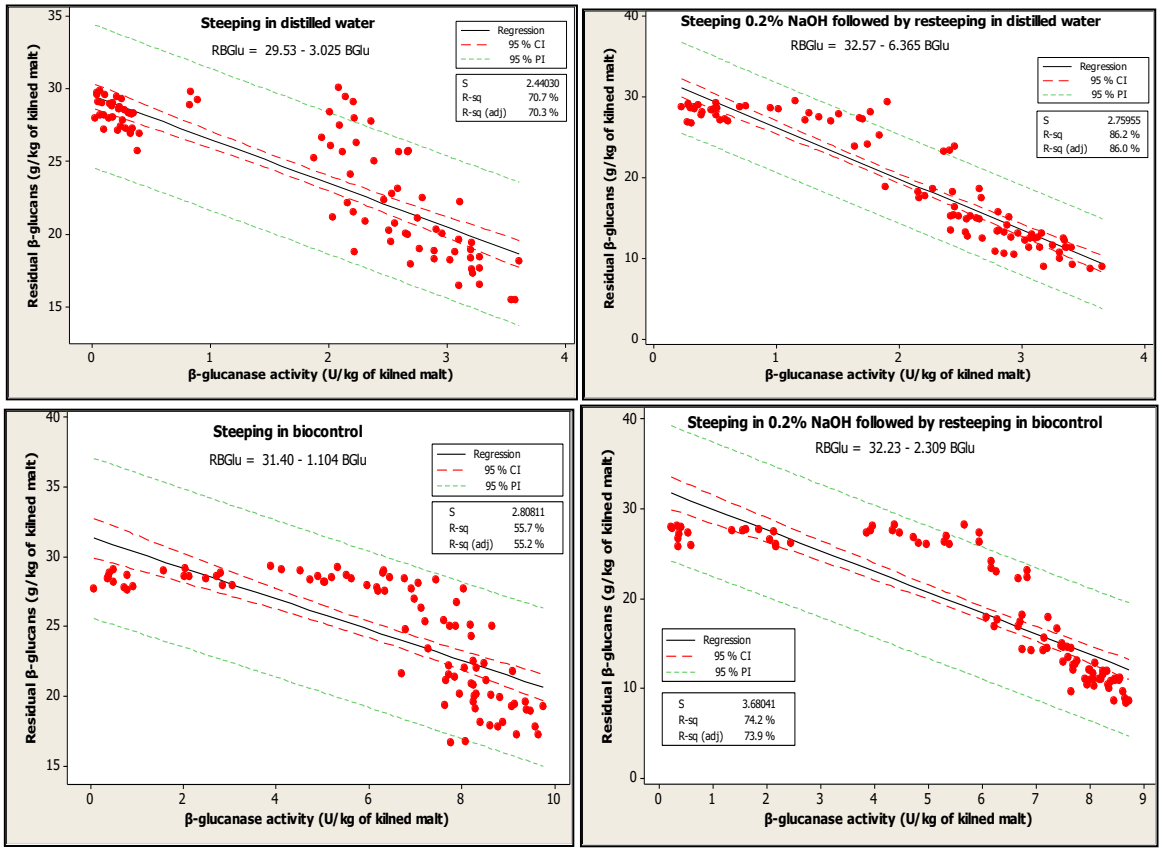


**Figure 20. The regression lines of the grains residual *β*-glucans content vs the *β*-glucanase activity for each steeping treatment**

### 5.4.3. Effect of the use of 0.2% NaOH prior to resteeping on red sorghum residuals *β*-glucans (assayed using methods 1 and 2) and *β*-glucanase activity (assayed both using methods 1, 2 and 3)

84 h-germinated red sorghum malts *β*-glucanase activities assayed using method 1, 2 and 3 and their residual *β*-glucans content assayed by methods 1 and 2 are presented in Figures 21 and 22 respectively and the Tukey's HSD test was used to compare treatments in terms of their *β*-glucanase activity and residual *β*-glucans dor malts obtained at the same germination temperature.

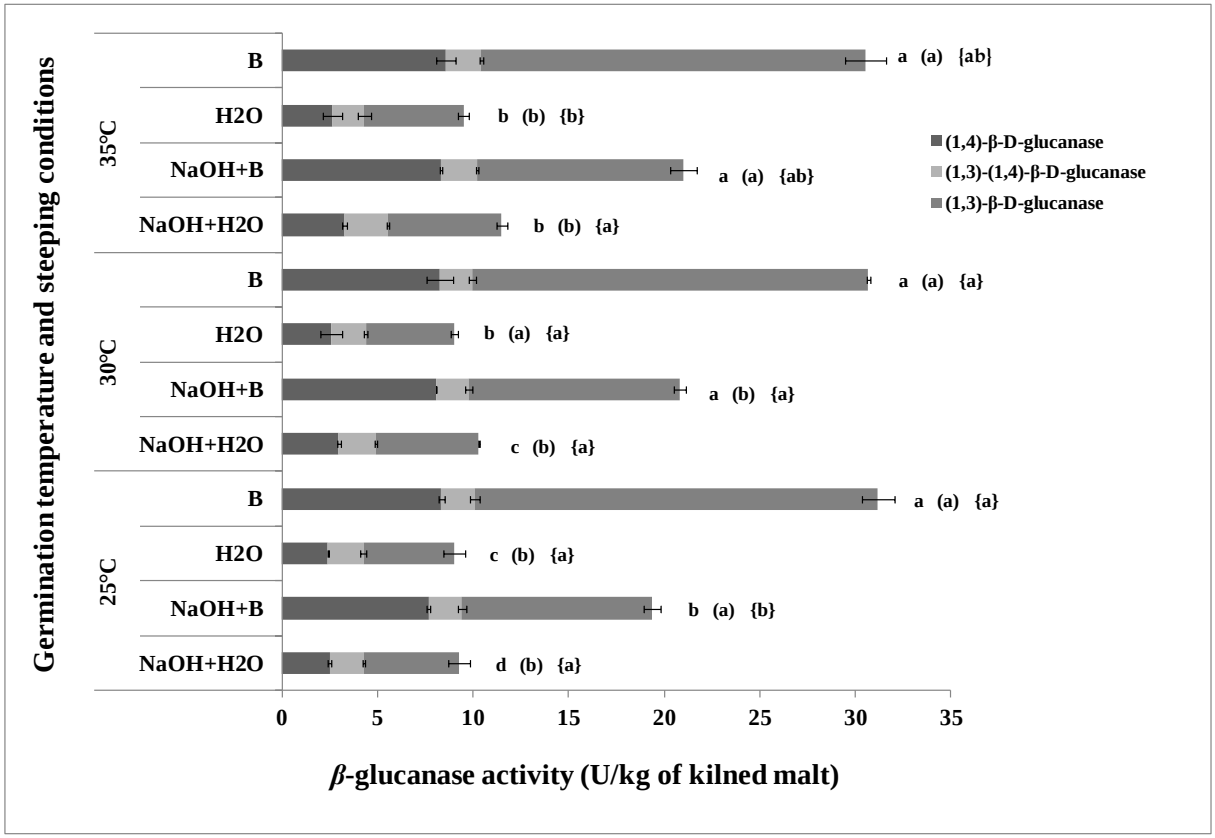


**Figure 21. Kilned malt *β*-glucanase activity (U/kg of kilned malt) for malts obtained after 84 h of germination as assayed using Methods 1, 2 and 3. For each germination temperature, treatments having a letter in common [letters in parentheses for method 2 and letters braces for method 3] are not statistically different according to Tukey's HSD test ($p<0.05$)**

These results show that (Figure 21):

- **At germination temperature of 25 °C**: there is no increase in the *β* -(1,3)-(1,4)-glucanase activity with the biocontrol steeping (treatments "B" and "NaOH+B"), while the *β*-(1,3)-glucanase increases of 346.9 and 110.6% for the treatment "B" and "NaOH+B" respectively. The *β*-(1,4)-glucanase activity increases by 245 and 217.4% for the treatment "B" and "NaOH+B" respectively.
- **At germination temperature of 30 °C**: there is no increase in the *β* -(1,3)-(1,4)-glucanase activity with the biocontrol steeping (treatments "B" and "NaOH+B"), while the *β*-(1,3)-glucanase increases of 347 and 137.7% for the treatment "B" and "NaOH+B" respectively. The *β*-(1,4)-glucanase activity increases by 221 and 213.9% for the treatment "B" and "NaOH+B" respectively.
- **At germination temperature of 35 °C**: there is no increase in the *β*-(1,3)-(1,4)-glucanase with the steeping treatment "B", 14% increase being obtained with steeping treatment "NaOH+B" while the *β*-(1,3)-glucanase activity increases of 287 and 108% respectively for the steeping treatments "B" and "NaOH+B" respectively. The *β*-(1,4)-glucanase activity increased by 223 and 214% for the steeping treatment "B" and' "NaOH+B" respectively.

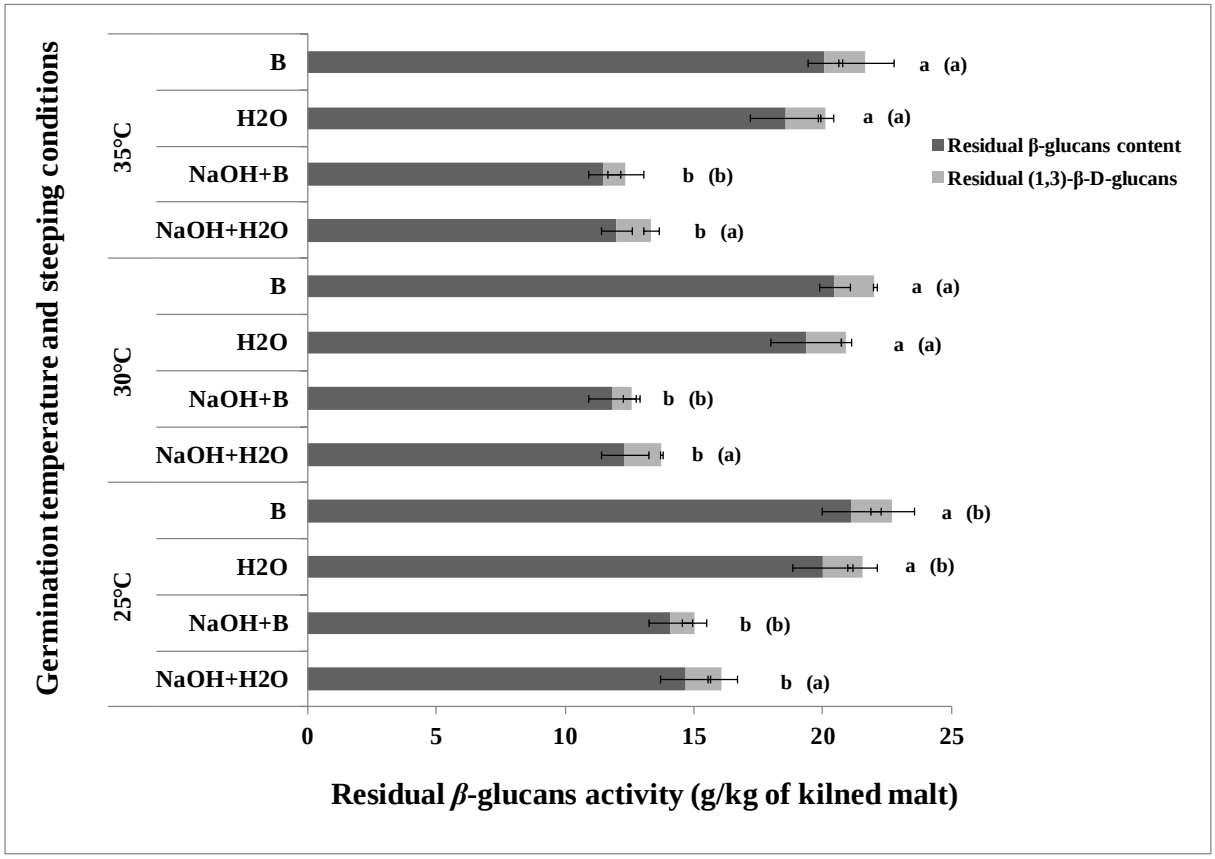


**Figure 22. Kilned malt residual *β*-glucans (g/kg of kilned malt) for malts obtained after 84 h of germination as assayed using Methods 1 and 2. For each germination temperature, treatments having a letter in common [letters in parentheses for method 2 and letters braces for method 3] are not statistically different according to Tukey's HSD test ($p<0.05$).**

The results in Figure 22 show that the steeping in 0.2% NaOH prior to resteeping induces:

- At the germination temperature of 25 °C: a further reduction of the residual *β*-glucans (assayed using method 1) of 26.7 and 33.3% with the treatments "NaOH+$H_2O$" and "NaOH+B" respectively when compared to the steeping in the corresponded control ("$H_2O$" and "B" respectively). The reduction of the residual *β*-glucans assayed by method 2 was 7.9 and 40.9% with the treatments "NaOH+$H_2O$" and "NaOH+B" respectively.
- At the germination temperature of 30 °C: a further reduction of the residual *β*-glucans (assayed using method 1) of 42.2 and 36.4% with the treatments "NaOH+$H_2O$" and "NaOH+B" respectively when compared to the steeping in the corresponded control ("$H_2O$" and "B" respectively). The reduction of the residual *β*-glucans assayed by method 2 was 8.2 and 52.5% with the treatments "NaOH+$H_2O$" and "NaOH+B" respectively.
- At the germination temperature of 35 °C: a further reduction of the residual *β*-glucans (assayed using method 1) of 42.7 and 35.2% with the treatments "NaOH+$H_2O$" and "NaOH+B" respectively when compared to the steeping in the corresponded control ("$H_2O$"

and “B” respectively). The reduction of the residual *β*-glucans assayed by method 2 was 14.1 and 47.6% with the treatments “NaOH+$H_2O$” and “NaOH+B” respectively.

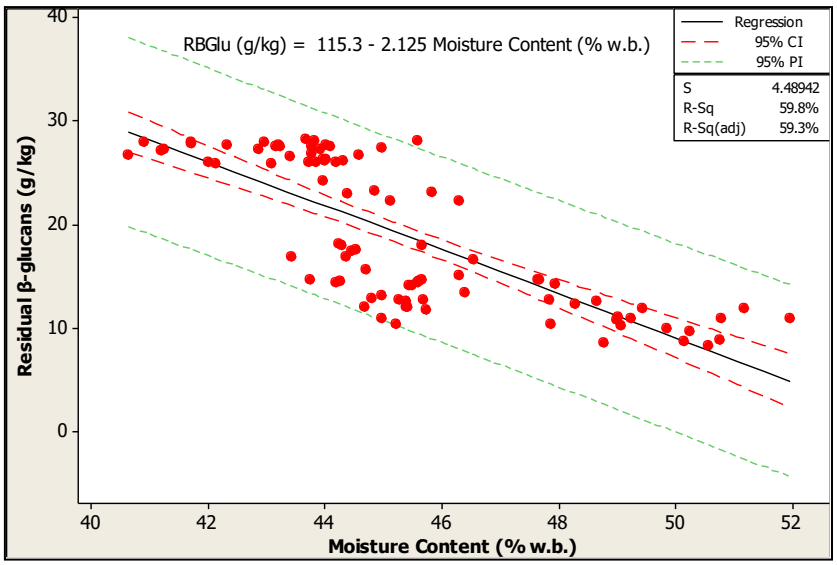


**Figure 23. Regression straight line of *β*-glucans (g/kg of kilned malt) versus green malt moisture**

The residual *β*-glucans was found to be correlated with the green malt moisture content (w.b) (Figure 23).

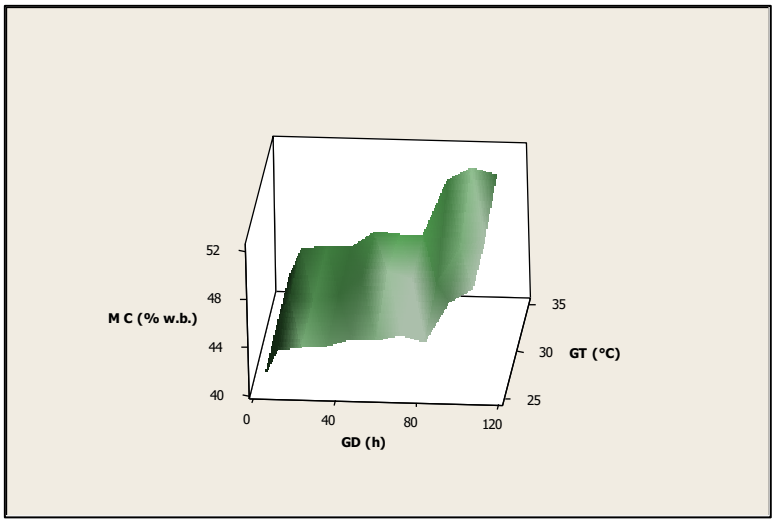


**Figure 24. Grain moisture content (M C) (% w.b.) for steeping treatment B (combined use of dilute alkaline and biocontrol) as affected by germination duration (GD) and germination temperature (GT)**

### *5.5. Discussion*

Figure 19 shows that the use of *Bacillus subtilis* leads to malt with highly improved *β*-glucanase activity (treatments "B" and "NaOH+B") and that the differences between treatments "$H_2O$" and "NaOH+$H_2O$", in terms of *β*-glucanase activity for malts obtained at the same GT, does not justify that between their residual *β*-glucans content. In fact, the only difference between treatments "$H_2O$" and "NaOH+$H_2O$" for example lies in soaking grains in 0.2% NaOH during the first 8 hours, suggesting that the use of dilute alkaline prior to steeping probably affects *β*-glucans hydrolysis leading to malt with low *β*-glucans content. This effect can also be observed between treatments "B" and "NaOH+B". This difference between combined use 0.2% NaOH treatments and the corresponded controls ("NaOH+$H_2O$" vs "$H_2O$" and "NaOH+B" vs "B") would be due, in part, to the observation by Dewar and Orovan (1997) that the dilute alkali causes the endosperm to open up, thus creating more favourable conditions for the hydrolysis of grain *β*-glucans. Elsewhere, NaOH is used to extract *β*-glucans (Bielecki and Galas, 1991; Beer *et al.*, 1997; Rimsten *et al.*, 2003). This confirms the report of Lewis and Bamforth (2006); the dissolution of *β*-glucans, which is in the non-living part of the grain, would be strongly affected by enzyme concentration, temperature, *β*-glucans availability, water and pH. The enzyme responsible for this hydrolysis (*β*-glucanase), works under the following conditions: pH 3.5−7.0 and optimum temperature 30−60 °C (Bielecki and Galas, 1991). This partially explains the high percentage of *β*-glucans reduction achieved with treatments "NaOH+$H_2O$" and "NaOH+B" (Figure 22). If the temperature conditions can be fixed, the acidification of the endosperm and the reducing environment necessary for the solubilization of certain macromolecules are possible during germination (reviewed by Simpson, 2001). The positive effect of steeping in 0.2% NaOH prior to resteeping could possibly due, among others, to the increased grain steep-out moisture. It has been shown that, when 0.2% NaOH was used as the steeping liquor, the red sorghum steep-out moisture was higher when compared with biocontrol-treated grain (Bwanganga *et al.*, 2012). A linear relationship can be obtained between the residual *β*-glucans content and the green malt moisture with an R-sq (adj) of 59.3% (Figure 23). When NaOH is used prior to resteeping, the malt residual *β*-glucans (assayed using method 1: with barley *β*-(1,3)-(1,4)-D-glucans used as the substrate) decreased significantly (Figure 19 and 22). This result suggests that sorghum malt residual *β*-(1,3)-(1,4)-D-glucans do not only depend on the amount of *β*-glucanases but also on the availability of grain *β*-D-glucans, which can be affected by the steeping conditions. It is for example known that grain moisture is important metabolic and physiological parameter in the sense that its availability affects the hydrolysis of grain constituents (Bewley and Black, 1994; Bewley, 1997, Lewis and Bamforth, 2006). This partially explains the difference between the treatments "$H_2O$" and "NaOH+$H_2O$" and that between treatments "B" and "NaOH+B": the use of NaOH increases the rate of rehydration of the red sorghum, this increase being even greater when the temperature is high (Bwanganga *et al.*, 2012). During the rehydration of grain, the grain reserve polymers are initially hydrated (Nonogaki *et al.*,

2010) and this hydration is important for subsequent hydrolysis. The results in Figure 19 allow the distinguishing of the existence of phases in the degradation of grain *β*-glucans. A first quasi-stationary phase (6 to about 24 h of germination at 35 °C and until 36 h at 25 and 30 °C) during which the percentage reduction of the grain *β*-glucans content does not exceed 12%; then comes a second phase where the rate of hydrolysis of *β*-glucans increases (63.8−70.7% reduction achieved after 108 h of germination. The highest percentage of *β*-D-glucans reduction obtained was approximately 71% after 108 h of germination with treatment NaOH+B at 35 °C (Figure 19), corresponding to 0.86% of residual *β*-glucans. All percentages of reduction were lower than those required for good barley malts (around 85%), for a final concentration of residual *β*-glucans, by approximately 0.5% (Lewis and Bamforth, 2006). When distilled water was used during the steeping step, the lowest residual *β*-glucans content obtained was 1.6% which was achieved after 108 h germination at 35°C. These results are consistent with those reported by Etokakpan (1992), where sorghum grain contains less *β*-D-glucans than barley, but sorghum malts contain more *β*-D-glucans than those of barley.

In our opinion, the increase in *β*-(1,3)- and *β*-(1,4)-glucanases observed with treatments "B" and "NaOH+B" is possibly due to the interaction between the *Bacillus subtilis* population, used as starters, and the steeped grain. The use of bacterial starters during steeping is perceived by the steeped grain as a biotic stress. In fact, it is known that plant-environment interactions transform plants into extraordinary "chemical synthesis industries". Indeed, the cascade of reactions which ultimately lead to the synthesis and accumulation of secondary metabolites is triggered by a wide range of external stimuli (reviewed by Sudha and Ravishankar, 2002). The detection of stimuli by specific receptors induces signalling in the cell, through signalling pathways, leading to gene expression and a series of biochemical changes (Sudha and Ravishankar, 2002). It is known that one of the classical types of interspecies antagonisms, leading to control strategies of plant pathogens, is the mixed-path antagonism which can be due, among other mechanisms, to plant hydrolytic enzyme synthesis (chitinases, glucanases and proteases) (Pal and Gardener, 2006). The role of the cell wall in the signalling phenomena underlying plant-microorganism interactions is well known (Esquerré-Tugayé *et al.*, 2000). Hence, the high *β*-glucanase activity obtained with the treatments "B" and "NaOH+B" assumes the existence of a possible grain-*Bacillus subtilis* S499 interaction. The increase in *β*-glucanase activities observed with method 1 and method 3 (Figure 21) are possibly due to increased endo-(1-4)-*β*-D-glucanase and endo-(1-3)-*β*-D-glucanase activities respectively. In fact, while plant *β*-(1,3)-(1,4)-glucanases play an important role in the hydrolysis of storage polysaccharides, the *β*-(1,3)-glucanase role in plant defence, germination, microsporogenesis and embryogenesis is recognized (Cheong *et al.*, 2000 and others cited by McLeod *et al.* , 2003). It is known, for example, that endo-*β*-(1,3)-D-glucanase only degrades *β*-(1,3)-glycosidic bonds (Kumagai and Ojima, 2009) and are present in root, leaves and the aleurone, and is specific for (1,3)- and (1,3)-(1,6)-*β*-D-glucans (Hrmova and Fincher, 2009). Hrmova and Fincher (2009) also reported that endo (1,3)-*β*-D-glucan hydrolases

require relatively extended regions of an unsubstituted or unbranched (1,3)-*β*-D-glucan backbone for activity and laminarin is their preferred substrate. Our results (Figure 21) confirm the report by Etokakpan and Palmer (1990) suggesting that malting sorghum developed mainly endo-*β*-(l,3)-D-glucanase. Figure 21 also shows an increased endo-*β*-(l,4)-glucanase activity (method 1), the role of which, in the endosperm cell wall hydrolysis of barley malt, has been questioned (see Palmer, 1989 cited by Etokakpan and Palmer, 1990). It had been suggested that this enzyme is possibly associated with contaminating fungal material and does not play an *in vivo* role in cell wall breakdown in either barley or sorghum (Etokakpan and Palmer, 1990).

Onwurah (2001) results suggest that sorghum *β*-glucan is a mixture of two polysaccharide chains: one bearing (1,3) linkages with (1,6) bonds at branch points while the other chain bears (1,4) linkages with (1,6) bonds at branch points, or a straight (single) chain with mixed (1,3) and (1,4) linkages with (1,6) bonds at branch points. Onwurah (2001) results also suggest the existence of a chain which is 93–94% of the *β*-glucan polysaccharide chains, and constitutes *β*-D-glucopyranosyl residues in (1-4) linkages and 4–5% (1-6) bonds at branch points. So, according to our results, we suggest that endo-*β*-(l,4)-glucanase can play a significant role in sorghum *β*-glucans hydrolysis (correlationship between red sorghum malt *β*-glucans content and malt endo-*β*-(l,4)-glucanase).

### *5.6. Conclusions*

In this work, we demonstrated that a significant increase in *β*-glucanase activity is not necessarily accompanied by a reduction in residual *β*-glucans. However, when dilute alkaline steeping is used prior to resteeping in the biocontrol, for approximately the same enzyme level, the residual *β*-glucans decreased significantly. This is not necessarily only due to the *β*-glucanase activity of the grain, but also the availability of the grain *β*-glucans during malting.

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## Chapter VI. Indole acetic and gibberellic acids production by *Bacillus subtilis* S499 and their effect on red sorghum malt enzymes synthesis

### *Présentation du chapitre*

Les résultats obtenus dans les chapitres précédents montrent que l'utilisation de *B. subtilis* S499 influence la synthèse des enzymes ($\alpha$-amylase, $\beta$-amylase et $\beta$-glucanases). Parmi les hypothèses qui expliquent ces résultats figurent la possibilité de synthèse, par *B. subtilis* S499, de molécules signalisatrices, en l'occurrence de phytohormones. Les résultats obtenus dans la littérature montrent que l'effet des phytohormones sur la synthèse des enzymes lors du maltage du sorgho n'est pas équivalent à celui sur le malt d'orge. Dans ce chapitre, nous nous sommes penchés sur ce problème en :

1. déterminant les quantités de deux phytohormones (l'acide gibbérellique et l'acide indole acétique) produites par *B. subtilis* S499.
2. comparant les activités enzymatiques des malts obtenus lorsque les biocontrôles sont utilisés et celles des ceux obtenus après trempage dans les solutions ayant des concentrations équivalentes en acide gibbérellique et en acide indole acétique.
3. évaluant l'effet du contexte développemental sur l'efficience de l'utilisation des phytohormones.
4. étudiant l'interaction entre l'acide gibbérellique et l'acide abscissique.

Ce travail discute des résultats, parfois contradictoires trouvés dans la littérature sur :

1. l'effet des phytohormones sur la synthèse des enzymes lors de la germination du sorgho.
2. la localisation des principaux sites de synthèse des enzymes.
3. la différence de réponse aux phytohormones en fonction du type d'enzyme étudié.

### *Abstract*

*Bacillus subtilis* was grown under optimal conditions for lipopeptide production (surfactins, iturins and fengycins) - molecules exhibiting antifungal properties. Two phytohormones, namely indole acetic acid and gibberellic acid were extracted from the *B. subtilis* culture and assayed using RP-HPLC-DAD. Two steeping treatments have been used: (1) *B. subtilis* starter (biocontrol steeping) and (2) a steeping liquor having the same IAA and GA3 concentration as the *B. subtilis* starter culture (phytohormone steeping). All enzyme activities were highly affected by biocontrol steeping when compared with the phytohormone steeping. Sorghum malt $\alpha$-amylase, $\beta$-(1,3)- and $\beta$-(1,4)-glucanases were found to be greatly affected by steeping treatments when compared with $\beta$-amylase and $\beta$-(1,3)-

(1,4)-glucanase. The results also show that the phytohormone application time, steeping liquor renewal and their interaction affect sorghum malt enzyme activities. ABA and GA3 cross-talk affects the amylase synthesis, with greater effect on $\alpha$-amylase than on $\beta$-amylase.

Key words: *Bacillus subtilis*, Indoleacetic acids, Gibberellic acids, Abscisic acid, cross-talk, red sorghum, malting

### *6.1. Introduction*

The use of *B. subtilis* as a starter culture during red sorghum steeping exerts an increase in malt enzyme activities ($\alpha$-amylase, $\beta$-amylase and $\beta$-glucanase) (chapters 2, 3 and 4). Similar results (increased sorghum malt diastatic power) have also been obtained by Lefyedi and Taylor (2007) upon the use of lactic acid bacteria starter cultures (*Lactobacillus plantarum* and *Pediococcus pentosaceus*) or yeast (*Saccharomyces* sp.). One of the assumptions outlined for this increase is the grain biotic stress due to the presence of microbes in the malted grain ecosystem. In fact, germination is the stage during which the majority of physical, chemical and biochemical transformations occur in the grain, and the effect of signalling molecules on plant growth and differentiation, as biotic and/or abiotic interactions, is well known. One of the classic models of plant-microbe interaction is the recognition of the molecules produced by microorganisms (pathogen/microbe-associated molecular patterns) by surface receptors (surface pattern-recognition receptors) thus inducing an immune reaction in the plant (pattern-triggered immunity) (Jones and Dangl, 2006). The role of phytohormones (salicylic acid, jasmonic acid, auxins, ethylene, gibberellic acid (GA), various cytokinins and abscisic acid (ABA)) during the molecular dialogue is recognized (Robert-Seilaniantz *et al.*, 2011). These phytohormones are not only produced by plants, but also by certain microorganisms (bacteria and fungi) grouped into the often-called Plant Growth Promoting Rhizobacteria (PGPR). Phytohormones produced by microorganisms can be absorbed by the plant and then affect plant metabolism (Bottini *et al.*, 2004). Some microorganisms (fungi and bacteria), belonging to the malted barley microbial ecosystem, have been recognized as capable of producing phytohormones (Gibberellic acid (GA), indole-3-acetic acid (IAA) and ABA) (Tuomi *et al.*, 1995).

One of the most important phytohormones implicated in seed germination is gibberellic acids familly. It has been shown that the deficient Arabidopsis mutant lacks the important enzyme, ent-DP synthase (ent-copalyl diphosphate synthase), for GA biosynthesis and is non-germinating, dwarf and male sterile (see Cheng *et al.*, 2004; Koornneef and van der Veen, 1980; Sun and Kamiya, 1994 cited by Cao *et al.*, 2005 for details).

ABA is also very important during seed germination and is known to inhibit the effect of gibberellic acid and induce the embryo synthesis of storage proteins (Finkelstein *et al.*, 2002; Kagaya *et al.*,

2005). In addition, ABA is implicated in plant biotic and abiotic stresses such as: drought- or osmotica-induced stomatal closure; induction of tolerance to water, salt, hypoxic and cold stress; and wound or pathogen response (see Finkelstein *et al.*, 2002). Its role in pathogen defence is also recognized with the induction of gene transcription specifically for proteinase inhibitors in response to wounding (Pena-Cortis *et al.*, 1989).

Cytokinins constitute a very important component of the phytohormone family. It has been shown that the cytokinins content is reduced in response to a decreased water availability and maintaining high levels of cytokinins promotes stomatal opening (Davies *et al.* 2005). It was also suggested that cytokinins inhibit root growth and can therefore support the carbon allocation from the shoots to the roots, enhancing the ability to penetrate the soil. Under stressful conditions this inhibition may be limited due to a decrease in cytokinins content (Arkhipova *et al.*, 2007). The role of two members of this family (zeatin and zeatin-9, beta-ribonucleoside) in enhancing the germination of dormant embryos is well known (Tzou *et al.*, 1973).

While cytokinins are the main root signal hormones, auxins (IAA) are considered as the primary shoot signal hormones (Aloni *et al.*, 2006). Auxins play an important, but poorly defined, role in seed germination (Jung and Park, 2011); in contrast, the role of auxins as mediators of the tropistic responses of bending in response to gravity and light; general root and shoot architecture; organ patterning; vascular development and growth in tissue culture is well known (reviewed by Woodward and Bartel, 2005). All these phytohormones play an important role in malt properties because of their effect on grain metabolism and physiology.

The aim of this study is the assessment of the production of IAA and GA by *B. subtilis* and its impact on red sorghum malt enzyme activities ($\alpha$-amylase, $\beta$-amylase and $\beta$-glucanases). RP-HPLC-UV analysis was used for phytohormones assays (GA, IAA and ABA). GA and ABA diffusion time course was also evaluated to facilitate the understanding of the effect of their cross-talk on $\alpha$- and $\beta$-amylase activities.

## *6.2. Materials and methods*

### 6.2.1. *B. subtilis* propagation

*B. subtilis* S499 strain was obtained from the Walloon Center of Industrial Biology (CWBI) and grown as described by Bwanganga *et al.* (2012; 2013). After cell counting under a microscope in a Burker cell, the culture was diluted in distilled water to obtain $10^8$ cells $ml^{-1}$.

### 6.2.2. Phytohormone extraction and assay

The extraction of phytohormones (IAA and GA3) was performed as described by Ünyayar *et al.* (1996). 10 ml of *B. subtilis* $10^8$ cells $ml^{-1}$ culture was obtained (pH adjusted to 2.5). Extraction was performed 3 times with 15 ml of ethyl acetate and the ethyl acetate phase containing the free form of IAA, GA3 and ABA was recovered. The aqueous phase was adjusted to pH = 7 and the extraction was performed 3 times with ethyl acetate (15 ml); the ethyl acetate phase containing free zeatin was recovered. The aqueous phase was adjusted to pH = 11 and hydrolysed for 1 hour at 70°C: the ethyl acetate phase containing bound zeatin was recovered. The aqueous phase was adjusted to pH = 7 and the extraction was performed 3 times with ethyl acetate (15 ml); the ethyl acetate phase containing bound forms of IAA, GA3 and ABA was recovered. All recovered phases were mixed and the solvent was removed under vacuum. The extract was lyophilized and resuspended in 10 ml of 50% methanol prior to analysis by RP-HPLC-DAD (Agilent 1200 series) on Prevail Select C-18 column (15 cm × 3.0 mm, 3 µm) thermostatized at 30°C: an isocratic gradient with 35% MeOH (in 1% acetic acid) for IAA and GA3 with a flow rate of 0.4 ml/min was used and detection was performed by UV (280, 254 nm, respectively). Calibration curves were obtained with GA3 and IAA from Sigma-Aldrich and spiked solutions were prepared before phytohormone extraction by adjusting 2 bottles containing 0.3125 and 0.625 mg of IAA plus 0.281nd 0.563 mg of GA3 to 10 ml with $10^8$ cells $ml^{-1}$ *B. subtilis* culture (obtained as described above). The phytohormone extraction and assay were performed as described previously.

### 6.2.3. Red sorghum malting

The red sorghum cultivar used was obtained from the D.R. Congo and has been described previously (Bwanganga *et al.*, 2012; 2013). Four steeping treatments were used hencefold 'bio- steeping', 'phyt. steeping', 'GA3 treatment' and IAA treatment. Sorghum malts were obtained as described by Bwanganga *et al.* (2012; 2013) by manual sorting, steeping at 30°C for 8 h in 0.2% NaOH followed by resteeping for 8 h in the biocontrol steep liquor, containing a *B. subtilis* culture at $10^8$ cells $ml^{-1}$, for the 'bio. steeping', in distilled water containing 6.8 ppm of IAA plus 2.7 ppm of GA3 for the 'phyto steeping' treatment, in distilled water containing 2.7 ppm of GA3 for the 'GA3 treatment' and in distilled water containing 6.8 ppm of IAA for the 'IAA treatment' (50 g of grain was steeped in 100 ml of steeping solution); 8 h resteeping in distilled water was used as the control. Considering the predicted values of the models proposed in Chap. IV and Bwanganga *et al.* (2013), respectively, for $\alpha$- and $\beta$-amylase activities (Figure 31 A and B), a germination temperature of 30°C and germination time of 84 h were used for this study ($\alpha$-amylase activity + $\beta$-amylase activity > 300 U/g for $\beta$-amylase activity value > 50 U/g). Green malts were kilned for 48 h at 40°C. In fact, according to Bwanganga *et al.* (2013) at around 48 h of germination, the total malting loss increases almost exponentially and beyond 84 h of germination there is no further considerable increase in $\beta$-glucanase activity (see Chap.

VI). The effect of phytohormone application time and steeping liquor renewal on *α*-amylase activity was evaluated as follows: steeping was conducted in distilled water and IAA and GA3 was added after 4, 8, 12 h in steeping liquor so as to obtain 6.8 and 2.7 ppm respectively, for the not-renewed steeping liquor treatment (N-RSL). For the renewed steeping liquor treatment (RSL), the distilled water used as the steeping liquor was substituted after 4, 8, 12 h steeping by a solution containing 6.8 and 2.7 ppm of IAA and GA3 respectively, in distilled water.

#### 6.2.4. Enzyme activity assays

*α*- and *β*-amylase activities were assayed using Megazyme methods (K-CERA Ceralpha method for *α*-amylase activity and Betamyl Method for *β*-amylase). *β*-glucanase extraction was carried out using 3 methods as described in Chap. VII. For method 1, the assay was performed at 40°C in a reaction mixture containing 0.5 ml extract and 0.5 ml of 2% carboxymethylcellulose as a substrate. For method 2, *β*-glucanase activity was assayed using the Megazyme Azo-Barley glucan - a chemically modified (to increase solubility), dye-labelled barley *β*-glucan (1% w/v) in 0.02% sodium azide was used as the substrate (Megazyme S-ABG100 03/11). The Megazyme assay procedure for cereal flours and malt endo-(1,3)-*β*-glucanase activity was used as method 3.

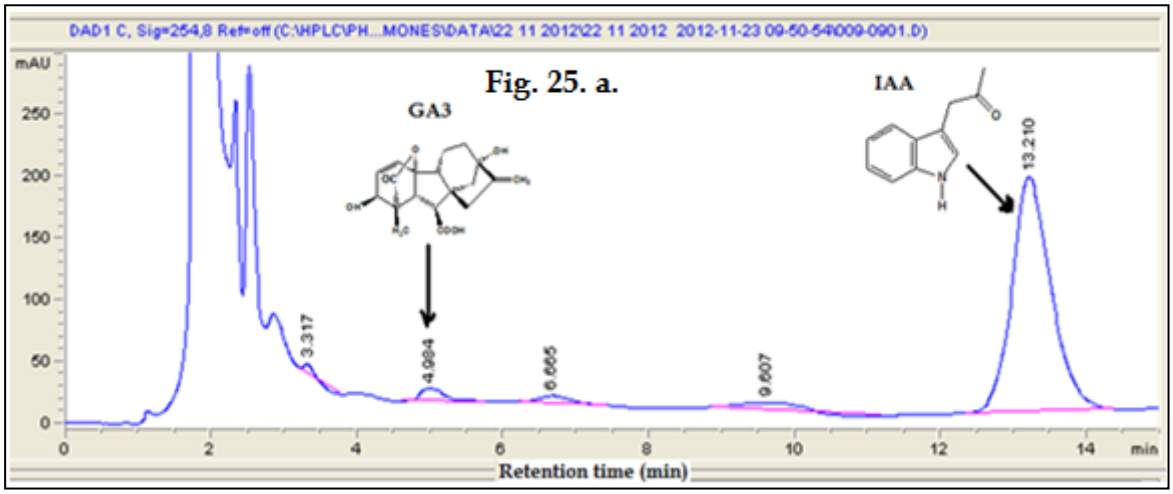
#### 6.2.5. ABA and GA diffusion and cross-talk

Diffusion of molecules in the grains is an important phenomenon that can help to explain in part why two signalling molecules that are present in the grain behave differently over time. Knowledge of modes of phytohormones diffusion may also elucidate the response of different parts of the grain to signalling molecules. To evaluate ABA and GA3 diffusion and cross-talk in the malted grain, red sorghum grains (150 g) were steeped in soaking liquor containing respectively 1000 ppm of GA3 and 1000 ppm of ABA. Grains steep-out moisture was calculated as described by Bwanganga *et al.* (2012). At the corresponding steeping time, steep-out grains were dried à 40°C for 48 h. 100 g of dried grains flour was obtained by grinding kilned malt in an IKA mill followed by sieving (mesh size: 0.5 mm) and the extraction of phytohormones [Abscisic acid (ABA) and gibberellic acid (GA3) was performed as described previously. All recovered phases were mixed and the solvent was removed under vacuum. The extract was lyophilized and resuspended in 10 mL of 50% methanol prior to analysis by RP-HPLC-DAD on Prevail Select C-18 (15 cm × 3.0 mm, 3 µm) thermostatized at 30°C: isocratic gradient with 35% MeOH (in 1% acetic acid) for GA3 and 55% MeOH (in 0.1M acetic acid) for ABA with a flow rate of 0.4mL/min and detection was performed by UV (254 and 265 nm), respectively. Calibration curves were obtained with ABA and GA3 from SIGMA-ALDRICH and spiked solutions were prepared before phytohormone extraction by adding ABA and GA3. In order to study ABA and GA3 cross-talk, 100 g of red sorghum were soaked in 200 mL of GA3 (1000 ppm) to which was added ABA so to obtain respectively 62.5, 125, 250, 375, 500, 625, 750, 875, 1000 ppm of ABA. Malting

was carried out as described above (16 h steeping at 30°C, 84 h germination at 30°C and 48 h kilning at 40°C) and malt $\alpha$- and $\beta$-amylase activities were extracted and assayed using the corresponding cited Megazyme method.

### *6.3. Results*

Phytohormones retention times were around 14 and 5 min respectively for IAA and GA3 (Figure 25.a.) and around 6 min for ABA (Figure 25.b.).



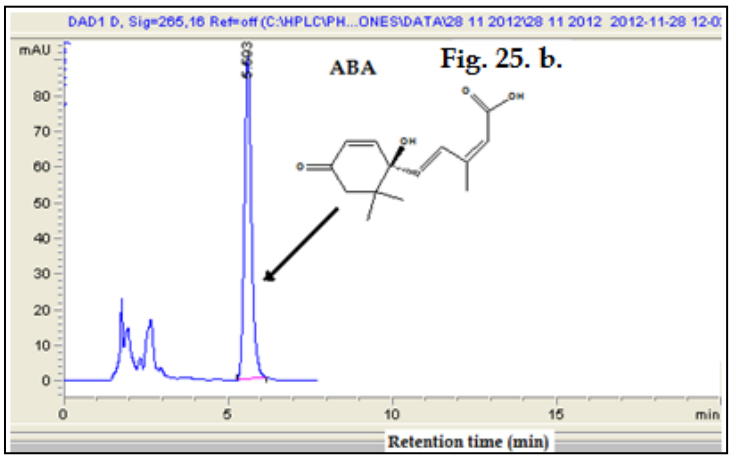




**Figure 25. (a) *B. subtilis* $10^8$ cells/ml culture spiked with 125 ppm IAA and 125 ppm GA3 chromatogram (UV detection at 254 nm). Calibration curves equations were: pick area = 57.407 × concentration (ppm) with R-sq = 0.9992 for IAA and pick area = 2.2104 × concentration (ppm) with R-sq = 0.9997 for GA3 and ; (b) ABA retention time, calibration curve equation was: pick area = 188,74 × concentration (ppm) with R-sq = 0,9997.**

Lipopeptide levels in the *B. subtilis* cell-free supernatant were approximately: surfactins (385.21 µg/ml); iturins (138.38 µg/ml); fengycins (264.54 µg/ml) (Bwanganga *et al.*, 2012). Spiked solutions were prepared as described in the materials and methods section and the recovered percentages (spiking and calibration curve slope ratios) were 47.8, 50.1 and 62.5% respectively for IAA, GA3 and ABA (see Figure 26 a and b for IAA and GA3, and Figure 32 for ABA).

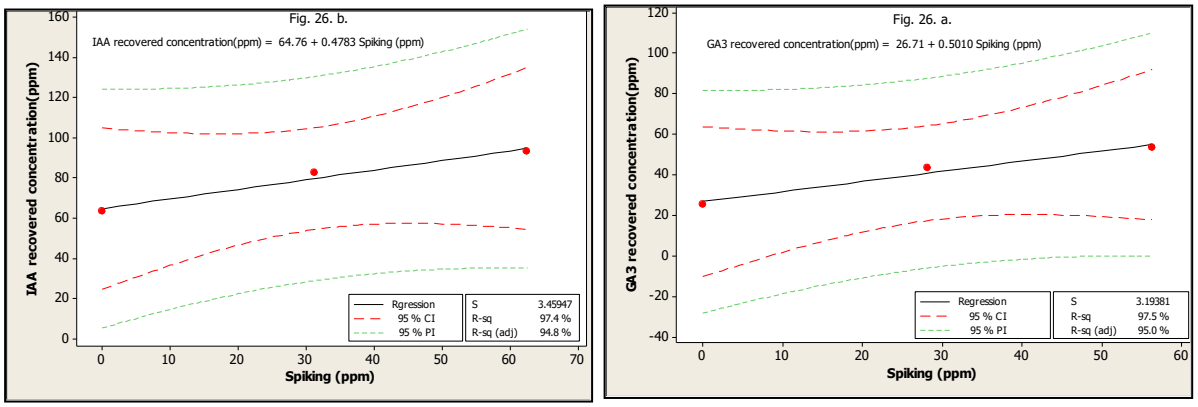



**Figure 26. IAA and GA3 concentration in spiked *B. subtilis* $10^8$ cells/ml culture**

Malt properties in terms of *α*-amylase, *β*-amylase and *β*-glucanase activities are presented in Figure 27. The most abundant phytohormone produced by the *B. subtilis* strain was IAA (around 6.8 ppm) against approximately 2.7 ppm for GA3 (Figure 26). Figure 27 shows that, except for *β*-(1,3)-(1,4)-glucanase, all studied enzyme activities were influenced by the malting treatment. The use of *B. subtilis* as well as phytohormones induced an increase in the synthesis of the studied enzymes dependent upon their type (Figure 27). *α*-amylase, *β*-(1,4)- and *β*-(1,3)-glucanase activities were strongly influenced by the steeping treatments - (256, 635 and 167% increase respectively when the 'biocontrol steeping' was used as opposed to a 20, 202 and 45% increase respectively when the 'phytohormone steeping' was used – compared with $H_2O$). *β*-amylase and *β*-(1,3)-(1,4)-glucanase activities were the least influenced (44 and 9% increase respectively when the biocontrol was used as opposed to a 11 and 6% increase respectively when the 'phytohormone steeping' was used, compared with $H_2O$).

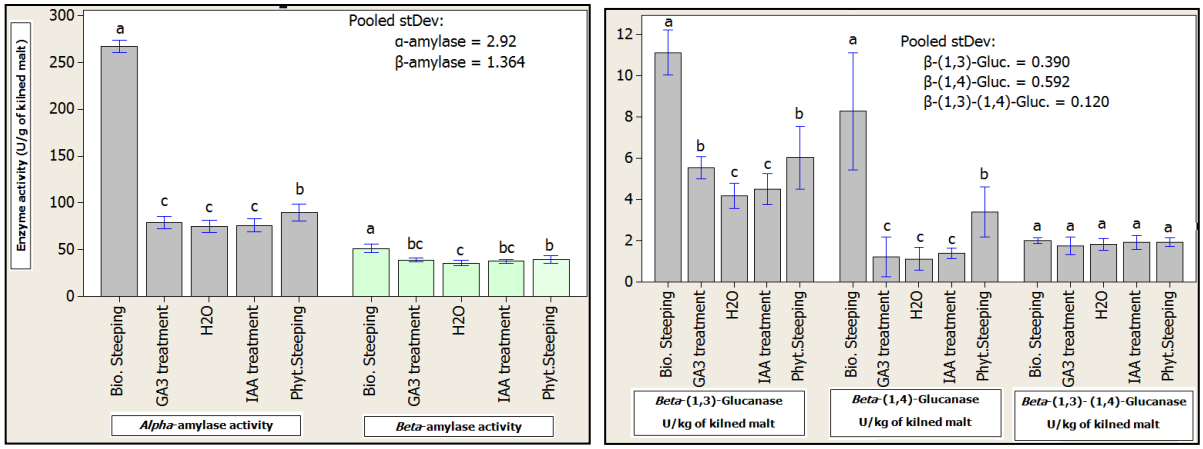



**Figure 27. Enzyme activities (*α*-amylase, *β*-amylase, *β*-(1,3)-, *β*-(1,4)- and *β*-(1,3)-(1,4)- glucanase activities) as affected by steeping treatments**

We tried then to answer the question of why some enzymes are they affected more than others. The problem of the diffusion of the signaling molecules, the possible interactions between the phenolic compounds and the phytohormones and GA-ABA cross-talk were then studied. The effect of the

application time of IAA and GA3, and the renewal or not of the steeping liquor on *α*- and *β*-amylase activities is presented in Figure 28, and the associated 2-Way ANOVA are presented in Table 25. Sorghum grains diffused ABA and GA3 concentrations are presented in Figure 29 (see Figure 32 for ABA spiked solution concentration). To elucidate how GA and ABA cross-talk affects the *α*- and *β*-amylase activities, the effect of the addition of ABA in a soaking liquor containing 1000 ppm of GA3 on *α*- and *β*-amylase activities is presented in Figure 30.

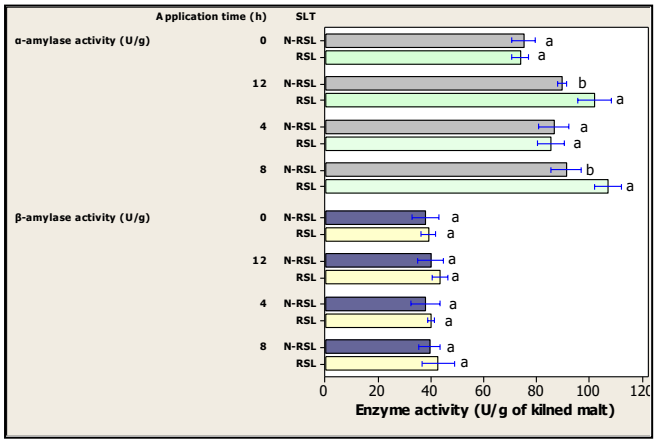



**Figure 28. *α*-and *β*-amylase activities as affected by IAA and GA3 application time and steeping liquor treatment (SLT). N-RSL: Not Renewed Steeping Liquor; RSL: Renewed Steeping Liquor. For each application time, SLT having a letter in common are not statistically different according to Tukey's HSD test (p<0.05)**

**Table 25. 2-way ANOVA for *α*-and *β*-amylase activities (95% confidence interval)**

| Source | DF | *α*-amylase activity | | *β*-amylase activity | |
|---|---|---|---|---|---|
| | | F | p | F | p |
| **Application time (h)** | 3 | 194.23 | 0.000 | 4.63 | 0.016 |
| **Steeping liquor treatment** | 1 | 64.62 | 0.000 | 11.88 | 0.003 |
| **Interaction** | 3 | 31.64 | 0.000 | 0.67 | 0.585 |
| **Error** | 16 | | | | |
| **Total** | 23 | | | | |
| | | | S = 1.954 | | S = 1.775 |
| | | | R-sq = 97.89% | | R-sq = 63.44% |
| | | | R-sq (adj) = 96.97% | | R-sq (adj) = 47.45% |

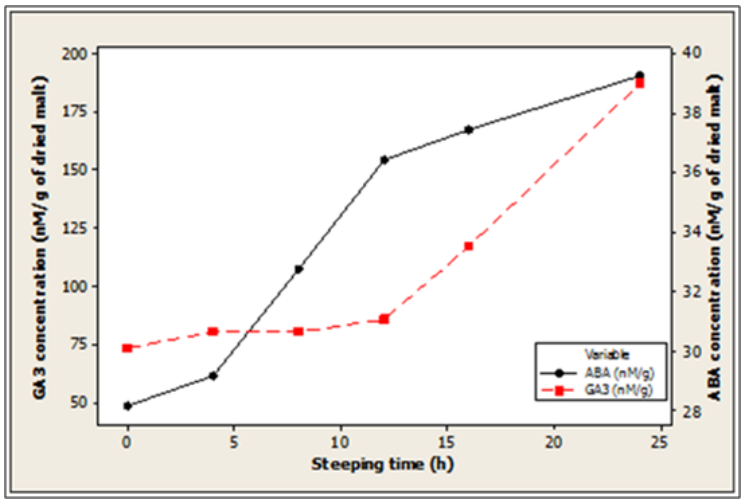



**Figure 29. GA3 and ABA diffusion during red sorghum steeping**

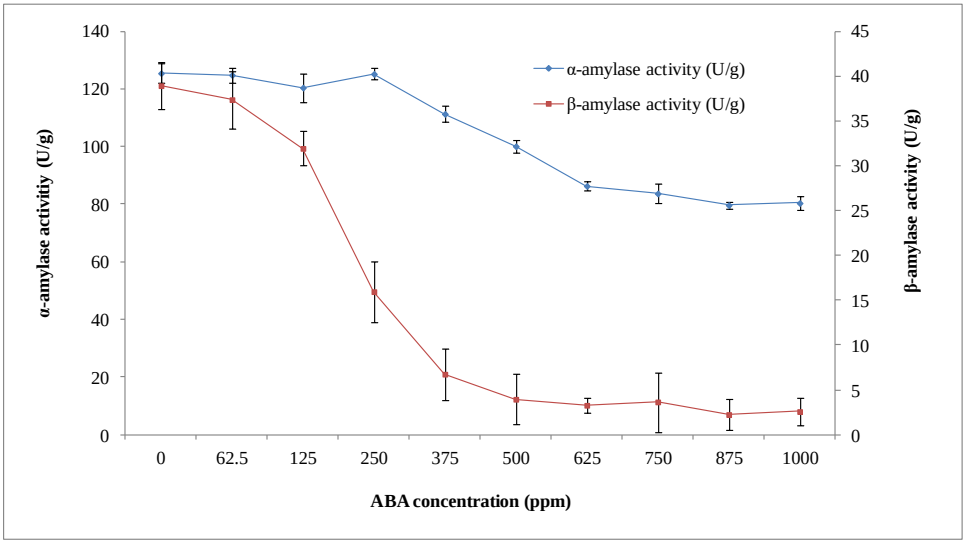



**Figure 30. Effect of GA3 and ABA cross-talk on *α*-and *β*-amylase activities**

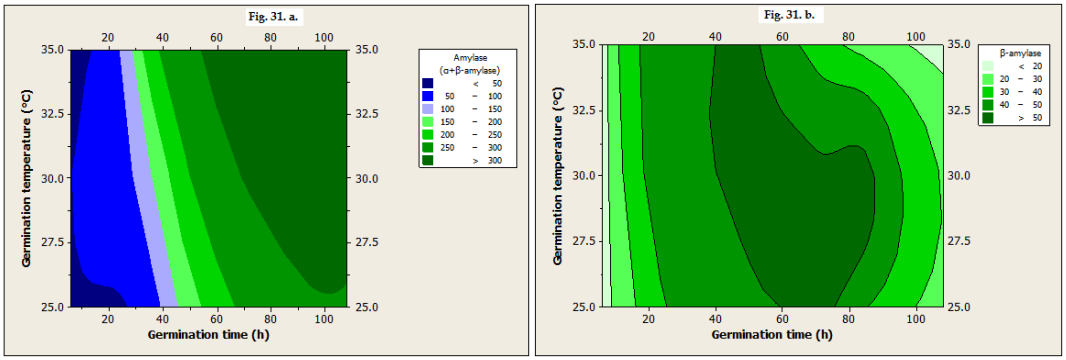



**Figure 31. *α*+*β*-amylase (a) and *β*-amylase (b) surface plots as affected by germination time and temperature (Bwanganga *et al.*, 2013, and Chap IV)**

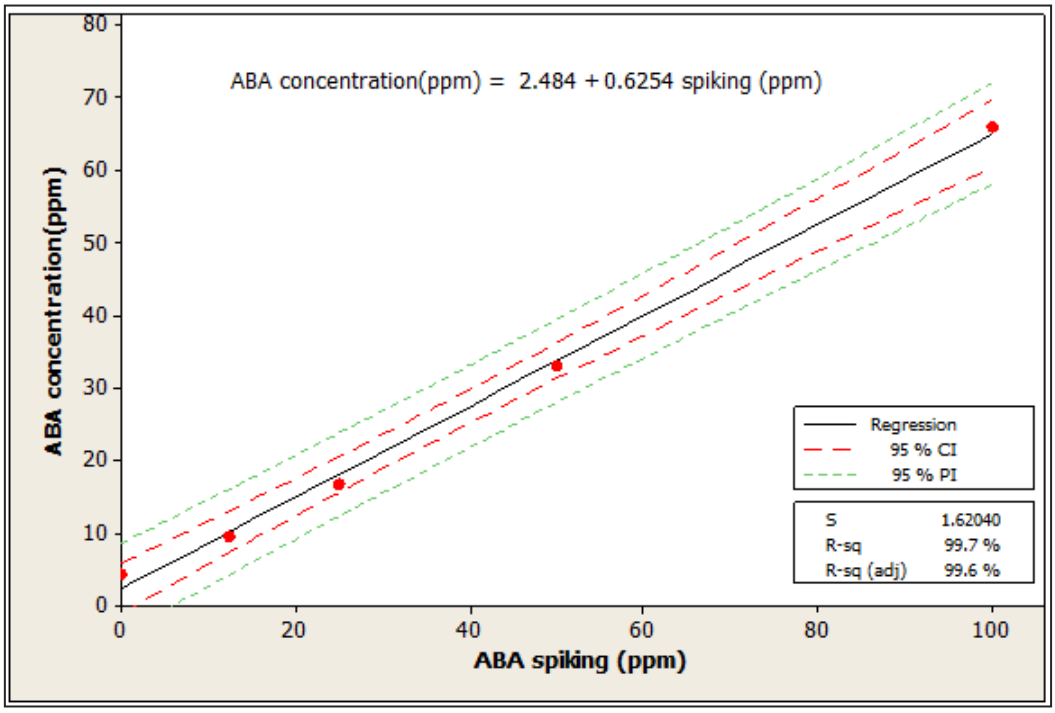



**Figure 32. ABA concentration in spiked red sorghum steeped grains flour [pick area = 188.74 × concentration (ppm) with R-sq = 0.9997]**

### *6.4. Discussion*

Plant-microbe interactions induce changes in metabolic, physiological and gene regulation in both plant and microbes and are summarized in what is often called 'molecular dialogue'. Indeed, phytohormones are considered to play a major role in these interactions. An important phenomenon is related to the difference between enzymes, in terms of the degree of activation of their synthesis, in different malting conditions. This difference is possibly due to location of their production (i.e. aleurone and/ or scutellum). Aisien *et al.* (1983) and Aisien and Palmer (1983), reported that the scutellum is the major site of *de novo* $\alpha$-amylase synthesis during sorghum malting. This location of enzyme synthesis was determined by separately incubating the embryo and the endosperm of soaked sorghum. However, enzyme synthesis by the cells of the aleurone layer is also related to the metabolism of the embryo - these two parts of the grain do not work in isolation. This is shown by the results obtained by Aisien *et al.* (1983); using whole grain, the development of the starch-degrading enzyme in the endosperm was found to be dependent on its synthesis in the embryo tissue of the germinated grain. According to the results of Figure 27 we suggest that enzymes are synthesized in one part of the grain where the cells are more sensitive to activation by exogenous signalling molecules (which, in our opinion, would be the aleurone) and another part where the effect of exogenous signalling molecules is perceived to be less (which, in our opinion, would be the scutellum). This is in agreement with the results obtained by Bwanganga *et al.* (2013) and in Chap. IV which show that the repression phase of $\beta$-amylase synthesis starts faster than that of $\alpha$-amylase synthesis under the same malting conditions, suggesting that these two enzymes do not have the same

major expression site. In fact, aleurone and scutellum cells don't have the same sensitivity to hormone signalling. It is known, for example, that gibberellins promote *α*-amylase expression in both the aleurone and the scutellum, but sugars repress GA-induced expression only in the scutellum and that this sugar repression cannot be counteracted by additional GA (Perata *et al.*, 1997). In addition, as can be seen (Figure 30), the repression of *β*-amylase synthesis by exogenous ABA for example is greater than that of *α*-amylase. The *β*-amylase activity was dramatically affected by the concentration of ABA (up to 94% reduction of its synthesis when the soaking liquor contains 750 ppm ABA against approximately 57% reduction in the synthesis of *α*-amylase). Our results show also that, *β*-(1,3)-(1,4)-glucanase isn't strongly influenced by the steeping treatment while *β*-(1,3) - and *β*-(1,4)-glucanases are. The efficiency of the use of phytohormones, perceived as exogenous stress by the grain during malting, whether produced by microorganisms or not, would be highly influenced by the sensitivity of the major site of the enzyme synthesis. Indeed, the expression sites of isoenzymes may differ. While the isoform EI (*β*-(1,3)-(1,4)-D-glucanase) is transcribed in the scutellum and aleurone of germinated grain, the expression site of the isoenzymes EII (*β*-(1,3)-(1,4)-D-glucanase) and GII (*β*-(1,3)-D-glucanase) is the aleurone (Hrmova and Fincher, 2009). The fact that the expression sites of *β*-(1,3)-D-glucanase are root and leaves for isoforms EI and EII, and aleurone for the isoform EII (Hrmova and Fincher, 2009), in addition to the fact that this enzyme presents no specificity for grain *β*-(1,3)-(1,4)-D-glucan, combined with the fact that its synthesis is greatly improved by the presence of microbes suggests that it is an enzyme involved in plant-microbe interactions (the often called *Pathogenesis-Related* Proteins).

Ours results show also that the time of phytohormone application and the renewal or not of steeping liquor have a significant effect on the grain *α*- and *β*-amylase activities while their first interaction affects only the *α*-amylase activity (Table 25). Several hypotheses can be taken into account when considering this effect on enzyme synthesis; among others are the physiological state of the grain and the negative effect of polyphenols leached in steeping liquor on phytohormone perception. In fact, the response of the germinated grain can be different depending on the diffusion kinetics of signalling molecules in the malted grain (Figure 29 for example shows that ABA and GA3 diffuse differently during steeping). Indeed, the developmental context of the cell is recognized to have an influence on hormone signalling in plants which can also impinge on hormone cross-talk but, according to Chow and McCourt (2003) this developmental context effect has seldom been questioned in hormone signalling. It can be seen that from 16 h soaking the diffusion rate of GA3 increases, while the germ absorbs almost no additional ABA (Figure 29). Indeed, at around 16 h soaking, the percentage of chitted grains increases (when steeping is conducted with aeration) (Bwanganga *et al.*, 2012), thus facilitating the diffusion of GA3 due to partial hydrolysis of both the aleurone layer and endosperm cell walls by synthesized hydrolytic enzymes. During steeping, for example, it is known that grain rehydration in the first stage of germination is physical, resulting in softening or rupturing of the seed

coat and an increase in the seed volume; when the plant metabolic reactions start, the absorption of water becomes more regulated (Chong *et al.*, 2002). So, it is therefore understandable that the diffusion of molecules dissolved in the soaking liquor is dependent on the physiological state of the seed. The knowledge of the phytohormones transport mechanisms across different plant tissues has been a major advance in the understanding of their effects on the target cells. It is known that, during germination, hormones are synthesized or activated in the embryo and then transported into the aleurone layer mainly through apoplastic and symplastic pathways (a tiny percentage of GA4 only being found in the starchy endosperm) (Atzorn and Weiler, 1983). Phytotormones can then trigger the production of enzymes within the aleurone layer. As we mentioned earlier, after enzymes are synthesis, they hydrolyze both the aleurone layer and the endosperm cell walls, thus facilitating the free diffusion of phytohormones and enzymes in the grain partially modified. Also, the chemiosmotic model of polar auxin transport, for example, is well known (Hopkins, 2003). The acidification necessary for this transport is achieved in the endosperm by acids secreted from the aleurone layer only during germination (Simpson, 2001). Therefore, it is understandable that the chemiosmotic transport of auxins is only possible during germination. Additionally, during sorghum steeping, high quantities of phenolic compounds are released in the steeping liquor and can play an important role in the plant-microbe dialogue. Certain plant phenolics are suggested to be cofactors of peroxidase functioning as an IAA oxidase which destroys the IAA (e.g. monohydroxy B-ring flavonoids), whereas dihydroxy B-ring forms act as inhibitors of the IAA degrading activity (Lattanzio *et al.*, 2006, Tsavkelova, 2006). It has been shown that the process of polar auxin transport can be inhibited by a group of phenolic compounds which apparently act by binding to auxin-binding proteins known as the naphthylphthalamic acid (NPA) receptors (Jacobs and Rubery, 1988). Hence, the difference between the N-RSL and RSL is probably due, among other factors, to the removal of phenolic compounds during steeping and the effect of the phytohormone application time due to the physiological state of the grain (endosperm acidification being possible after acids released by the aleurone layer cells during grain germination), and the interaction of these two factors.

As shown in Figure 25. a, *B. subtilis* S499 produces both IAA and GA3. It is universally accepted that *B. subtilis* produces IAA but the GA3 production is rather controversial. Indeed, very few studies have reported the production of GA3 by *B. subtilis* (Tang, 1994 cited by Kilian *et al.*, 2000; Sgroy *et al.*, 2009).

When such a biocontrol strategy is in place, grain-microbe interactions are far from unsophisticated. This makes it difficult to determine the significance of the difference found between the use of the biocontrol and that of phytohormones (IAA and/or GA3) (Figure 27) in the sense that, even if a complete molecular screening of the steeping liquor was performed, it would be utopian to believe in the replacement of a living being, in this case the population of *B. subtilis* used, by a mixture of

molecules. Indeed, it is known that the cross-talk is a source of signal amplification as well as signal complexity and explains how the small number of plant hormones can elicit such a wide variety of cellular responses (Bethke *et al.*, 1995). In fact, the dynamic nature of plant-microbe molecular dialogue makes the understanding of the sequence of events more complex, when considering: the secretion of virulence factors and various resistance effector responses in the plant, changes in the microorganism virulence, regulation of gene expression and a whole series of reactions - from simple to more complex. In the other hand, it is also conceivable that when *B. subtilis* is in contact with the malted grain, the production of the phytohormones by *B. subtilis* is probably higher than that obtained after growth on optimized Landy broth.

### *6.5. Conclusions*

*B. subtilis* can produce a range of phytohormones such as IAA and GA3; when these phytohormones are dissolved in the steeping liquor, malt enzyme activities are improved but are still lower than those obtained with the biocontrol steeping. The effect of the biocontrol as well as distilled water containing phytohormones (IAA and GA3) on malt enzyme activities is dependent on the enzyme in question. The time of application of GA3 and IAA and its interaction with the renewal of the steeping liquor have been found to have significant effects on $\alpha$-amylase synthesis. In the case of sorghum, given the high content of phenolic compounds, a phenomenon yet to be elucidated is phytohormone diffusion through the aleurone layer, when the biocontrol or phytohormones are used, and the possible interaction between polyphenols and phytohormone diffusion. Phytohormone diffusion has already been partially studied in the case of barley for abscisic acid and gibberellic acid with a view that the diffusion takes place along the apical-basal axis of the grain (Bruggeman *et al.*, 2001). Recently, it has been shown that the growth, yield, antioxidant activity and phenolic content of lentil plants, for example, were affected by IAA, GA3 and others plant growth regulators (Giannakoula *et al.*, 2012).

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# Chapter VII. Enhancing indole 3-acetic acid production by *Bacillus subtilis* S499

## *Présentation du chapitre*

Les résultats du chapitre 6 nous ont montré que *B. subtilis* S499 produit de l'acide indole acétique. Cette production reste pourtant faible comparée aux quantités produites par d'autres souches de *B. subtilis*. Dans ce chapitre (chapitre 7) un essai d'optimisation la production de l'acide indole acétique par *B. subtilis* S499 est réalisé. Les effets de la durée d'incubation, du pH, de la concentration en tryptophane et du moment d'application du tryptophane sont élucidés.


## *Abstract*

Several studies have shown that *Bacillus subtilis* produces indole 3-acetic acid (IAA) (Swain and Ray, 2008; Sgroy *et al.*, 2009). It has also been shown that this production can be improved by the addition of L-tryptophan and the choice of the growth conditions of the strains used (see Swain and Ray, 2008). Our previous work showed that when *B. subtilis* S499 is grown on Landy medium, the production of IAA is very low. In this work we attempt to enhance the production of IAA by *B. subtilis* using a Box-Behnken experimental design (factors = 3 and replicates = 1). The factors selected in this study were: the incubation duration (*DI*), the tryptophan concentration ([*Tryp*]) and *pH*. The bacterial growth ($OD_{600\ nm}$), the percentage of sporulation and the production of IAA were examined for an incubation period of 168 h. The effect of each factor was studied and the results show that the following factors: [*Tryp*], [*Tryp*]*[*Tryp*], *pH*DI* and *pH*[*Tryp*] don't have a significant effect on the production of IAA by *B. subtilis* S499. The effect of *pH* and that of the L-tryptophan application time on the production of IAA were also determined.


### *7.1. Introduction*

Our previous work showed that when *B. subtilis* is grown in Landy medium, the production of indole-3-acetic acid (IAA) is low (about 7µg/mL). Some strains of *B. subtilis* have also been used as biofertilizers and one of the molecules presumed to cause the effect of *B. subtilis* on plant physiology is IAA (Kilian *et al.*, 2000). So, the objective of this study is to improve the production of IAA by *B. subtilis* S499.

### *7.2. Choice of factors and experimental design*

The biosynthesis of IAA is a tryptophan-dependent pathway (both in plants and microorganisms) despite the highlighting of tryptophan-independent pathways (Baca and Elmerich, 2007, Spaepen *et al.*, 2007). Apart from genetic factors, among the environmental factors which influence the production of IAA in bacteria include acidic pH, osmotic and matrix stress, and carbon limitation

(Spaepen *et al.*, 2007). To study the effect of different factors on the production of IAA, *B. subtilis* S499 was obtained from the Walloon Center of Industrial Biology (CWBI) and grown on Luria broth agar at 37 °C for 24 h. An inoculating loopful was transferred to 100 mL Landy broth and incubated for 16 h. Finally, 3 mL was transferred to 100 mL Landy broth and incubated at 30 °C (with rotary shaking at 130 rpm) for 168 h.

In this study the production of IAA is described as dependent on the incubation duration (*DI*), the *pH* and the concentration of L-tryptophan added to the medium ([*Tryp*]) − the basic medium being Landy broth. An experimental Box-Behnken design was first used to assess the effect of factors (*DI*, *pH* and [*Tryp*]) on the production of IAA by *B. subtilis*. The study of regression allowed the determination of the conditions of *DI*, *pH* and [*Tryp*] which maximize the production of IAA. The effect of [*Tryp*] was then studied throughout the incubation period. The effect of *pH* was studied for an incubation period of 168 h at pH 5, 6, 7, 8 and 9. After selection of the optimum *pH*, the effect of the L-tryptophan application timing was then examined at *pH* = 7.3 for an incubation period of 168 h. To study the effect of L-tryptophan application timing, L-tryptophan was added after 48 and 72 h of incubation for the total incubation duration of 168 h.

### *7.3. Results*

The effect of *DI*, *pH*, [*Tryp*] and their first order interactions on the production of IAA is presented in Table 26. This table shows that at a 5% threshold the following factors don't have significant effects statistically, namely: [*Tryp*], [*Tryp*]*[*Tryp*], *pH***DI* and *pH**[*Tryp*]. The regression analysis of the IAA with *DI*, *pH* and [*Tryp*] as factors is presented in Table 27. The effect of the *pH* on the production of IAA by *B. subtilis* S499 is shown in Figure 33.

**Table 26. ANOVA for indole 3-acetic acid using Box-Behnken experimental design (factors = 3; replicates = 1)**

| Source | DF | F | p |
|---|---|---|---|
| **Regression** | **9** | **234.52** | **0.000** |
| **Linear** | 3 | 52.07 | 0.000 |
| *DI* | 1 | 81.63 | 0.000 |
| *pH* | 1 | 49.51 | 0.001 |
| [*Tryp*] | 1 | 0.06 | 0.821 |
| **square** | **3** | **52.88** | **0.000** |
| *DI***DI* | 1 | 79.26 | 0.000 |
| *pH***pH* | 1 | 48.05 | 0.001 |
| [*Tryp*]*[*Tryp*] | 1 | 0.77 | 0.421 |
| **Interaction** | **3** | **4.70** | **0.008** |
| *DI***pH* | 1 | 13.19 | 0.775 |
| *DI**[*Tryp*] | 1 | 0.09 | 0.002 |
| *pH**[*Tryp*] | 1 | 38.52 | 0.372 |
| **Residual Error** | 5 | | |
| **Lack of fit** | 2 | 0.87 | 0.504 |
| **Pure Error** | 3 | | |
| **Total** | 14 | | |

The effect of *DI* and [*Tryp*] on the production of IAA by *B. subtilis* S499 was evaluated at *pH* = 7.3 and results are presented in Figure 34, parallel to the growth of *B. subtilis* and the sporulation

evaluated as the percentage of resistant cells to a heat treatment of 80 °C for 15 mins. The model equation associated with the ANOVA of Table 26 is:

$$[\boldsymbol{AIA}] = -\mathbf{12.6682} + \mathbf{0.0755}\,\boldsymbol{DI} + \mathbf{4.0237}\,\boldsymbol{pH} - \mathbf{0.0134}\,[\boldsymbol{Tryp}] - \mathbf{0.0002}\,\boldsymbol{DI}^2 - \mathbf{0.2775}\,\boldsymbol{pH}^2 - \mathbf{0.0009}\,[\boldsymbol{Tryp}]^2 - \mathbf{0.0003}\,\boldsymbol{DI} \times \boldsymbol{pH} + \mathbf{0.0012}\,\boldsymbol{DI} \times [\boldsymbol{Tryp}] - \mathbf{0.0056}\,\boldsymbol{pH} \times [\boldsymbol{Tryp}] \quad \text{Eq. 22.}$$

This equation is derived from Table 27 and is used to calculate the values of *DI*, *pH* and [*Tryp*] which maximize the production of IAA by *B. subtilis* S499 ($\partial$[*IAA*]/$\partial$*DI* = 0, $\partial$[*IAA*]/$\partial$*pH* = 0 and $\partial$[*IAA*]/$\partial$[*Tryp*] = 0 under the conditions of this study: *DI* = 0 to 168 h, *pH* = 5-9 and [*Tryp*] = 0 to 25 ppm). The experimental and predicted values of Eq. 22 are presented in Table 28. The root mean square error (RMSE) calculated as described in Chapters II, III and IV is 0.17 ppm. Figure 35 shows the effect of the L-tryptophan application time on the production of IAA by *B. subtilis* S499.

**Table 27. Analysis of the regression of indole 3-acetic acid concentration as affected by the incubation duration (*DI*), *pH* and the L-tryptophan concentration ([*Tryp*])**

| Terme | Coeff | Coef ErT | T | P |
|---|---|---|---|---|
| Constante | -12.6682 | 1.92811 | -6.570 | 0.001 |
| *DI* | 0.0755 | 0.00835 | 9.035 | 0.000 |
| *pH* | 4.0237 | 0.57182 | 7.037 | 0.001 |
| [*Tryp*] | -0.0134 | 0.05614 | -0.239 | 0.821 |
| *DI*DI* | -0.0002 | 0.00002 | -8.903 | 0.000 |
| *pH*pH* | -0.2775 | 0.04004 | -6.932 | 0.001 |
| [*Tryp*]*[*Tryp*] | -0.0009 | 0.00102 | -0.876 | 0.421 |
| *DI*pH* | -0.0003 | 0.00085 | -0.301 | 0.775 |
| *DI**[*Tryp*] | 0.0012 | 0.00019 | 6.206 | 0.002 |
| *pH**[*Tryp*] | -0.0056 | 0.00573 | -0.979 | 0.372 |

S = 0.286470 ; R carré = 99.76 % ; R carré (ajust) = 99.34 %

**Table 28. Experimental and predicted values of indole 3-acetic acid concentration ([IAA]) produced by *B. subtilis* S499 as affected by the incubation duration (*DI*), the *pH* and the L-tryptophan concentration ([Tryp]) (Box-Behnken design, factors = 3, replicates = 1)**

| OrdEssai | *DI* | *pH* | [*Tryp*] | [*AIA*] exp. | [*AIA*] pred. |
|---|---|---|---|---|---|
| **1** | 84 | 7 | 12.5 | 6.7 | 7.1 |
| **2** | 0 | 5 | 12.5 | 0.0 | -0.1 |
| **3** | 84 | 5 | 0.0 | 5.2 | 5.3 |
| **4** | 84 | 7 | 12.5 | 7.1 | 7.1 |
| **5** | 84 | 7 | 12.5 | 7.3 | 7.1 |
| **6** | 168 | 9 | 12.5 | 9.1 | 9.2 |
| **7** | 84 | 9 | 25.0 | 6.3 | 6.1 |
| **8** | 84 | 9 | 0.0 | 5.9 | 5.8 |
| **9** | 168 | 5 | 12.5 | 9.3 | 9.1 |
| **10** | 84 | 5 | 25.0 | 6.1 | 6.2 |
| **11** | 0 | 9 | 12.5 | 0.0 | 0.1 |
| **12** | 84 | 7 | 12.5 | 7.4 | 7.1 |
| **13** | 168 | 7 | 25.0 | 11.7 | 11.7 |
| **14** | 168 | 7 | 0.0 | 8.6 | 8.6 |
| **15** | 0 | 7 | 25.0 | 0.0 | 0.0 |
| **RMSE = 0.17 ppm** | | | | | |

n.d. = non détecté

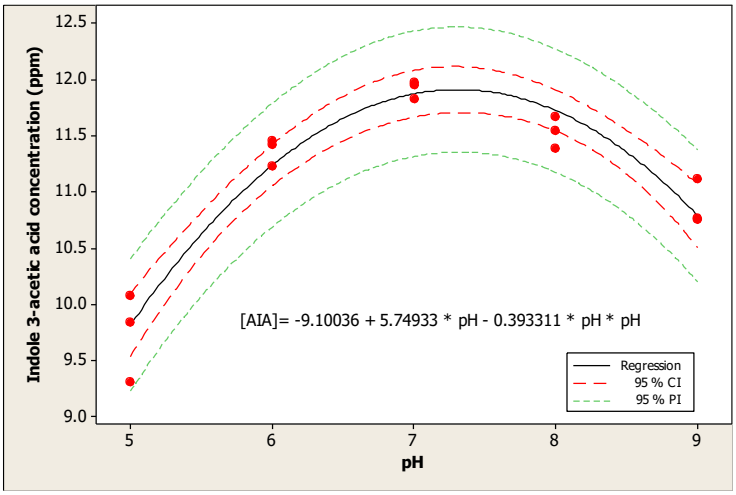


**Figure 33.Effect of *pH* on the production of indole 3-acetic acid by *B. subtilis* S499 (*DI* = 168 h and [*Tryp*] = 25 ppm)**

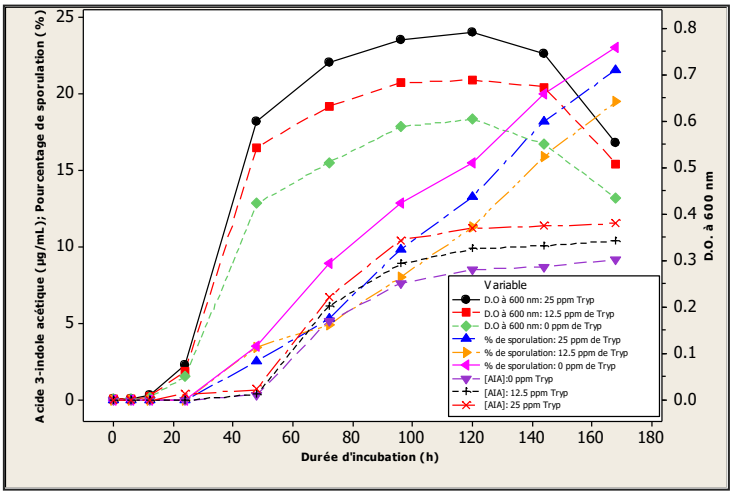


**Figure 34. *B. subtilis* S499 indole 3-acetic acid production (µg.mL$^{-1}$), cells growth ($OD_{600nm}$) and sporulation (the percentage of resistant cells to a heat treatment of 80 °C for 15 minutes) as affected by the incubation duration (*DI*) (h) and the L-tryptophan concentration (ppm) ([*Tryp*])**

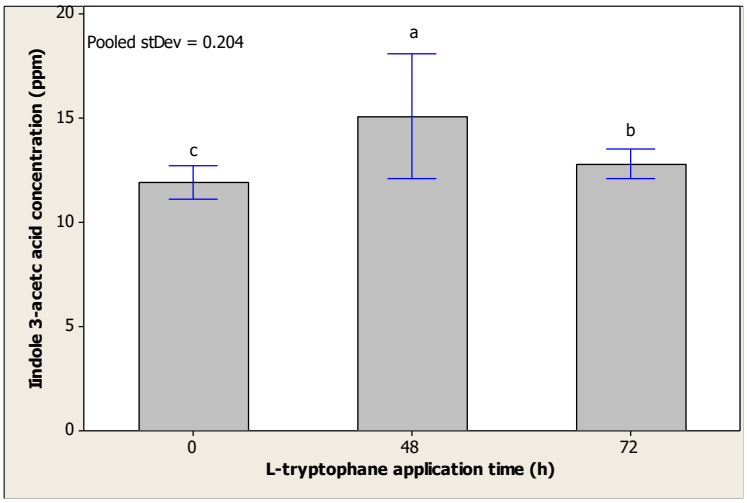


**Figure 35. *B. subtilis* S499 indole 3-acetic acid production as affected by the L-tryptophan application time (*DI* = 168 h, *pH* ≈ 7.3 and [*Tryp*] = 25 ppm)**

**Table 29. Analysis of the regression of indole 3-acetic acid concentration as affected by the *pH***

| Lack of fit | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **SS** | **MS** | **F** | **P** |
| Error | 12 | 0.677530 | 0.056461 | | |
| Lack of fit | 2 | 0.204695 | 0.102347 | 2.16 | 0.166 |
| Pure Error | 10 | 0.472836 | 0.047284 | | |

### *7.4. Discussion*

Our results show that the synthesis of IAA is effective in large quantities only from about 72 h of incubation duration (Figure 33). This is probably due to the decrease in the availability of nutrients in the medium − a decrease due to the increase in the density of the bacterial population, here expressed by a significant increase in $OD_{600\ nm}$. As shown in Figure 33 (percentages of sporulation), the production of IAA coincides with the sporulation phase of *B. subtilis* S499. In fact, it is known that the production of secondary metabolites by microorganisms is made possible in large quantities when the microbial population reaches a certain population density in the environment. In fact, the micro-organisms are able to perceive their density in an environment − a phenomenon referred to as "quorum sensing". This induced modulation of gene expression controls a number of phenomena such as the virulence and synthesis of secondary metabolites (Bernard *et al.*, 2007). The production of secondary metabolites starts when bacterial growth is limited by the supply of key nutrients (carbon, nitrogen or phosphorus) (Barrios-González *et al.*, 2003). Although the *B. subtilis* strain is known as non-hazardous to humans as well as plants, the reasons of IAA production should be considered carefully because they can be linked to pathogenesis as well as phytostimulation (Spaepen *et al.*, 2007). Our results (increased production of IAA concomitantly with sporulation) lead us to believe that sporulation plays a major role in the biosynthesis of IAA by *B. subtilis* S499. Indeed, in moulds for example, it has been clearly established that the transition from the vegetative to spore phase is highly regulated − and its initiation is strongly driven by the perception of the environment as well as microbial physiology (Yu and Keller, 2005; Brodhagen and Keller, 2006; Fox and Howlett, 2008) − and that the synthesis of secondary metabolites is highly dependent on the physiological state of the mould. In *B. subtilis*, perception of environmental and physiological signals, due to the depletion of nutrients, cell density, the Krebs cycle, synthesis and DNA damage, leads to the activation of a protein Spo0A regulatory transcription and ultimately leads to the process of sporulation (Stragier and Losick, 1996). However, it has been shown, for example, that the same G-protein allowed the transfer of information within the cell to regulate sporulation as well as the biosynthesis of secondary metabolites in *Aspergillus nidulans* (Hicks *et al.*, 1997). It thus becomes necessary to question the real physiological link between the production of IAA and *B. subtilis* S499 sporulation. It has been reported that the production of IAA by microbes is one of a number of different strategies to seduce

the plant partner (Spaepen *et al.*, 2007). The production of IAA in particular, and that of other phytohormones in general is, in our opinion, both an expression of the seduction as described by Spaepen *et al.*, 2007, but also a cry of distress that *B. subtilis* sends to its environment (the habitat of *B. subtilis* being the soil). Our results (Tables 26, 27 and 28; Figures 33, 34 and 35) show that the addition of L-tryptophane induces increased synthesis of IAA, but the differences between the different concentrations appear significant later (at around 72 h) (see Figure 33). It is known that tryptophan is the main precursor of auxin biosynthesis in bacteria (Spaepen *et al.*, 2007). It has been shown, for example, that the addition of 25 µg $mL^{-1}$ of tryptophan to the culture medium induced an increase in IAA production of approximately 256% by a strain of *B. subtilis* (Erturk *et al.*, 2010). Our results show, in addition, that the timing of the addition of L-tryptophan is important (Figure 35). Indeed, when the addition of tryptophan is carried out at *DI* = 48 h and *DI* = 72 h, the production of IAA increases by 27 and 8% respectively. This is possibly due to what has been shown by Smets *et al.* (2004): "when tryptophan is present in the culture medium, its concentration decreases significantly at the beginning of the growth of *Azospirillum brasilense* and achieves very low levels during the stationary phase of growth". Our results (Figure 34) also show that the production of IAA is a polynomial function of the *pH* and the optimal IAA production by *B. subtilis* S499 is achieved at *pH*≈7.3 when [*Tryp*] = 25 ppm and *DI* = 168 h. It has also recently been shown that the *pH* affected the growth, the sporulation and the production of secondary metabolites in *Aspergillus umbrosus* (Sood, 2011).

### *7.5. Conclusion*

The choice of *pH*, [*Tryp*] and *DI* is important to improve the production of IAA. Depending on the *pH*, IAA production follows a polynomial function with a maximum around *pH* 7.3. When tryptophan is applied to the medium at *DI* = 48 h and *DI*= 72 h, the production of IAA by *B. subtilis* S499 increases significantly when compared with the application at the beginning of *B. subtilis* growth. In summary, the application of 25 ppm of tryptophan at 48 h of incubation for an incubation period of 168 h at *pH* 7.3 can be considered useful for the production of IAA by *B. subtilis* S499.

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## General discussion

### *Bacillus subtilis* S499 biocontrol of mould

When malted sorghum is used, notably in making products for local consumption, such as beer, infant porridge and non-fermented beverages, a problem encountered is the presence of moulds liable to produce mycotoxins, representing a health hazard to consumers. The control of mould growth during sorghum malting is by chemical means (using acid or alkaline liquors) (Taylor *et al.*, 2006). The first attempt of the biocontrol of mould during sorghum malting is attributed to Lefyedi and Taylor (2007), who used lactic acid bacteria and yeasts, and achieved an acceptable reduction of total mould growth. The presence of lactic acid bacteria in the brewing environment is disadvantageous as this family of bacteria contains a large number of beer spoilage microorganisms: *Lactobacillus* spp. (*Lb. brevis*, *Lb. brevisimilis*, *Lb. bucheneri*, *Lb. casei*, *Lb. coryneformis*, *Lb. curvatus*, *Lb. lindneri*, *Lb. malefermentans*, *Lb. parabuchneri* and *Lb. plantarum*) and *Pediococcus* spp. (*P. damnosus*, *P. dextrinicus* and *P. inopinatus*) (Sakamoto and Konings, 2003; Asano *et al.*, 2009). The only fact that lactic acid bacteria have in common is that they synthesize lactic acid from various carbon substrates (Desmazeaud, 1998), so the isolation of a lactic acid bacterium, known as a potential beer spoilage bacterium, is made even more difficult because the detection and identification of lactic acid bacteria in the brewery are both difficult and expensive.

In that context, we have focused on this problem, and specifically on the possibility of using a harmless microbe, *B. subtilis*, to control mould development (chap. 2). This study has shown that the use of *B. subtilis* S499 causes a significant reduction of this issue. Our results showed also that during malting, when the bacterial cells are used alone (i.e. the washed *Bacillus* cells), mould growth was still inhibited. Three areas of dilution have been identified: a first zone of strong inhibition, where the dilution of the culture of *B. subtilis* does not have much impact on the reduction of mould growth (DF> minID), a second dilution zone where the inhibitory effect of the steeping liquor is almost proportional to the dilution of the *B. subtilis* culture (maxID <DF <minID), and a third dilution zone where treatment is simply ineffective: no significant decrease in the total mould growth was observed upon increasing the concentration of *B. subtilis* (DF< maxID).

### Effect of malting conditions on red sorghum malt characteristics

We found treatments based on this biocontrol agent (*B. subtilis*), when applied at the steeping step of the malting process, to provide a good level of mould reduction. The malts obtained, however, had low levels of key hydrolytic enzymes whose presence is desirable in malts. We have thus sought to improve these levels, with some success (chap. 3). In fact, when *B. subtilis* is used during sorghum malting, the synthesis of certain key hydrolytic enzymes is not guaranteed ($\alpha$-and $\beta$-amylases),

whereas the *β*-(1,4)-glucanase activity is significantly improved; the choice of optimal conditions then becomes crucial. It is known that microbes play an important role during malting and the change in their dynamic can significantly affect malt properties (Laitila *et al.*, 2007).

Thus, parameters that have been studied by others are examined here in relation to their bearing on this particular biocontrol approach and by comparison with a well-studied chemical approach to mould control: steeping in dilute alkaline solution. For example, although the germination-favoring effect of aeration is well known, we did not know how aeration during steeping would affect mould control (both moulds and *B. subtilis* are aerobes) or how crucial it would be in a situation of competition for oxygen between the grain and the biocontrol agent.

The aeration of the soaking liquor becomes an important factor in the sense that the competition for oxygen between the malted grain and the high microbial population is not favourable to germ development. When the steeped grain is well aerated, the time and temperature conditions for obtaining the optimum moisture necessary for proper grain germination must be determined. Our results showed that when the grain is aerated during germination, chitting is favoured, hence restricting the use of certain models to monitor rehydration (Peleg model), to only the soak time less than the time start of chitting. This aeration proved essential to the chitting in the case of the biocontrol (even when the mesophilic aerobic flora is low).

The grain moisture, often considered as an important predictor of the malts diastatic power (Dewar *et al.*, 1997), proved less suitable in explaining the variations in enzyme activities between treatments. Also, the effect of the strain of *B. subtilis* used as the biocontrol is not only limited to that of the lipopeptides considered as antifungal molecules. This clarification should lead to new strategies for biological control (screening of all antifungal compounds produced by *B. subtilis* and the understanding of the interactions between the *B. subtilis* population, the other members of the grain microbial ecosystem and the malted grain itself). When soaking is carried out in dilute alkaline solutions, the polyphenol content is greatly reduced compared with the biocontrol. Steeping in NaOH followed by resteeping in the biocontrol has been considered as a strategy which combines the benefits of using dilute alkaline (improvement of amylase activities, reduction of the content of total polyphenols and condensed tannins) with those of the biocontrol (reduction of mould growth, improvement of malt *β*-glucanase activities), although we did not observe any synergy in terms of the reduction of mould growth.

When soaking conditions (temperature, duration and nature of the soaking liquor) are defined, a question to be addressed is: how long should germination proceed in order to take maximum advantage of the underlying phenomenon? The answer to this question is both simple and complex: the germination should be stopped when the optimal malt properties are achieved (maximal enzyme

activities, good grain modification, good reduction of mould growth, good reduction of the polyphenols content and condensed tannins, etc.). It is difficult, if not impossible, to satisfy all these requirements at the same time without compromise − compromise which is not always easy when the laws governing various properties are not clearly known. Hence, we set out to model the time course of the key starch hydrolysing enzyme activities, namely: *α*-amylase and *β*-amylase. These studies showed that the maximum values of these two enzyme activities were not achieved under the same malting conditions and the induction phase of their synthesis is followed by a repression phase much less influenced by the malting conditions.

The Weibull 4 parameters model offers the advantage of highlighting the effect of malting conditions on the synthesis of *α*- and *β*-amylase. The effect of germination temperature on the synthesis of these two enzymes is an important phenomenon: increasing the germination temperature leads to an increase in the rate of *α*-amylase synthesis and a decrease in the rate of *β*-amylase synthesis. These results are, in part, supported by the work of Nielsen *et al.* (1997); Seki *et al.* (2001) and Kaplan and Guy (2004), who reported an increase in the *β*-amylase synthesis rate when the plant tissues were exposed to low temperatures.

**_β_-Glucans management during malting**

The malt total *β*-glucans content is a useful indicator of malt modification.When sorghum is used in brewing, the role of the *β*-glucans as factors affecting the extraction efficiency is a major issue (Taylor *et al.*, 2006). High levels of residual *β*-glucan pose a range of problems during sorghum brewing such as low lauthering rate, low beer filtration, haze formation, etc. It is also known that malting is a good step to deal with the hydrolysis of *β*-glucans, failing to do so the remaining possibilities are limited (Lewis and Bamforth, 2006). We focused on this problem and studied, in particular, the effect of the combined use of a chemical treatment (steeping in dilute alkaline 0.2% NaOH) with the biocontrol (*B. subtilis* starters).

In this work we have shown that the combined use allowed taking advantage of the positive effects of the two treatments – opening of the endosperm cell walls, subsequent improvement in water absorption and improved synthesis of certain enzymes (*α*- and *β*-amylases) due to the use of 0.2% NaOH − and the reduction of mould growth and the improvement of the *β*-glucanase synthesis due to the use of the biocontrol. These results are interesting in the sense that, as reviewed by Ogbonna (2011), knowledge of the catalytic properties of *β*-glucanases will elucidate their mode of activity in the depolymerisation of the *β*-glucans of sorghum malts.

The development of *β*-(1,3) and *β*-(1,4)-glucanase activities, when *B. subtilis* S499 is used as a biocontrol of mould, opens the door to discussions regarding the role of these two enzymes during

sorghum germination. When malts are intended for brewing use, the development of a high activity of *β*-(1,3) and *β*-(1,4)-glucanase is debatable as there is no information concerning their sensitivity to the mashing temperature. Indeed, if these enzymes are more thermostable than the *β*-(1,3) - (1,4)-glucanase, they can cause *β*-glucans solubilization in the wort and affect the lautering rate.

**Phytohormones synthesis by *B. subtilis* S499 and effect on malt properties**

While the GA-dependency of *α*-amylase production by cereal aleurones is well known, there are obvious contradictions concerning the effect of exogenous GA on the synthesis of *α*-amylase during sorghum malting (see Pagano *et al.*, 1997; Dewar *et al.*, 1998). The results of Aisien and Palmer (1983) showed that GA3 failed to induce an increase in *α*-amylase production during sorghum germination and seedling growth. These authors (Aisien and Palmer, 1983) also suggested that IAA sequential treatments, first with IAA and then with GA3 failed to increase the *α*-amylase production. The results of Dewar *et al.* (1998) also showed that there did not appear to be a relationship between the amount of gibberellic acid detected and the amylase activity. These results differ from those obtained by Macleod and Palmer (1966), cited by Aisien and Palmer (1983), for barley malting.

In order to deepen the understanding of this phenomenon, we then evaluated the ability of *B. subtilis* to synthesize indole 3-acetic acid and gibberellic acid and their effect on red sorghum malts properties. The effect of these two phytohormones was compared with that of *B. subtilis* starters on the synthesis of *α*-amylase, *β*-amylase, *β*-(1,3)-glucanase, *β*-(1,4)-glucanase and *β*-(1,3)-(1,4)-glucanase during sorghum malting. When steeping in 0.2% NaOH is used prior to the phytohormones application or resteeping in *B. subtilis* starters, the *α*-amylase production was significantly improved. These results (chap. 6) also showed that the sensitivity of the synthesis of grain enzymes depends on the type of enzyme in question; as the main sites of the enzymes are not always the same. For example, it was shown that the synthesis of *α*-amylase is more repressed by abscisic acid than *β*-amylase. Another phenomenon that has been clarified is the developmental context of the grain which affects the efficiency of the use of exogenous phytohormones, especially for cereals such as sorghum which contain a lot of polyphenols. Ours results (chap. 6) have shown tha t the enzymes production during red sorghum malting can be improved by renewing the steeping liquor and choosing the optimal application time of phytohormones.

These results should encourage the interest of researchers to investigate the interactions between sorghum polyphenols and various phytohormone signalling molecules. Indeed, the inefficient use of phytohormones such as the gibberellic acid on the synthesis of enzymes during sorghum malting when compared with barley (Aisien *et al.*, 1986) could result from interactions between polyphenolic compounds and phytohormones. On the other hand, the results of Aisien and Palmer (1983) were obtained after dissecting ungerminated sorghum grains into embryos and endosperms after 2 h

steeping, and incubating the separated parts in various plant hormone solutions. The apparent contradiction between our results and those of Aisien and Palmer (1983) could probably be due to the fact that:

1. During grain germination, signalling molecules are secreted from the embryo and diffuse from the embryo to the endosperm. In such a situation, since a complete screening of the signalling molecules (types and quantity of signalling molecules) hasn't been carried out, it is impossible to replace the complex interactions between the living parts of the grain by a range of molecules (signalling molecules).
2. The use of NaOH prior to phytohormone application, in the case of our study, possibly allowed the elimination of some phytohormone inhibitors since it has, for example, been shown that the process of polar auxin transport can be inhibited by a group of phenolic compounds which apparently act by binding to auxin-binding proteins known as the naphthylphthalamic acid (NPA) receptors (Jacobs and Rubery, 1988).
3. As shown by Pagano *et al.* (1997), the GA-dependency of $\alpha$-amylase production during sorghum germination is difficult to analyse since the embryo is the site of both $\alpha$-amylase synthesis and endogenous GA production.

## Conclusions and outlook

The use of barley in brewing is a tradition in some countries strongly committed to the quality of malted barley-based beer, although it is possible to obtain a good quality beer with greater flexibility in the choice of raw materials. It is interesting to note that there is not just "one barley malt", but rather "several types of barley malts", and there is not "one barley beer", but rather "several types of barley-based beers"; it is therefore conceivable that the quality of malt is to be defined depending on the type of beer you want to produce.

Thus, our work comprised defining a series of conditions for obtaining malted sorghum with as diverse as possible characteristics: enzyme activities, polyphenols and condensed tannins content, total malting losses, degree of modification, etc. This work has enabled the achievement of sorghum malt slightly contaminated with mould, the definition of good conditions for soaking and germination has contributed to the understanding of phenomena such as the induction and/or repression of the synthesis of $\alpha$- and $\beta$-amylases during red sorghum malting, the sorghum-*Bacillus* dialogue during malting and the effect of phytohormones on red sorghum malt properties.

This study is far from answering all questions related to the use of *Bacillus-subtilis*-based biocontrol treatments and the elucidation of the biochemical and physiological phenomena underlying obtaining high quality malts. Studies determining the optimal conditions for the production of signalling

molecules by *B. subtilis*, the diffusion kinetics of phytohormones in sorghum grain, the isoformic profile of the synthesized enzymes and the location of their major sites of synthesis deserve to be performed to allow efficient use of this biocontrol strategy. The effects of possible interactions between polyphenols and phytohormones on enzyme synthesis and work during soaking, germination and kilning also deserve special attention.

Knowledge of the biochemistry of sorghum malting is expected to be reviewed in various brewing scenarios in order to choose the most realistic and adapted to this cereal, because the sensitivity of the different enzymes synthesized during malting to the brewing conditions, varies from one cereal to another. The interest of such a study lies in the use of sorghum as a raw material in the modern brewing industry and is expected to encourage local producers, which is a major asset for developing countries who are forced to be competitive in this dynamic economic environment.

Brewing with sorghum responds indeed to a comprehensive policy, which is the valuation of tropical products, because in this century − where the population growth is exponential, the requirements of production, processing, marketing and consumption are so entangled that they cannot escape, unless they break – the increase of cereal production, the improvement of the local cereal quality and processability are some of the major concerns for undeveloped countries.

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