ROSE DAPHNEE NGAMENI TCHONKOUANG

METHOD DEVELOPMENT & VALIDATION FOR THE EFFICIENT DETECTION OF SUPER-ATTENUATING (OVER-FERMENTING) YEAST CONTAMINANTS (*Saccharomyces cerevisiae* var. *diastaticus*) IN THE BREWERY INDUSTRY

UAlg ISE
UNIVERSIDADE DO ALGARVE
INSTITUTO SUPERIOR DE ENGENHARIA

2021

ROSE DAPHNEE NGAMENI TCHONKOUANG

METHOD DEVELOPMENT & VALIDATION FOR THE EFFICIENT DETECTION OF SUPER-ATTENUATING (OVER-FERMENTING) YEAST CONTAMINANTS (*Saccharomyces cerevisiae* var. *diastaticus*) IN THE BREWERY INDUSTRY

Master of Science in Food Technology

Dissertation made under the co-supervision of:

Dr. Célia Maria Brito Quintas & Dr. Liesbeth Derde

UAlg ISE
UNIVERSIDADE DO ALGARVE
INSTITUTO SUPERIOR DE ENGENHARIA

2021

METHOD DEVELOPMENT & VALIDATION FOR THE EFFICIENT DETECTION OF SUPER-ATTENUATING (OVER-FERMENTING) YEAST CONTAMINANTS (*Saccharomyces cerevisiae* var. *diastaticus*) IN THE BREWERY INDUSTRY

Work Authorship Declaration

I declare to be the author of this work, which is unique and unprecedented. Authors and works consulted are properly cited in the text and are included in the listing of references.

- -

(Rose Daphnee Ngameni Tchonkouang)

ACKNOWLEDGEMENT

First and foremost, I would like to thank the God of Abraham, Isaac and Jacob who has granted countless blessings and opportunities to extend my knowledge in science and finalize this dissertation.

Global Innovation and Technology Center, Anheuser-Busch InBev (AB InBev):
Mrs. Els Fonteyn, for opening the doors of AB InBev to me and allowing me to conduct my Master thesis project in the Global Yeast & Fermentation Laboratory. A special thanks for the support, guidance, supervision, and excellent hospitality.

Doctor Liesbeth Derde, for the supervision, support, assistance during laboratory trials, text corrections and constructive criticism. Your availability and willingness to help, give advice and share made my stay indelible.

I would like to thank the people at AB InBev (Belgium) and particularly the Yeast & Fermentation team members for the guidance and explanations regarding the laboratory work. The innumerable challenges faced in the Yeast & Fermentation Laboratory and GITEC have greatly impacted my personal and professional development. This internship represents the culmination of my studies.

Department of Food Engineering, University of Algarve:
Doctor Célia Quintas, my heartfelt thanks for the affectious support and intellectual assist. The past year has been very challenging, my heartfelt thanks for the encouragement, the endurance you channelled through me from afar and the suggestions provided during the writing of the dissertation.

Doctor Eduardo Esteves, special thanks for the time spent in providing support for completing the statistical analyses of the experimental data and presentation of data analyses results.

Doctor Margarida Vieira, thank you for being enthusiastic about research and for enabling me to publish my first scientific paper under your supervision.

My Family:
I am profoundly grateful to my father, mother, sisters, aunts, uncles, and relatives for being great enablers, the words of encouragement, inspirational conversations and for teaching me how to exercise patience. Thank you for your unconditional love, for setting me back on the right path and making me trust the process during difficult times.

My Heartfelt thanks for everything!

Rose Daphnee Tchonkouang

Leuven, Belgium

ABSTRACT

Saccharomyces cerevisiae var. *diastaticus* is the most dangerous spoilage yeast of fermented beverages in the brewery industry. In contrast to conventional brewing yeast, diastaticus yeast causes super-attenuation due to its ability to degrade residual dextrin and starch in beers, thereby causing degraded mouthfeel, over-carbonation, high alcohol levels, and package explosion. Usually, a diastaticus contamination can remain unnoticed several months after packaging. Diastaticus yeast is particularly difficult to detect with traditional microbiological analyses due to the common characteristics shared with brewing yeast. The objective of the present study was to optimize/develop the detection and growth control of diastaticus yeasts using three different assays: a) the modified Durham test, b) the dextrin agar test and, c) a novel test developed during this project, called the 'attenuation test'. Strain DSM 70487 and Strain-Y were used as positive and negative controls, respectively. The attenuation test was the most reliable assay because all the investigated diastaticus strains were detected by monitoring the reduction in density (°P) associated with residual saccharides consumption in fully attenuated beer medium. Although the spoilage yeast strain TUM 1-B-8 demonstrated mild super-attenuating activity in the attenuation test, this strain did not show spoilage potential when assayed for growth on dextrin agar plates and did not demonstrate gas production potential in the Durham test. The positive control DSM 70487 showed spoilage potential in all assays, with the fastest detection time of 2 days recorded in the Durham test. Growth on dextrin agar at pH 5.2 and 6.2 revealed faster growth and more rapid detection at pH 5.2. The lowest detection limit ($5x10^0$ cells/ml) was noted in the agar tests. This research demonstrated clear variations in the super-attenuating strength of *S. cerevisiae* var. *diastaticus* yeast strains and highlighted the necessity to combine multiple assays for reliable detection of diastaticus activity in investigated samples.

Keywords: *Saccharomyces cerevisiae* var. *diastaticus*, beer, spoilage, super-attenuation, quality control

RESUMO

Saccharomyces cerevisiae var. *diastaticus* é uma estirpe da espécie utilizada na produção de cerveja, *S. cerevisiae*. *S. cerevisiae* var. *diastaticus* é a levedura de deterioração mais perigosa nas bebidas fermentadas obtidas na indústria cervejeira. As espécies do género *Saccharomyces* utilizadas no processamento de cerveja, como *S. cerevisiae* e *S. Pastorianus* degradam carboidratos numa determinada ordem: primeiro a glicose, posteriormente a frutose seguida da maltose e, finalmente, o trissacarídeo maltotriose, que dificilmente é consumido pela maioria das leveduras de cerveja. Em geral, estas leveduras são incapazes de utilizar oligossacarídeos (3-10 resíduos de monossacarídeos), e dextrinas e polissacarídeos (Ex.: amido). Os carboidratos não fermentados permanecem na cerveja e contribuem para o corpo e a sensação na boca do produto final. Contudo, *S. cerevisiae* var. *diastaticus* é uma levedura amilolítica que possui o gene STA1 o qual codifica a síntese da enzima glucoamilase extracelular. Esta enzima degrada as dextrinas e o amido presentes na cerveja acabada, levando à sua degradação, situação denominada superatenuação. A superatenuação caracteriza-se pela existência de fermentações secundárias de carboidratos residuais podendo causar a produção de dióxido de carbono, o aparecimento de sabores estranhos, o desenvolvimento de uma sensação desagradável na boca e a produção de níveis anormalmente altos de álcool. As contaminações por leveduras diastásicas são dificilmente detetadas, sendo os sinais de deterioração identificados vários meses após o embalamento, quando o produto já se encontra no mercado.

Na última década tem sido reportado um aumento no número de incidentes resultantes de contaminações por *S. cerevisiae* var. *diastaticus* os quais estão associados a perdas financeiras que atingem bilhões de euros/ano na Europa. Esta estirpe é difícil de detetar e identificar através de análises microbiológicas tradicionais devido às características comuns com a levedura de cerveja. Além disso, o gene STA1 pode estar presente em estirpes de levedura que não apresentam potencial de deterioração e superatenuação.

O objetivo do presente estudo foi otimizar/desenvolver e comparar métodos para detetar o crescimento e capacidade diastásica de várias estirpes de leveduras, utilizando três abordagens diferentes: a) o teste de Durham modificado, b) o teste em meio de cultura sólido de agar com dextrinas (com azul de bromofenol ou roxo de bromocresol), e c) um teste desenvolvido durante o presente projeto designado “teste de atenuação”. As estirpes

DSM 70487 e Estirpe-Y foram utilizadas como controlos positivo e negativo, respetivamente. Foram também utilizadas misturas da estirpe diastásica TUM 1-B-8 com DSM 70487 (concentrações celulares variáveis de 1 x 10^8 a 5 x 10^0 células/ml) e com a estirpe de levedura lager (Estirpe-Y). Todos os ensaios foram realizados em duplicado. O teste de Durham e o "teste de atenuação" foram realizados no meio de cultura de "cerveja totalmente atenuado" com zinco (200 ppb) e sem zinco. A "cerveja totalmente atenuada" é um meio preparado pela reinoculação da cerveja embalada com uma levedura starter lager para garantir o consumo completo de qualquer açúcar fermentável residual. O teste de Durham permitiu avaliar a libertação de subprodutos gasosos (dióxido de carbono) em tubos Durham invertidos resultantes da fermentação de estirpes de levedura diastásicas que foram inoculadas em meio de "cerveja totalmente atenuado" e incubadas durante 30 dias à temperatura ambiente. O "teste de atenuação" consistiu em medições semanais do extrato aparente (EA) (°P) do meio de "cerveja totalmente atenuado" previamente inoculado e incubado à temperatura ambiente, durante 4 semanas. A presença das estirpes degradadoras provoca a diminuição do EA devido à hidrólise de sacarídeos residuais presentes no meio de cultura/cerveja. A capacidade de as leveduras utilizarem dextrina durante o seu crescimento foi estudada em meio de cultura sólido contendo dextrina, seguida de incubação aeróbia a 28 °C, durante 30 dias. Foi testado o meio de cultura sólido com os corantes azul de bromofenol (pH inicial 5,2) e roxo de bromocresol (pH inicial 6,2). A presença destes corantes indicadores de pH contribuiu para facilitar a deteção visual do crescimento das leveduras uma vez que uma alteração da cor do meio sólido resultava da sua acidificação devido ao crescimento das estirpes *S. cerevisiae* var. *diastaticus* previamente inoculadas.

A presença das estirpes investigadas foi confirmada no "teste de atenuação" após um período de duas semanas devido à redução dos valores do EA. Observaram-se reduções do EA entre a 2ª e a 3ª semanas de incubação no meio de "cerveja totalmente atenuado", suplementado com zinco, quando se utilizaram inóculos contendo 1x10^2 células/ml de DSM 70487. Apenas a estirpe utilizada como controlo positivo, DSM 70487 (1x10^8 células/ml), demonstrou potencial de deterioração no teste de Durham, tendo a primeira visualização do gás sido registada após 2 dias de incubação. Não houve diferenças nos resultados obtidos nos testes de Durham realizados com e sem zinco. Os ensaios realizados em meio sólido com dextrina (pH 5,2 e 6,2) revelaram que as estirpes superatenuantes cresceram mais rapidamente nos meios com valores de pH inicial de 5,2

pelo que a sua presença foi detetada mais rapidamente quando o pH inicial foi 5,2 em comparação com 6,2. Os testes realizados no meio de cultura sólido apresentaram um limite mínimo de deteção de $5x10^0$ células/ml. A estirpe DSM 70487 apresentou um potencial de deterioração mais alto do que TUM 1-B-8 em todos os estudos realizados. A estirpe DSM 70487 revelou capacidade de superatenuação em todos os testes, enquanto a estirpe TUM 1-B-8 mostrou atividade superatenuante nos estudos de refermentação em meio de "cerveja totalmente atenuado", mas não apresentou potencial de deterioração quando testada em meio sólido com dextrina e não foi detetada a produção de gás nos testes de Durham.

Com base nos resultados obtidos no presente estudo, o "teste de atenuação" permitiu obter melhores resultados para a deteção de estirpes de leveduras de degradação *S. cerevisiae* var. *diastaticus* quando comparado com os outros ensaios realizados. Este estudo permitiu também evidenciar a existência de variações na capacidade superatenuante das estirpes testadas e destacou a necessidade de combinar várias estratégias experimentais para a deteção destes contaminantes nas amostras a estudar durante o controlo de qualidade no ambiente de cervejaria.

Palavras chave: *Saccharomyces cerevisiae* var. *diastaticus*, cerveja, degradação, superatenuação, controlo de qualidade

Table of Contents

ACKNOWLEDGEMENT iv
ABSTRACT v
RESUMO vi
TABLE OF CONTENTS ix
LIST OF FIGURES xii
LIST OF TABLES xiv
LIST OF ABBREVIATIONS xv
1 INTRODUCTION 1
1.1 Overview of *Saccharomyces cerevisiae* var. *diastaticus* 1
1.2 Aim and Objectives 2
1.3 Thesis Organization 3
2 LITERATURE REVIEW 4
2.1 Industrial Brewing Yeast 4
2.2 Wort Production Process 5
2.2.1 Barley to Malt Processes – Malting 6
2.2.2 Conversion of Malt to Wort 11
2.2.3 Wort Sugar Composition 14
2.3 Sugar Uptake in Fermenting Wort 15
2.4 Overview of Beer Spoilage Microorganisms 17
2.4.1 Lactic Acid Bacteria 17
2.4.2 *Pectinatus* and *Megasphaera* 19
2.4.3 Wild Yeast 20
2.5 Super-attenuating Yeast 21
2.5.1 Sources of Diastaticus Yeast Contamination 22

2.5.2 Consequences of Diastaticus Yeast Contamination 23
2.5.3 Current Detection Methods for Diastaticus Yeast 25
3 METHODOLOGY 29
3.1 Yeast Strains 29
3.2 Propagation of Yeast Strains 29
3.3 Determination of yeast cell concentration 30
3.3.1 Preparation of concentrated aqueous yeast suspensions 31
3.4 Preparation of Fully Attenuated Beer Medium 32
3.4.1 Physicochemical Analyses of Fully Attenuated Beer Medium 32
3.5 Modified Durham Test in Fully Attenuated Beer: Gas Production assay 33
3.6 Attenuation test in Fully Attenuated Beer 34
3.6.1 Follow-up Analyses (Attenuation test) 35
3.7 Dextrin Agar Test 35
3.7.1 Dextrin Agar Medium Preparation 35
3.7.2 Dextrin Utilization Assay 36
3.7.3 Saccharide Profiling of dextrin agar medium components 37
3.8 Statistical Analyses 37
3.8.1 Attenuation Test 37
4 RESULTS AND DISCUSSION 38
4.1 Saccharide Profiling of Media 38
4.2 Modified Durham Test: Gas Production Assay 39
4.3 Attenuation Test 44

4.4 Dextrin Agar Test 49
5 CONCLUSION AND FUTURE STUDIES 54
5.1 Conclusion 54
5.2 Future Works 55
6 BIBLIOGRAPHY 56
7 APPENDIX 64
7.1 Appendix 1: SPSS output tables 64

List of Figures

Figure 1: Activities and changes at different malting stages (Fox, 2018) 7

Figure 2: Schematic representation of starch hydrolysis (Evans et al, 2009). 9

Figure 3: Process flow diagram of Wort Production (Eßlinger & Narziß, 2009)..... 12

Figure 4: Uptake of Wort Sugars by Fermenting Lager Yeast (Stewart, 2016) 16

Figure 5: Laboratory yeast propagation scheme 30

Figure 6: Representative diagram of culture bottles used for the attenuation test..... 34

Figure 7: Illustrative pictures of different gas formation intervals in Durham tubes. A= G0/4 (no gas formation), B = G1/4 (from >0.00 mL to 0.45 ml), C = G2/4 (from 0.45 ml to 0.9 ml), D = G3/4 (from 0.9 ml to 1.35 ml) and E = G4/4 (from 1.35 ml to 1.9 ml). 40

Figure 8: Gas formed in Durham tubes by DSM 70487 (duplicate samples) inoculated in fully attenuated beer with zinc adjunct (left) and without zinc adjunct (right) at day 4. 42

Figure 9: Evolution of the apparent extract (°P) of fully attenuated beer medium (without zinc) pitched with yeast strains for 4 weeks of incubation. Each point represents the mean of replications; n=2. Initial density of beer medium = 1.9 °P, Initial pH = 3.3. Error bars represent the standard deviation. The weekly AE values observed between the investigated samples were significantly different ($P < 0.05$)..... 44

Figure 10: Evolution of the apparent extract (°P) of fully attenuated beer supplemented with 200 ppb (µg/L) zinc, pitched with yeast strains for 4 weeks of incubation. Each point is the average of two measurements. Initial density of beer medium = 1.9 °P, Initial pH = 3.3. Error bars represent the standard deviation. The weekly AE values observed between the investigated samples were significantly different ($P < 0.05$)..... 45

Figure 11: Mean Apparent extract values (°P) of each inoculum per sampling time (with and without zinc). a) week 1, b) week 2, c) week 3, and d) week 4. Whiskers represent 95% confidence intervals. Results of the statistical analyses (Tukey's test, $P < 0.05$) are annotated as follows: A=significant differences among inoculated yeast(s), B=significant differences between trials with and without added zinc, AB = significant differences

among inocula & significant effect of zinc addition. There were significant differences in the apparent attenuation among strains and co-cultures for each sampling time, from week 1 to 4. 47

Figure 12: Apparent attenuation of fully attenuated beer medium by each inoculum during incubation. a) DSM 70487, b) Strain-Y, c) TUM 1-B-8, d) Mixture 1, e) Mixture 2, f) Mixture 3. Whiskers represent 95% confidence intervals. For significant differences (Tukey's test) among mean AE values with and without zinc addition; *$p<0.05$, **$p<0.001$, °=non-significant. For significant variations in the apparent extract values with incubation (sampling) time; a=$p<0.05$, b=$p<0.001$, c=non-significant. For existence of interaction effect between factors zinc and incubation time; X= interaction effect Y = No interaction effect. 48

Figure 13: Colour change of inoculated petri plates containing dextrin agar with bromophenol blue at pH 5.2 (left) and dextrin agar medium with bromocresol purple at pH 6.2 (right) after 30 days of incubation at 28 °C. 51

List of Tables

Table 1: Differences Between Ale and Lager Yeasts 5

Table 2: Wort Production Process from Barley (Adapted from Aroh, (2019) & Willaert (2007)). 6

Table 3: Optimum condition and amylolytic activities of enzymes during mashing (Modified from Vriesekoop et al (2010); Willaert (2007)) 13

Table 4: Carbohydrate Composition in Wort (Modified from Stewart, 2016) 15

Table 5: Thermal resistance of vegetative cells of *S. cerevisiae* var. *diastaticus* strains in various beer media 25

Table 6: Culture media and growth conditions tested for the detection of *Saccharomyces cerevisiae* var. *diastaticus* 26

Table 7: Strains used for diastaticus detection assays 29

Table 8: Cell concentrations of the Strains in Suspension of Pure Yeast and Yeast Blends 31

Table 9: Formulation of dextrin type III agar with bromocresol purple 35

Table 10: Formulation of dextrin type III agar with bromophenol blue 35

Table 11: Saccharide profiles (DP 1 – 7) of the agar medium components (1.5% dextrin and YNB without amino acids) and fully attenuated beer medium. Total sugar refers to the sum of the sugars: fructose, glucose, sucrose, and maltose. DP = degree of polymerization. 38

Table 12: Gas Formation and days required (medium with Zinc supplement). Durham tubes volume described in intervals from >0.00 mL to 0.45 ml as G1/4, from 0.45 ml to 0.9 ml as G2/4, from 0.9 ml to 1.35 ml as G3/4, from 1.35 ml to 1.9 ml as G4/4 and tubes without gas formation were described as G0/4. [a] = Strain-Y, [b] = DSM 70487. First appearance of gas in Durham tubes is highlighted in yellow 40

Table 13: Gas Formation by yeast strains inoculated in beer medium without zinc. Durham tubes volume described in intervals from >0.00 mL to 0.45 ml as G1/4, from 0.45 ml to 0.9 ml as G2/4, from 0.9 ml to 1.35 ml as G3/4, from 1.35 ml to 1.9 ml as G4/4 and tubes without gas formation were described as G0/4. [a] = Strain-Y, [b] = DSM 70487. First appearance of gas in Durham tubes is highlighted in yellow. 41

Table 14: Growth of yeast cells (resuspended in 0.9% NaCl) on dextrin agar media adjusted at pH 5.2 and pH 6.2 incubated at 28 °C recorded after 30 days incubation. [a] = Strain-Y, [b] = DSM 70487 50

Table 15: Days Required for observing visible growth on dextrin agar media inoculated with pure yeast strains and mixed cultures of lager yeast (Strain-Y) and spoilage yeast (*S. cerevisiae* var. *diastaticus*). [a] = Strain-Y, [b] = DSM 70487 51

List of Abbreviations

AE: Apparent extract

ATP: Adenosine triphosphate

DNA: Deoxyribonucleic acid

EPS: Exopolysaccharides

FAN: Free amino Nitrogen

FPDM: Farber Pham Diastaticus Medium

HPLC: High Performance Liquid Chromatography

ISE: Instituto Superior de Engenharia (*Institute of Engineering*)

LAB: Lactic Acid Bacteria

LCSM: Lin's Cupric Sulphate Medium

MYGP: Malt extract, Yeast extract, Glucose, and Peptone media

PCR: Polymerase chain reaction

POF: Phenolic-off-flavours

ppb: Parts per billion

ppm: Parts per million

PTFE: Polytetrafluoroethylene

RPM: Revolutions per minute

YNB: Yeast Nitrogen Base

YPS: Yeast extract, peptone, Lintner Starch

ºP: Degree Plato (Units of apparent extract)

CHAPTER ONE

1 INTRODUCTION

1.1 Overview of *Saccharomyces cerevisiae* var. *diastaticus*

Saccharomyces cerevisiae var. *diastaticus* is the most dangerous spoilage yeast of fermented beverages in the brewery industry (Hutzler et al, 2012). Diastaticus is a wild yeast contaminant that has a high beer spoilage potential (Farber & Pham, 2019). In the event of contamination, diastaticus yeasts degrades unfermented oligosaccharides and polysaccharides, and thus over-fermenting or, in brewer's jargon, "super-attenuating" the beer. These prolonged fermentation activities by *S. cerevisiae* var. *diastaticus* is caused by the secretion of one of the three isozymes of an extracellular glucoamylase I, II or III, normally absent in brewer's yeast. Three unlinked homologous STA genes (STA1, STA2 and STA3) code for the expression of the glucoamylase isozymes I, II and III respectively (Ogata et al, 2017). Glucoamylase breaks down dextrins and starch into simple sugars that can be taken into the yeast's cell, leading to a very high degree of attenuation (Abbet, 2019). Some diastaticus strains are true wild yeast contaminants, but other strains have been selected and commercialized as saison ale strains, recognized for very high attenuation rates and strong phenolic profile during beer fermentation (Farber & Pham, 2019).

Detecting contaminations in the final packed products by this subspecies of *S. cerevisiae* has led to costly product recalls as infection may lead to off flavour production, over-attenuation, and over-carbonation, potentially causing gushing, exploding packages, or non-compliance with regulatory guidelines (particularly alcoholic beverages labelling regulations) due to abnormally high alcohol by volume (Farber et al, 2019). Their potential to build up high pressure due to their CO_2 production from secondary fermentation in packaged products, especially with a risk for glass bottles and cans to explode represents one of the greatest threats of this contaminant due to exposure to physical injury (Meier-Dörnberg et al, 2017).

In the last decade, an increase in contamination incidents have been reported with certain contamination events associated with financial losses ranging from millions to billions of euros per year in Europe (Suiker et al, 2021). Up to date, there is no existing method that

is widely approved for the detection of low concentrations of diastaticus strains, and the European Brewery Convention (EBC) is currently working towards this goal. Enrichment methods cannot be utilized solely for detecting a low level of contamination, as conventional brewing yeast species will outcompete the growth of diastaticus yeasts, and there is no visual difference in their cell structure (Vogeser, 2019). There is, thus, a need to develop formulations and methods for the efficient detection of diastaticus contaminations, to ensure the proper prevention and control of contaminations in the brewing industry. This project aimed to develop a reliable method for detecting *Saccharomyces cerevisiae* var. *diastaticus* in the presence of the brewing yeast.

1.2 Aim and Objectives

Beer is generally recognized as a microbiologically stable beverage. An explanation for this is that beer's composition does not favour the growth of most microbial spoilers due to the presence of ethanol, high carbon dioxide content, low oxygen content, a relatively low pH value (approximately 4) and the antibacterial effects of hop bitter compounds. However, the amylolytic yeast *Saccharomyces cerevisiae* var. *diastaticus* survives in fermented beer due to the action of secreted glucoamylases on residual dextrin and starches. The main aim of this project was to assess and develop methods to detect contaminations by *Saccharomyces cerevisiae* var. *diastaticus*. The specific objectives of the present study include:

-To assess the gas production potential of pure yeast strains and mixed cultures through the Durham test (fermentation test) in fully attenuated beer medium.

-To evaluate the apparent attenuation of fully attenuated beer medium inoculated with pure yeast strains and mixed cultures of diastaticus and non-diastaticus strains via measurements of the apparent extract in degrees Plato (°P).

-To evaluate the effect of zinc on the fermentative performance of yeast strains inoculated in fully attenuated beer medium

-To investigate the ability of pure yeast strains and inocula consisting of mixtures of diastaticus and non-diastaticus strains to grow on solid media with dextrin as the sole carbon source.

-To determine the ability of the inoculated cultures to degrade dextrin on a solid media at varying pH conditions.

1.3 Thesis Organization

The present dissertation is divided into 5 chapters. The organization of this dissertation and the content of each chapter is described below:

The first chapter presents a brief introduction on which this study is based and describes the main aim of the study.

In chapter two, the literature review is presented to show the study's relevance and ease the interpretation of the results obtained.

In the third chapter, the methodology used in the study is described with the objective to compare various microbial analyses procedures to efficiently detect diastaticus yeast.

Chapter four presents the results of the investigation and detailed interpretation of the results obtained.

In chapter five, the study's conclusion, together with the future studies to be performed, are presented.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Industrial Brewing Yeast

Beer brewing is an elaborate process that involves the mixing and processing of four (or five) raw materials, including barley malt, cereal adjuncts (maize, rice, buckwheat, oats, triticale), brewing water, hops, and yeast (Fox, 2018). Added as the last ingredient of beer, yeast has a crucial role in beer production and has the uniqueness of being the only living ingredient in beer (Pires et al, 2014). It is responsible for the conversion of wort carbohydrates into ethanol and CO_2, as well as the synthesis of flavour compounds. The traditional brewing process involves fermentation of wort by domesticated yeast strains (Gibson et al, 2017). A major characteristic of this traditional process is that yeast is usually the sole microbial agent, and any deviation is considered a flaw, although other yeast strains and microbial agents such as bacteria can occur during the beer fermentation process, when desired by the manufacturer (Capece et al, 2018).

The major brewer's yeasts strains commonly employed in beer production belongs to the genus *Saccharomyces* (Capece et al, 2018), which include two main yeast species that correspond to ale and lager beers. The species *Saccharomyces cerevisiae* is used for the production of ale beers whereas the species *Saccharomyces pastorianus,* previously named by Hansen as S. c*arlsbergensis* (Lattici et al, 2020), is used for lager beers fermentations. *S. pastorianus* is an allopolyploid hybrid of *S. cerevisiae* and *S. eubayanus* (Libkind et al, 2011). This interspecific hybrid is the most commonly used yeast in breweries worldwide, with more than 90 % of beers produced using this strain (Capece et al, 2018; Krogerus et al, 2015).

The origins of lager yeast are unclear, however, Krogerus et al (2015) reported that the original hybridization event was probably due to a contamination by *S. eubayanus* in a traditional ale beer fermentation with *S. cerevisiae.* Ale and lager yeasts are classified as top-fermenting and bottom-fermenting yeasts, respectively, based on their flocculation properties at the end of the wort fermentation process. Ale yeast strains rise to the surface of the fermented wort, while lager yeast aggregates at the bottom of the fermentation vessel (Capece et al, 2018). Beers produced using ale yeasts are brewed at fermentation temperatures ranging from 15-25 °C, while beer production using lager yeast is conducted

at temperatures ranging from 8-12 °C (Lattici et al, 2020). Table 1 summarizes the differences between ale and lager yeast.

Table 1: Differences Between Ale and Lager Yeasts

Yeast type	Ale	Lager
Species name	*Saccharomyces cerevisiae*	*Saccharomyces pastorianus*
Fermentation temperatures	15-25 °C	8-12 °C
Flocculation	Top fermenting	Bottom fermenting
Melibiose uptake	Does not ferment melibiose	Ferments melibiose

During beer production, brewer's yeast is commonly cultured in an acidic aqueous sugar-rich liquid medium derived from malt, called "wort". Brewing strains are capable of fermenting numerous carbohydrates in the wort such as glucose, sucrose, fructose, maltose, galactose, raffinose and maltotriose (Stewart, 2016). Lager yeasts are differentiated from ale yeasts due to their ability to ferment the disaccharide melibiose (glucose-galactose). In addition, bottom-fermenting yeast strains exhibit a higher affinity for galactose and maltotriose compared to ale strains (Naydenova et al, 2012).

2.2 Wort Production Process

The main ingredients required in producing wort are malted barley and hops. Wort production includes six main steps: grinding of malt and adjuncts, mashing, wort filtration, wort boiling, wort clarification, and wort cooling and aeration (Willaert, 2007) Table 2 describes the principal steps involved in the production of wort.

Table 2: Wort Production Process from Barley (Adapted from Aroh, (2019) & Willaert (2007)).

	Step	Main Activities	Aim/goal	Duration	Temperature(°C)
1)	**Malting**				
	Barley	Cleaning & grading	Removal of impurities and sorting the grains according to size (small to large)	/	/
	Steeping	Hydration & aeration of barley	Initiation of barley germination	48 h	12-22
	Germination	Growth and modification of the grain	Production of hydrolytic enzymes	3-5 days	22
	Kilning	Heat treatment & dehydration of the green malt. Removal of unwanted rootlets, husk, and dust from the kilned malt.	Preventing further germination, growth, grain modification. Loss of enzyme activity	24-48 h	22-110
2)	**Milling**	Malted grain crushing without disintegrating the husks	Enzyme release through endosperm exposure (increase of surface area)	1-2 h	22
	Cleaning	Removal of unwanted rootlets, husk, and dust	Malted barley free of impurities	/	/
3)	**Mashing & Lautering**	Boiling of wort and added hops	Enzymatic conversion of starch into fermentable sugars, extraction nutrients & separation of nutrient-rich wort extract from malt	1-2 h	30-72
4)	**Wort boiling**	Malt mixing with hot water & mash heating	Wort sterilization & concentration. Colour, flavour & aroma development. Enzyme inactivation	0.5-1 h	>98
5)	**Whirlpool**	Recirculation of hopped wort through whirlpool effect	Elimination of residual hops and wort clarification (trub sedimentation)	<1 h	100-80
6)	**Wort Cooling & aeration**	Cooling of wort through heat exchanger, injection of air	Preparation of the wort for pitching yeast	1 h	12-18

2.2.1 Barley to Malt Processes – Malting

The malting process involves the conversions of barley to malt. Malt is a key raw material in brewing that gives rise to the wort extract which is subsequently used as fermentation medium by the yeast. As shown in Figure 1, malting encompasses the three main steps:

steeping, germination, and kilning (Lodolo et al, 2008). The purpose of this process is to release a spectrum of enzymes in the grains that can hydrolyse the cereal constituents to produce the wort extract (Stewart, 2016). Malt is derived from cereals, with barley (*Hordeum vulgare*) being the most used grain. Other cereals such as corn, wheat, rice, sorghum, and cane and beet sugar may be used as well (Stewart, 2016).

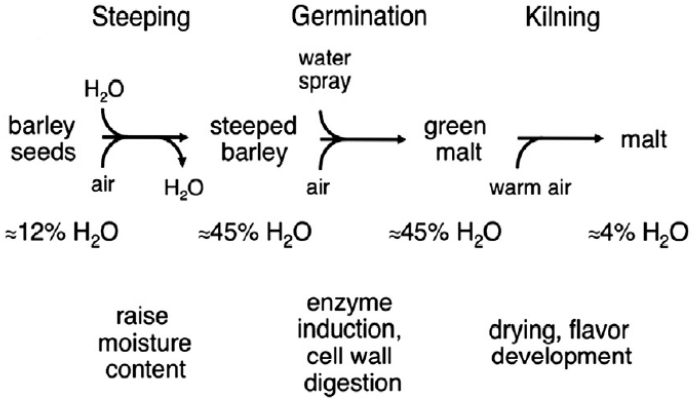

Figure 1: Activities and changes at different malting stages (Fox, 2018)

Steeping

Steeping is performed at a controlled temperature to stimulate germination. The soaked barley (or other cereals) grains absorbs water at temperatures between 14-16 °C resulting in the grain swelling by approximately one third. This promotes a gradual increase in respiration, resulting in CO_2 accumulation and rise in temperature of the grain (Guido & Moreira, 2014).

Steeping is characterized by a minimum of two to three water changes that are interspersed with ventilated air rests between tank refills over a period of 24-40 hours. Following the water immersions and air rests, the grain reaches 35% moisture content which is sufficient to stimulate germination. Nevertheless, steeping is continued until a final moisture content of 42-48% is reached (Figure 1). A higher moisture is necessary for uniform enzyme distribution throughout the endosperm, to ensure a proper modification of grain resources to support the germination step (Oser, 2015). Guido & Moreira (2014) stated that the germination and modification rates are controlled by regulating the moisture content and temperature of the grain.

The barley grains are subjected to a controlled germination after steeping, these germinated barley grains are called ‘malt’ (De Keukeleire, 2000). It is crucial to ensure that the grain remains sufficiently moist and has adequate ventilation at the correct temperatures (Oser, 2015).

Germination

Germination is characterized by embryo development of the grain, manifested by the rootlets growth, and the elongation of the shoot (acrospire), with the modification of the complex raw materials of the starchy endosperm to make them available for fermentation (Guido & Moreira, 2014). To support the development of the embryo, the essential components within the endosperm are released by cell wall degradation along with the disintegration of the protein matrix surrounding starch granules. This releases the starchy complexes from the protein matrix of the endosperm, making them available for partial degradation by enzymes (Fox, 2018).

The starch granules in the endosperm are composed of amylose and amylopectin. Amylopectin is a highly branched molecule, made up of many short (6-20 glucose units) clusters of glucose molecules linked by α-1,4 and α-1,6 glycosidic bonds. On the other hand, amylose is composed of unbranched chains of glucose monomers connected by α-1,4 glycosidic linkages. Amylopectin is by far more abundant in starch granules (Oser, 2015). The purpose of germination is to induce the synthesis and release of amylolytic (starch-degrading) enzymes.

According to Willaert (2007), the enzymes involved in the conversion of starch to fermentable sugars are: α-amylase, β-amylase, limit-dextrinase (also known as 6-alpha-glucanohydrolase), maltase (also known as α-glucosidase) and saccharase (also known as β-d-fructofuranosidefructohydrolase, β-fructofuranosidase, sucrase and invertase). The enzymes α-amylase, α-glucosidase, and limit dextrinase are synthesized *de novo* in the barley grain during germination. On the other hand, β-amylase is already present in the cereal grains as it is produced and stored during barley grain development. β-amylase is present in the hydrated grain in both the bound and free states (Vinje et al, 2015). In the initial stages of germination, the enzyme alpha amylase is produced and released by the grain’s aleurone layer to catalyse the subsequent hydrolysis of the long chain polysaccharides, amylopectin, and amylose (Guido & Moreira, 2014). The bound β-

amylase is released between day 1 and 3 of germination (Vinje et al, 2015). Figure 2 demonstrates the action of the amylolytic enzymes on the starch polymers.

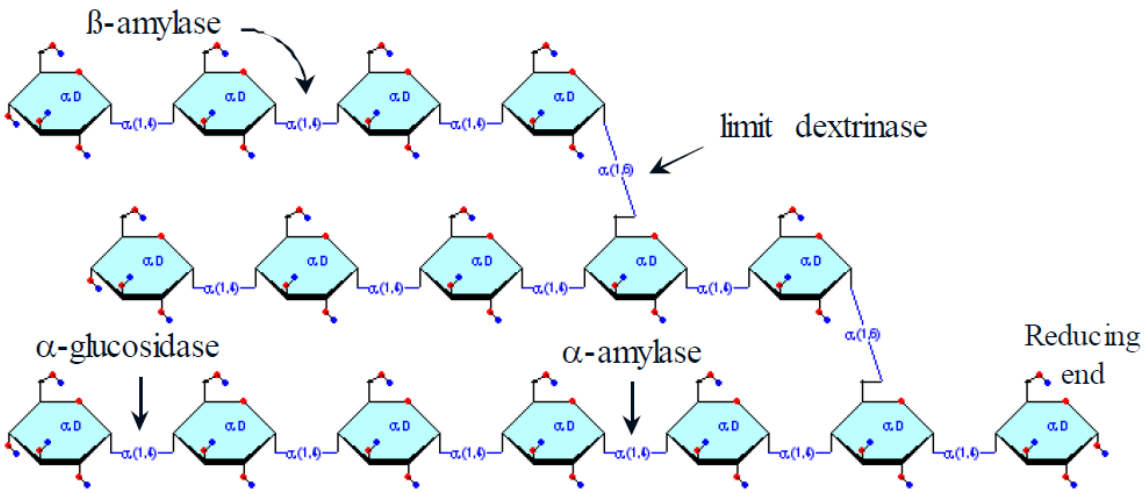

Figure 2: Schematic representation of starch hydrolysis (Evans et al, 2009).

Limit dextrinase is a debranching enzyme that possesses the unique ability to catalyse the hydrolysis of α-1,6 glycosidic linkages in amylopectin, producing oligosaccharides. This enzyme is present in the developing and quiescent barley kernel. Its *de novo* synthesis is equally induced in the germinating grain. It is synthesized in an inactive form bound to a proteinaceous inhibitor molecule by the aleurone cells of germinated grains (Hu et al, 2014). Maltases are specialised α-glucosidases that act on maltose and other short maltooligosaccharides produced by amylases and limit dextrinase, producing glucose (Stanley et al, 2011). They have a high affinity for maltose and release a-D-glucose from the non-reducing end of their substrate. The predominant form present in the germinating barley are exo-acting enzymes (Andriotis et al, 2016). Saccharase is derived from the developing embryo and is linked to root development during germination (Vriesekoop et al, 2010).

α-Amylase cleaves the starch polymers (amylose and amylopectin) at various points along the chain by hydrolysing the α-1,4 glycosidic linkages, giving rise to molecules of variable length (i.e., partially fermentable polysaccharide fractions such as dextrin and maltotriose) that can subsequently be utilized as substrates for the amylolytic enzymes (limit dextrinase, saccharase and β-amylase) mentioned above (Vinje et al, 2015). β-

amylase catalyze the hydrolysis α-1,4 at the ends of the glucose sub-units that are within three glucose molecules of an α-1,6 glycosidic bonds, this results in the release of the disaccharide, maltose (Oser, 2015).

Germination of the malted barley continues for a period of 4 to 6 days at average temperatures of 16 to 20 °C (Fox, 2018). Ideally, starch degradation during malting should be minimal. Typical starch hydrolysis during malting is about 18%. The concentration of glucose, fructose, maltose, sucrose, maltotriose, and total sugars significantly increase in the malted barley during germination (Vinje et al, 2015).

Kilning

To prevent the depletion of the accumulated sugars, dextrins, and other components (amino acids, and proteins) in the resultant malt extract by the embryo, the malt is kilned to terminate germination (Oser, 2015).

The green malt is heat treated to reduce the moisture content from approximately 44% to 12%. This is followed by a second phase of drying that occurs at a slower pace, in which the malt is dried from 12% to 4% (Guido & Moreira, 2014). This kilning process kills the embryo, halts germination, and prevents further modification while preserving the malt for long-term storage and retaining its enzyme activity (Oser, 2015). Kilning of malted barley is imperative to prevent malting loss that can be caused by the heterotrophic shoots and roots of the developing seedling (Vinje et al, 2015). The enzyme activity declines during kilning, unwanted flavours are eliminated, and the colour, aroma and flavour are developed (Aroh, 2019).

The dried barley malt is subjected to milling which involves carefully breaking down the barley husk leading to the exposure of the starchy endosperm and the content of the embryo, which are mainly hydrolytic enzymes (Pereira de Moura & Rocha dos Santos Mathias, 2018). Milling malt renders the starchy endosperm more accessible for the malt enzymes and this considerably improves the extraction process (Willaert, 2007). The resulting malt product provides all the nutrients required for yeast metabolism (Fox, 2018).

2.2.2 Conversion of Malt to Wort

The conversion of malt to wort is initiated during mashing. Mashing is performed for the dissolution of components of the malt into soluble end products, the disintegration of the malted grain cell wall structure, extraction and hydrolysis of starch, sugars, proteins and non-starch polysaccharides and the determination of the fermentable sugar profile (Aroh, 2019). During wort production, the enzymatic hydrolysis of starch is a step of paramount importance for the efficient release of digestible carbohydrates (glucose, fructose, maltose, maltotriose) which brewer's yeasts can utilize during wort fermentation (Hodzic et al, 2008).

Mashing involves mixing of milled barley malt and brewing water (termed 'mashing in') at controlled temperatures. The mixture of malt and water is termed 'mash' in brewer's jargon. The addition of brewing water activates malt enzymes, mainly α and β-amylases but also proteases for the hydrolysis of starch and proteins respectively, thereby giving rise to a mixture of sugars and peptides or amino acids (De Keukeleire, 2000). The diagram below (Figure 3) shows an overview of all major stages of wort production.

Mashing methods can be divided into infusion and decoction mashing. Infusion mashing is the simplest method, it involves gradually heating the mash in the same vessel (Jurková et al, 2012). In decoction mashing, a portion of the mash (the decoction) is quickly heated to conversion temperatures, and the mash is boiled to inactivate enzymes and returned into the main mash to increase the temperature of the mixture (Montanari et al, 2005). Mash conversion temperatures of around 65 °C will ensure the gelatinization of starch (Evans et al, 2009). Infusion mashing is predominantly used in ale beers production. The decoction method was developed to deal with issues associated with under-modified or enzymatically weak malts, it is generally used in the production of lager beers (Montanari et al, 2005). There are three-step, two-step, and single-step mash decoction systems (Jurková et al, 2012).

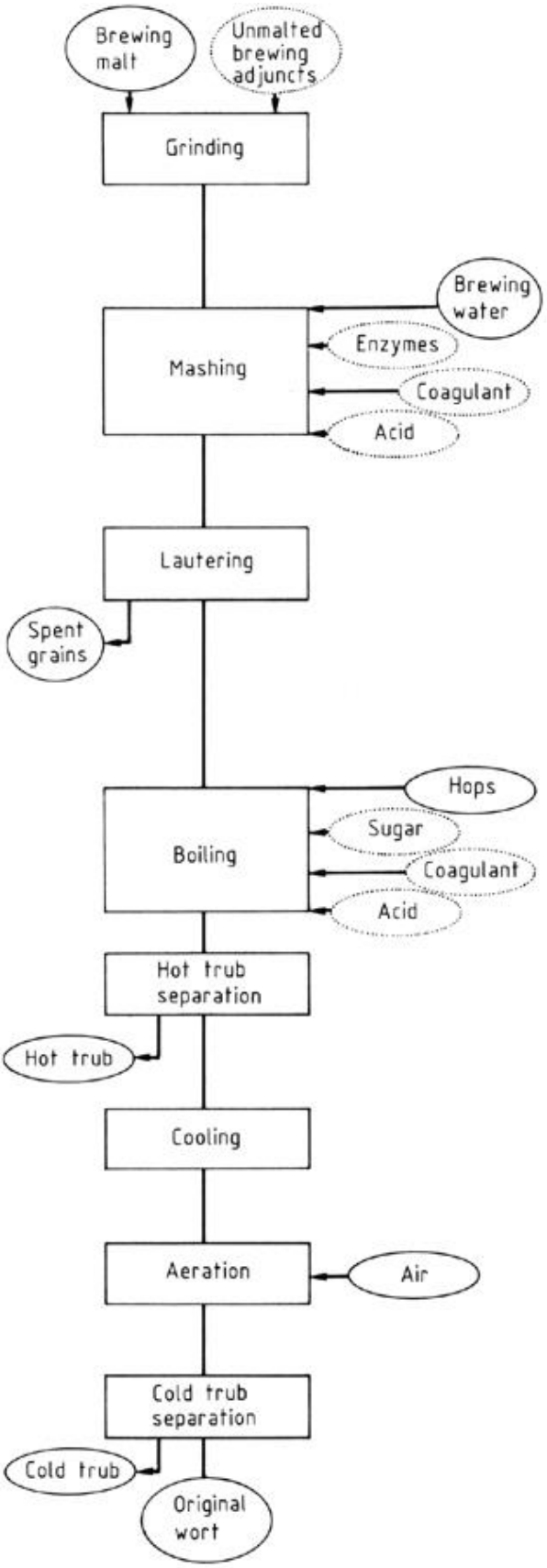

Figure 3: Process flow diagram of Wort Production (Eßlinger & Narziß, 2009)

Different mashing times and temperature regimens are applied in breweries, but the main goal is to gelatinize starch and optimize enzyme activity for starch hydrolysis (Fox, 2018). The intensity of the mashing process is influenced by the mashing-in temperature. Temperatures of 35-40 °C are required for an efficient dissolution of the substrate and the enzymes, to ensure proper enzyme functioning at their optimum temperature. Highly modified malts can be mashed in even at 62 °C, the optimum temperature of β-amylase (Eßlinger & Narziß, 2009). As the temperature increases in the mash, the starch granules begin to swell and absorb water irreversibly (gelatinization), which disrupts their intermolecular bonds and renders them vulnerable to attack by the starch hydrolytic enzymes: α-amylase, β-amylase, and limit dextrinase. Increasing the temperature during mashing can lead to an increased rate of enzymatic reactions, decrease in viscosity (which enhances mixing and separation of dissolved substances), starch gelatinization, and rapid diffusion and dissolution of malt components. On the other hand, high temperatures will equally accelerate enzyme denaturation, and the increased rate of dissolution may lead to extraction of undesirable compounds (e.g. tannins) from the malt (Saarni et al, 2020). The optimum temperatures under which these enzymes act on their substrates are shown in Table 3. Therefore, care should be taken to reach a sufficiently high mash temperature to achieve full gelatinization, but also low enough to prevent the rapid degradation of amylolytic enzymes (Saarni et al, 2020).

Table 3: Optimum condition and amylolytic activities of enzymes during mashing (Modified from Vriesekoop et al (2010); Willaert (2007))

Enzyme	Optimum temperature	Optimum pH	Inactivation Temperature	Substrate	Reaction	Product
α-Amylase	65-75 °C	5.5 -5.8	80 °C	amylose, amylopectin	Endohydrolysis of the α-1,4 glycosidic linkages	Dextrins, limit dextrin, maltotriose
β-Amylase	60-65 °C	5.4 - 5.6	70 °C	dextrins, amylose, amylopectin	Successive exohydrolysis of the penultimate α-1,4 glycosidic linkages at the non-reducing end of chains	Maltose
Limit dextrinase	55-60 °C	5.1 - 5.5	65-70 °C	amylopectin	Hydrolysis of a-1,6 bond in starch	Dextrin (unbranched chain)

Maltase	35-40 °C	6	40 °C	maltose	Hydrolysis of maltose	2 glucose molecules
Saccharase	50 °C	5.5	55-67 °C	sucrose	Hydrolysis of sucrose	Glucose and fructose

Mashing temperatures that may be employed during rest periods include 50 °C for proteolysis, 62-65 °C for maltose release by the action of β-amylase, 70-75 °C for saccharification (α-amylase); and 78 °C for mashing-off and malt enzyme inactivation (Aroh, 2019; Willaert, 2007). Saccharification is the complete degradation of starch to maltose and dextrins by amylases (Willaert, 2007).

After mashing, the sugary liquid (now called sweet wort) is separated from the residual malted barley and insoluble components (husks, the seedling, and other insoluble material) by lautering. The lautered wort is then boiled (100 °C) for at least an hour with hops (*Humulus lupulus* L.), clarified (removal of the hot trub and insoluble hop residues), and cooled (Aroh, 2019). Hops is added as a preservative thereby preventing microbial spoilage due to the action of iso-α-acids on Gram-negative bacteria, additionally hops imparts bitterness (Lodolo et al, 2008). The boiling process facilitates the extraction and transformation of hop components, the removal of unwanted flavour compounds, the thermal destruction of malt enzymes to fix the wort composition as well as sterilization of the wort (Vriesekoop et al, 2010).

The resulting wort contains a plethora of nutrients such as amino acids, peptides, proteins, carbohydrates, vitamins, inorganic ions, and lipids. Nevertheless, the principal components are free amino nitrogen (FAN), fermentable sugars, and unfermentable dextrins. FAN represents the sum of bioavailable nitrogenous compounds in the fermenting wort namely amino acids, small peptides (dipeptides & tripeptides) and ammonium ions (Stewart, 2016). Wort composition can vary due to differences in raw materials composition and processing factors, with potential negative impacts on yeast performance (Lodolo et al, 2008).

2.2.3 Wort Sugar Composition

Wort is composed of several carbohydrates including the simple sugars, glucose, sucrose, maltose and maltotriose. Maltose is the most abundant sugars in the wort (Stewart, 2016). A typical wort sugar profile is depicted in Table 4. The three major fermentable sugars

are glucose, and the α-glucosides; maltose and maltotriose (Willaert, 2007). The wort equally contains small amount of fructose derived from free cereal saccharose and complex carbohydrates such as dextrin (Hodzic et al, 2008).

Table 4: Carbohydrate Composition in Wort (Modified from Stewart, 2016)

Sugars	Percentage (%)
Glucose	10-15
Fructose	1-2
Sucrose	1-2
Maltose	50-60
Dextrins	20-30

2.3 Sugar Uptake in Fermenting Wort

Fermentation is the most time-consuming process within the brewing process. A typical fermentation takes about one week (De Keukeleire, 2000; Naydenova et al, 2012). The *Saccharomyces* brewing yeast is added to the aerated hopped wort during this phase, this is followed by the anaerobic conversion of wort sugars to the primary by-products, ethanol, and CO_2 by the yeast cells (Aroh, 2019). The mode of sugar consumption during fermentation is dependent on the sugar being used, the yeast species, and the fermentation conditions (Walker & Stewart, 2016).

According to Capece et al (2018), the yeast cells follow an increasing order of complexity in sugar consumption. The simplest sugars (glucose and fructose) are consumed first, followed by disaccharides (maltose) and trisaccharides (maltotriose). In other words, glucose is consumed first, followed by fructose, maltose, and finally maltotriose.

Wort sugars are assimilated either by their intact passage across the yeast cell membrane, or their hydrolysis outside the cell membrane by extracellular enzymes, followed by partial or complete absorption of the hydrolysis products into the cells (Stewart, 2016). For instance, the sugar assimilation is usually initiated by the extracellular hydrolysis of sucrose by invertase, causing an increase in glucose and fructose concentrations (Lodolo et al, 2008). On the other hand, maltose and maltotriose pass intact across the plasma membrane. Energy in the form of ATP is required for the uptake (active transport) of

disaccharides such as maltose (most abundant wort sugar) and maltotriose, whereas monosaccharides (glucose and fructose) are transported passively into the cells (Stewart, 2016). Thus, glucose is transported by facilitated diffusion and maltose by active transport. Figure 4 illustrates the consumption of sugars during fermentation by a lager yeast.

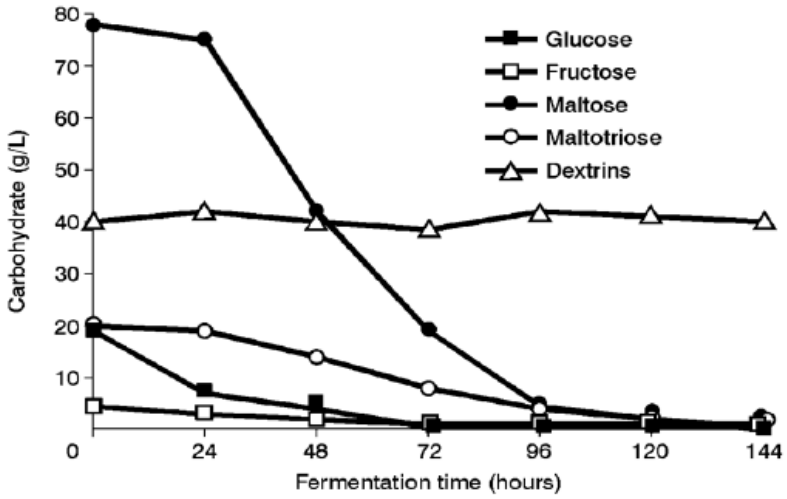

Figure 4: Uptake of Wort Sugars by Fermenting Lager Yeast (Stewart, 2016)

In fermentation media such as malt wort, the uptake of other sugars (such as maltose) is repressed in the presence of glucose, which is preferentially consumed during fermentation by *S. cerevisiae*, a phenomenon known as catabolite repression. This may lead to a slower or incomplete fermentation and the development of off-flavours (Walker & Stewart, 2016). As a result, maltose uptake is repressed at high glucose concentrations. Brewing yeast gradually begins maltotriose uptake once glucose and maltose are almost depleted, maltotriose uptake usually remains incomplete (Lattici et al, 2020). The maltotetraose and higher dextrins are unfermented by majority of brewing yeast strains. The enzyme glucoamylase (amyloglucosidase) catalyzes the hydrolysis of dextrin (Stewart 2016). However, glucoamylase enzymes contained in barley malt are not active at typical mashing regimens because of its significantly lower optimum temperature (35-40 °C) for activity (Vriesekoop et al, 2010). As a result, the remaining unfermented saccharides and dextrins contribute to the body, mouthfeel, drinkability, and palate fullness in the resulting beer (He et al, 2014).

2.4 Overview of Beer Spoilage Microorganisms

In addition to wild yeast contaminants such as *S. cerevisiae* var. *diastaticus*, the most prevalent microbial contaminants that present a major risk in the brewing industry include some Gram-positive and Gram-negative bacteria (Bokulich & Bamforth, 2013). These microorganisms spoil the beer by increased turbidity and acidity as well as the production of undesirable odors, such as the buttery off-flavour associated with diacetyl or rotten eggs associated to hydrogen sulfide (Sakamoto & Konings, 2003).

These microbial contaminants may originate from a variety of sources. Primary contaminants originate from raw materials and unclean brew house vessels while secondary contaminants are introduced during filling processes such as bottling, canning, or kegging in the packaging hall. Although majority of the documented cases of microbiological instability are attributed to secondary contaminations, the effects of primary contaminations may be more catastrophic, with the potential loss of an entire production batch (Obi, 2017).

Among the beer spoilage microorganisms, Gram-positive bacteria are considered the most damaging microbial group to brewers, which mainly include lactic acid bacteria (LAB) belonging to the genera *Lactobacillus* and *Pediococcus*. Gram-negative spoilers mainly include the strict anaerobes belonging to genera *Pectinatus*, *Megasphaera*, *Selenomonas* and *Zymophilus*. Other significant Gram-negative aerobic and facultative anaerobe beer spoilers belong to genera *Acetobacter*, *Zymomonas*, *Selenomonas*, and *Obesumbacterium* (Ashtavinayak & Elizabeth, 2016). In terms of prevalence and the negative effects on the flavour profiles of beer, the most hazardous spoilage bacteria belong to the genera, *Pectinatus*, *Megasphaera* and most importantly the LAB genera*;* *Lactobacillus* and *Pediococcus* (Suzuki, 2011).

2.4.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are the most prevalent beer-spoilage organisms. It has been reported that they account for 60-90% of the microbiological problems in breweries. They may render the beer undrinkable due to the production of lactic acid, diacetyl, and turbidity (Deng et al, 2014). In addition, LAB beer spoilage is characterized by

sedimentation, haze, biogenic amine production, gas formation and ropiness (slime formation), depending on species and strains (Geißler, 2016).

LAB are capable of growing in beer and spoiling it due to their resistance to hop compounds (Esmaeili et al, 2015). The iso-alpha-acids comprise the greatest fraction of hop compounds in beer, they are formed as a result of the heat induced oxidation during wort boiling (Geißler, 2016). They are responsible for imparting a bitter taste to the beer. In addition, the iso-alpha-acids exert a bacteriostatic effect (De Keukeleire, 2000). Majority of Gram-positive bacteria are inhibited by hop compounds, although Gram-negative bacteria are unaffected (Obi, 2017). The bacteriostatic property of iso-alpha-acids is derived from their action as proton ionophores. They cross the bacterial cell membrane undissociated and release protons inside the cell, thereby causing a decrease in the intracellular pH (Geißler, 2016). This proton influx disrupts the proton gradient (pH gradient) across the cytoplasmic membrane of susceptible cells (Suzuki, 2011; Obi, 2017).

The hop-resistance of certain LAB strains is presumably due to the presence of hop efflux transporters, HorA and HorC (Geißler, 2016). The activities of HorA and HorC most likely lead to a reduction in the net influx of the membrane-permeable hop acids into the cytoplasm, thereby counteracting the protonophoric effect of hop acids and maintain the transmembrane pH gradient (Suzuki, 2011; Geißler, 2016; Obi, 2017). Hop-resistant LAB species with a very high hazard potential include *Lactobacillus backii*, *L.* (*para*) *collinoides, L. paucivorans, L. brevis, L. lindneri* and *Pediococcus damnosus*, with the last three regarded as the dominant beer spoilers (Geißler, 2016).

L. brevis strains has been reported as the most prevalent LAB species in spoiled beer, some strains spoil almost all beer types, causing haziness, sedimentation, and acidification, but no buttery off-flavour is produced (Suzuki, 2011). *L. brevis* is physiologically versatile and can also cause super attenuation due to its ability to ferment starch and dextrins (Obi, 2017).

L. lindneri is highly resistant to hop compounds and is tolerant to high thermal treatments and sometimes survives mild pasteurizations. Furthermore, *L. lindneri* grows poorly in many detection media described in the brewing industry, and often causes spoilage incidents without being detected in microbiological quality control assays. *L. lindneri*

forms a relatively faint haze and little sediment with no off-flavour formation in beer (Suzuki, 2011).

P. damnosus is a major spoilage agent in beer with very high hazard potential (Obi, 2017). Contamination may lead to the production of acetoin and diacetyl resulting in a buttery off-flavour, as well as the production of exopolysaccharides (EPS) which causes ropiness in beer (Geißler, 2016). Nevertheless, some selected strains of LAB might be applied intentionally by the brewer, as starter cultures in malting or for mash and wort bio-acidification due to their potential desirable effect (Esmaeili et al, 2015).

2.4.2 *Pectinatus* and *Megasphaera*

Pectinatus and *Megasphaera* are regarded as the most important Gram-negative beer spoilage agents, mainly in unpasteurized beer. These strict anaerobes are mainly encountered as contaminants in large, modern breweries equipped with effective filling technology due to the concerted efforts towards the reduction of the oxygen content present during brewing. The reduction in oxygen content is intended to improve the chemical stability of beer and prevent the negative effects of beer oxidation. In addition, the growing demand for more 'natural' beers with little to no pasteurization treatment might be a possible explanation of their rising incidence (Haikara & Helander, 2006). *Pectinatus* and *Megasphaera* are equally tolerant to hop compounds (Esmaeili et al, 2015).

Spoilage by *Pectinatus* is characterized by high turbidity, high concentrations of propionic acid (>1000 mg/L), some acetic acid and sulfur compounds (such as dimethyl trisulphide, hydrogen sulfite, methyl mercaptane). The spoilage is perceived through the unpleasant smell of rotten eggs (Esmaeili et al, 2015).

Megasphaera on the other hand, causes low turbidity in beers and negligible sediments but causes the release of severe off-flavours. Bad smell-producing compounds, including butyric acid, caproic acid and hydrogen sulphide, are formed and the beer becomes undrinkable. *Megasphaera* is intolerant to ethanol, thus low alcohol beers and alcohol-free beers are particularly at risk (Suzuki, 2011).

2.4.3 Wild Yeast

Wild yeasts are generally defined as yeast species not deliberately introduced by the manufacturer, and therefore uncontrolled by the brewer during the production process. They may be detected at all stages of the production, from raw materials to the packaged beer, and from bar dispense equipment. Nonetheless, they are typically primary contaminants in the pitching yeast (Moretti, 2013). Wild yeasts are generally classified into non-*Saccharomyces* and *Saccharomyces* species (Esmaeili et al, 2015).

The non-*Saccharomyces* wild yeasts include genera such as *Brettanomyces*, *Candida*, and *Debaryomyces Filobasidium*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Torulaspora* and *Zygosaccharomyces* (Esmaeili et al, 2015). These yeasts spoil beer through the production of the highly volatile phenolic compounds such as 4-ethylguaiacol (4EG) and 4-ethylphenol (4EP), thereby releasing the unpleasant off-odours of bandages, sweat, and smoke. Several other metabolites, including significant acetate production in the presence of oxygen, result in a number of off-flavours produced by these yeasts (Bokulich & Bamforth, 2013).

The *Saccharomyces* wild yeasts present the greatest risk, given their physiological and morphological similarity to the production yeast (Latorre et al, 2020). They are equally the most frequent contaminants (Bokulich & Bamforth, 2013), with majority of the strains belonging to *S. cerevisiae* (Esmaeili et al, 2015). Beer spoilage originating from contaminations by *Saccharomyces* wild yeast is characterized by ester or phenolic off-flavour production (POF), increased turbidity or sedimentation, as well as super attenuation leading to over carbonation and diminished body (Bokulich & Bamforth, 2013). There is a rising concern in contaminations by *S. cerevisiae* var. *diastaticus* (Meier-Dörnberg et al, 2017). Conventional brewing yeasts are unable to ferment dextrins and these persist in the final beer product where they contribute to the body and mouthfeel (Moretti, 2013). However, *S. cerevisiae* var. *diastaticus* is able to utilize the unfermented dextrins due to the secretion of glucoamylase enzymes, leading to a high degree of attenuation (Latorre et al, 2020).

2.5 Super-attenuating Yeast

Diastatic strains of *Saccharomyces cerevisiae*, also referred as *S. cerevisiae* var. *diastaticus* for super-attenuating, hyper-attenuating or highly fermentative yeasts possess the unique ability to super-attenuate the resulting beer product, meaning that they can ferment residual long-chain oligosaccharides like dextrins and soluble starch that are unfermented by industrial brewing yeast strains (Krogerus & Gibson, 2020). Oligosaccharides are carbohydrate chains containing 3-10 sugar units (Laurentin & Edwards, 2013). Dextrins are mixtures of D-glucose polymers originating from cereal grain starches used in beer production. The dextrin composition of wort varies according to the malts, adjuncts and the mashing regimens used. For this reason, dextrin concentrations can vary significantly between beer styles. The hydrolysis of dextrin by super-attenuating yeasts, liberates glucose molecules and digestible carbohydrates (Burns et al, 2020).

S. cerevisiae var. *diastaticus* strains are considered to be obligatory spoilage microorganisms (wild yeast) in beer and beer-mixed beverages (Meier-Dörnberg et al, 2017). They are part of a sub-population of *S. cerevisiae* that are ubiquitous in the brewing industry but are far less common among the overall *S. cerevisiae* population (Burns et al, 2020).

According to Capece et al (2018), *S. cerevisiae* var. *diastaticus*, formerly known as *S. diastaticus*, can utilize dextrin and starch due to their ability to produce the extracellular enzyme, glucoamylase (α-1,4 glucan glucohydrolase). Conversely, as the STA genes are not present in ale and lager brewing yeast strains, they are unable to produce this extracellular enzyme (Stewart, 2016).

The hydrolytic activity of yeast glucoamylase was first reported in the 1940s. The first isolation of a diastatic strain of the genus *Saccharomyces* was reported in 1952, this strain was first considered a different species based on its unique phenotypic property, and the proposed taxonomic name was *Saccharomyces diastaticus.* The scientific name of *S. diastaticus* was later modified to *S. cerevisiae* as var. *diastaticus* due to the ability of these cells to mate and produce fertile hybrids with *S. cerevisiae*. This taxonomic classification has now been confirmed by whole-genome sequencing (Krogerus & Gibson, 2020).

The expression of glucoamylase is encoded by the homologous genes; STA1, STA2 and STA3 (Capece et al, 2018). The nucleotide sequences of the STA genes are nearly identical, but they were given different names as they have different chromosomal locations; STA 1, 2 and 3 genes are located on chromosomes II, IV, and XIV (Krogerus & Gibson, 2020). The *STA* genes are reported to have originated from the *S. cerevisiae* gene *SGA1*, a gene encoding the sporulation-specific intracellular glucoamylase Sga1p. STA1 gene is better characterized than STA2 and STA3, and its detection in yeast strains has become the criteria for categorizing strains as diastaticus using molecular techniques (Abbet, 2019). *S. cerevisiae* var. *diastaticus* strains are used as production yeast in the manufacture of Belgian beer styles such as Saison, Belgian Golden Strong, and Bière de Garde (Burns et al, 2020). When applied as the main fermenting microorganism according to established protocols, their presence is not considered a threat (Abbet, 2019).

2.5.1 Sources of Diastaticus Yeast Contamination

S. cerevisiae var. *diastaticus* is a widespread and absolute beer spoilage organism, as mentioned before, that can tolerate the selective environment in beer, particularly in smaller breweries where beers are seldom pasteurized, quality control is less stringent, and experimentation with different yeast strains is more frequent (Krogerus et al, 2019).

Diastatic strains of *S. cerevisiae* can occur as a primary contaminant. Primary contaminations include contamination of the wort, the yeast (pitching yeast), the fermentation and storage tanks, and of the filtration systems used (Meier-Dörnberg et al, 2017). During this primary contamination, diastaticus yeast can compete with the brewer's yeast species in culture. This may lead to a strong increase in the spoilage yeast cell concentration in the fermentation medium (Latorre et al, 2020).

Diastatic yeast strains may occur as secondary contaminants during the beer filling process (Latorre et al, 2020). Contamination in filling lines usually occurs as scatter contaminations and therefore, only single or several bottles may be infected with the diastaticus strain. Such contamination may originate from aerosol infection due to improper hygiene in the bottling area or by a so-called wash-out effect of biofilms formed in the pipework system of the filling machine. These kinds of trace contaminations that occur before releasing the product to the market are very difficult to detect (Meier-

Dörnberg et al, 2017). Secondary contaminations may equally originate from residues in bottles, air circulation in the filling lines and the capper (Latorre et al, 2020).

S. cerevisiae var. *diastaticus* cells exhibit higher spore formation from sexual reproduction, in contrast to ale and lager brewer's yeast strains. This ability increases the probability for an efficient spread within and between breweries (Latorre et al, 2020).

2.5.2 Consequences of Diastaticus Yeast Contamination

Super-attenuation is characterized by a lower residual extract, as well as an abnormal increase in CO_2 and ethanol concentration in packaged beer. Changes in taste, sediment formation, turbidity, gushing, and swelling of the package are all noticeable effects in contaminated products. The CO_2 accumulation in filled containers (such as bottles, cans, kegs, or disposable drums) causes pressure build up, this may lead to explosion or bursting of the package. Microbiological contamination with *S. cerevisiae* var. *diastaticus* negatively impacts breweries and consumers. The elevated alcohol content caused by this secondary fermentation renders the product non-compliant. Product recalls due to contaminations with these hyper-attenuators can cause economic losses and expose the consumer to risk of physical injury due to bursting resulting from over-carbonation (Meier-Dörnberg et al, 2017).

A rising incidence in contaminations by *S. cerevisiae* var. *diastaticus* starting from 2015 has been reported in a study performed by Meier-Dörnberg et al (2017). In this study, 62 confirmed cases of contaminations by diastaticus yeast were observed from a total of 126 investigated cases. These contaminations were detected as primary contaminations namely in the pitching yeast, and secondary contaminations in finished beers and beer-mixed beverages, among others.

Abbet (2019) equally reported an increased incidence in contaminations across Sweden. Moreover, the secondary contamination of beers with strains with high diastatic strength present a strong spoilage potential even at refrigerated conditions (8 °C). In the event of a contamination, the brewery should issue a recall for the products released to the market. A highly publicized case of product recall from diastaticus yeast contamination was observed in 2016 in the USA, the brewery 'Left Hand Brewing Company' situated in

Longmont, Colorado, recalled at least 20 000 cases of their craft beer called 'Nitro Milk Stout' following customers' complaints of beer gushing (Meier-Dörnberg et al, 2018).

Microbial stability of beer is usually achieved with a process of thermal pasteurization (Buzrul, 2007). The aim of pasteurization is to inactivate the brewing yeast along with potential spoilage microorganisms (molds, yeast and bacteria) that might deteriorate the beer product (Reveron et al, 2003; Milani et al, 2015). Nevertheless, pasteurization of the final product cannot always prevent the effects of a primary or secondary contamination by diastaticus yeast (Meier-Dörnberg et al, 2018).

The pasteurization measure for beer is PU (pasteurization unit). One PU is equivalent to 1 min at 60 °C (Milani et al, 2015). A pasteurization process of 5-15 PU (equivalent to 5 to 15 minutes at 60 °C reference temperature) has been suggested for beer (Reveron et al, 2003). Although laboratory trials indicate that pasteurization regimes ranging from 1 to 5 PU are effective in achieving commercial sterility, the time-temperature relationship of 15 minutes at 60 °C has been commonly used for commercial pasteurization (Buzrul, 2007). Nevertheless, the glucoamylase enzyme produced by diastaticus strains exhibit heat stability (optimum temperature of 55-65 °C) and may not be destroyed by the commonly applied beer pasteurization temperatures (60-62 °C). Thus, the released enzymes can continue their hydrolytic activities on starches and dextrins even after inactivation of the spoilage yeast by pasteurization (Reed & Nagodawithana, 1991).

In addition, a previous study revealed that flash pasteurization at 71 °C for 30 seconds did not fully inactivate the wild yeast *Saccharomyces diastaticus* (Milani et al, 2015). Minimal thermal processes (with lower temperatures) are preferred in order to reduce undesirable organoleptic changes (adverse effect of high temperature exposure on beer flavour, reduction of carbon dioxide solubility due to heat) in the finished beer and to minimize the energy expenditure for beer pasteurization (Buzrul, 2007). Table 5 presents data of the thermal inactivation of vegetative cells of previously studied diastaticus strains estimated in terms of D-value and Z-value parameters.

Table 5: Thermal resistance of vegetative cells of *S. cerevisiae* var. *diastaticus* strains in various beer media

Strain name	Medium	D-value (min)	Temperature (°C)	Z-value (°C)	Reference
***Saccharomyces diastaticus* - strain AB183**	Alcohol-free beer	0.51	60	7.8	Kilgour & Smith (1985)
***Saccharomyces diastaticus* - strain AB183**	Alcoholic beer (3.7% alcohol v/v)	0.06	60	5.4	Kilgour & Smith (1985)
Saccharomyces diastaticus	Wheat beer	0.00076	84	N/A	Zufall & Wackerbauer (2002)

D-value describes the time required for a 90% reduction of the investigated microbial population at a specific temperature and z-value is the temperature required for a one-log reduction (one tenth of the original value) in the D-value (Milani et al, 2015).

2.5.3 Current Detection Methods for Diastaticus Yeast

The detection and control of wild strains from the genus *Saccharomyces* is challenging due to their physiological and morphological similarity to the commercial brewing yeast (Latorre et al, 2020). The majority of the current detection methods for diastaticus yeast are based mainly on either the microbiological detection through culturing on specific selective growth media, or the molecular detection of the STA1 gene through conventional or quantitative PCR (Krogerus et al, 2019). Growth detection methods are time-consuming while the molecular methods are more rapid. A detection test commonly used is the Durham test using fully attenuated beer medium and monitoring possible gas production due to fermentation activities by the inoculum. A preliminary indication of an infection can be established by measuring the beer's apparent extract (Meier-Dörnberg et al, 2018). The detection methods can be divided into three groups: growth-based methods (culture dependent), physiological screening, and molecular/genetic method.

2.5.3.1 Growth Based Methods

Growth-based methods investigate the ability of the inoculated yeast to grow in a selective culture media that suppresses the growth of conventional brewer's yeast. Copper-containing media such as commercial LCSM (Lin's Cupric Sulphate Medium), originally developed for the detection of wild yeast (non-brewing yeast) and MYGP+copper (Malt

extract, Yeast extract, Glucose, and Peptone media with copper), are usually used for this purpose. Copper actively inhibits the growth of ale and lager yeasts while diastaticus yeast strains are copper-tolerant. However, this method is based on copper-tolerance rather than the super-attenuating ability (Krogerus & Gibson, 2020).

A selective medium known as the Farber Pham Diastaticus Medium (FPDM) was recently developed for the detection and enrichment of diastaticus yeast. This novel medium promotes the growth of diastaticus yeast while suppressing the growth of conventional brewing yeast. The composition of FPDM has not been disclosed (Burns et al, 2020). Detection using liquid and solid growth media containing dextrins or starch as the sole fermentable carbohydrate have equally been demonstrated. A pH indicator dye may be included in the media preparation to obtain a colour change associated with the fermentation activities (Meier-Dörnberg et al, 2018, Burns et al, 2020). Examples of such starch or dextrin-containing formulations include yeast nitrogen base (YNB), agar, bromophenol blue (Meier-Dörnberg et al, 2018) and, maltodextrin, YNB with ammonium sulphate, dipotassium ortho-phosphate (K_2HPO_4), and bromocresol green (Burns et al, 2020).

Table 6 summarizes the media formulations applied in the detection of diastaticus yeast strains. However, false positive could be observed since dextrins are a hydrolysis product of starch and may contain fermentable sugars. This method is time consuming as growth on dextrin or starch plates is reported to be very slow (Meier-Dörnberg et al, 2018).

Table 6: Culture media and growth conditions tested for the detection of *Saccharomyces cerevisiae* var. *diastaticus*

Medium	Composition	Incubation conditions	Reference
Omega-optimized LCSM	0.4 % yeast extract, 0.2 % dried malt extract, 0.2 % yeast peptone, 0.1 % K_2HPO_4 (dipotassium ortho-phosphate), 0.05% ammonium sulphate, 0.06% cupric sulphate, 1 % dextrose 2 % agar	30 °C 3 days	Burns et al, 2020

Starch agar + chloramphenicol	1.5 % agar, 1.5 % soluble starch, 0.67 % YNB, 0.005 % chloramphenicol pH 5.2	30 °C Incubation until significant growth was observed	Abbet, 2019
Starch agar + bromophenol blue	1.5 % agar, 1.5 % soluble starch, 0.67 % YNB, 0.004 % bromophenol blue, pH 5.2	Aerobic & anaerobic incubations 25 °C 37 days (888 hours)	Meier-Dörnberg et al, 2018
Starch agar + bromophenol blue	1.5 % agar, 1.5 % soluble starch, 0.67 % YNB, 0.004 % bromophenol blue, pH 5.2	Anaerobic incubation 25 °C 4 weeks	Krogerus et al, 2019
Dextrin agar	1.5 % agar, 1.5 % dextrin, 0.67 % YNB, pH 5.2	Aerobic & anaerobic incubation 25 °C 37 days (888 hours)	Meier-Dörnberg et al, 2018
Liquid BGM (Bromocresol Green Maltodextrin)	2% maltodextrin, 0.67% YNB with ammonium sulphate, 0.014 % K_2HPO_4, 0.022 % bromocresol green, pH 5.8 - 6	Room temperature 70 RPM 20 days	Burns et al, 2020
FPDM	Commercial formulation (agar)	30 °C 3 days	Burns et al, 2020
LCSM	Commercial formulation (agar)	30 °C 3 days	Burns et al, 2020

2.5.3.2 Physiological Screening

Physiological screening relies on the examination of yeast potential to express characteristics such as the production of phenolic-off-flavours (POF) or CO_2 formation during fermentation. The screening of diastaticus yeasts strains for their ability to produce off-flavours from the metabolism of phenolic acids have been developed by Meier-Dörnberg et al (2018). The fermentation media was supplemented with one of the phenolic precursor acids; ferulic acid, coumaric acid or cinnamic acid. The phenolic flavours tested are influenced by the formation of 4-vinyl guaiacol (4VG) from ferulic acid, with “clove-like” descriptor, 4-vinylstyrene (4VS) from cinnamic acid with “styrofoam-like” descriptor, and 4-vinyl phenol (4VP) with “medicine-like” descriptors. A sensory evaluation known as the sniff-test is then performed to detect and identify the flavours produced by the inoculated yeast.

Diastaticus yeast strains have been screened by monitoring CO_2 production by inoculating the yeast in sterile fermented beer medium contained in a test tube with an inverted Durham tube. The gas formed as a result of the fermentation of residual oligosaccharides by the super-attenuating yeast strains is collected in the inverted Durham tubes. Nevertheless, this method is equally time consuming (Krogerus & Gibson, 2020).

2.5.3.3 Molecular Methods

Genetic characterization has the advantage of being more rapid in comparison to the growth-based methods. These techniques require DNA to be extracted from the yeast strains. The most widespread molecular detection technique is the detection of the STA1 gene using PCR, with several commercial kits being marketed for this purpose. Some strains that carry the STA1 gene may show no to negligible spoilage potential, leading to false positives as they may not super-attenuate the beers. This phenomenon is associated with the deletion in the STA1 promoter region, resulting in no or minimal expression of the gene. Primers that detect the deletion in the STA1 promoter region have been developed to improve the reliability of detection (Krogerus & Gibson, 2020). This could equally be explained by the fact that the non-super-attenuating yeast carrying the STA1 gene either possesses altered function or localization of the enzyme or expresses the gene only in conditions not encountered in the brewery environment (Abbet, 2019).

The absence of STA1 gene correlates with the elimination of diastatic activity, and disruption in the STA1 promoter region leads to a reduction in the diastatic ability, both suggesting that STA1 encodes for the main super-attenuating phenotypic expression. On the other hand, another study reported that the disruption of STA2 also leads to a loss in diastatic activity, highlighting the complexity of the phenotype (Burns et al, 2020).

Real-time or quantitative PCR amplification has also been applied for the detection of the STA1 gene. This method is advantageous because lower levels of contaminations can be detected (Krogerus & Gibson, 2020). However, PCR detection methods are susceptible to user error and extraneous variables that can lead to false positives and false negatives. PCR genotyping can detect the presence of STA1 gene only but does not assess the viability of the cell from which the DNA was extracted (Burns et al, 2020).

CHAPTER THREE

3 METHODOLOGY

3.1 Yeast Strains

All strains used in this study are listed in Table 7. The strains were obtained as isolates on agar slants from the culture collection of the Global Yeast & Fermentation Laboratory of Anheuser-Busch InBev (Leuven, Belgium).

Table 7: Strains used for diastaticus detection assays

Strain Code	Strain Name/Species	Details	Origin
DSM 70487	*Saccharomyces cerevisiae* var. *diastaticus*	Spoilage yeast (positive control)	Leibniz Institute DSMZ culture collection (Germany)
Strain-Y	[No name]	Lager yeast (negative control)	Industrial yeast isolate (Belgium)
TUM 1-B-8	*Saccharomyces cerevisiae* var. *diastaticus*	Spoilage yeast	Microbiology & Yeast Center Weihenstephan (Germany)

3.2 Propagation of Yeast Strains

The strains used in the present study were propagated in wort medium obtained from the Stella Artois Brewery situated in Leuven (Belgium). The initial density (°P) of the wort was measured using a DMA 35 density meter (Anton Paar GmbH, Graz, Austria) and the wort was diluted using brewing water to a density of 15 °P. The wort was supplemented with zinc by adding 1 ml of a 200 ppm (200 mg/L) zinc chloride ($ZnCl_2$) stock solution per litre of the wort, for a final concentration of 200 ppb (200 µg/L) in the wort medium. Appropriate volumes of the wort were transferred to propagation flasks as follows: 40 ml of wort in a 100 ml Schott bottle, 160 ml of wort in a 1000 ml flask and 800 ml of wort in a 2000 ml flask. Following this, the containers with wort were sterilized in a horizontal Touchclave system MP autoclave (LTE Scientific Ltd., Oldham, United Kingdom) at 105 °C for 10 minutes.

The propagation was done in three steps by transferring the yeast cell suspension to a larger wort volume in a ratio of 1:4. A loopful of each strain was inoculated in 40 ml of sterile wort in a 100 ml Schott bottle and incubated at 26 °C for 30 hours with continuous

stirring at 100 RPM in a Multitron HT-AJ125TC floor incubator shaker (Infors HT, Bottmingen, Switzerland). After the incubation period, 40 ml of the yeast cells suspension from the previous culture were inoculated into 160 ml of sterile wort, in a 1000 ml flask, and further incubated at room temperature (22 °C to 25 °C) for 24 hours.

The yeast cultures were swirled manually for 15 minutes every one to two hours to promote aeration during incubation. The upscaling of the second step to the next propagation step was performed by transferring the 200 ml yeast culture to 800 ml of sterile wort in a 2000 ml flask, with similar incubation conditions to the second step. The laboratory propagation was done as shown in the scheme below (Figure 5).

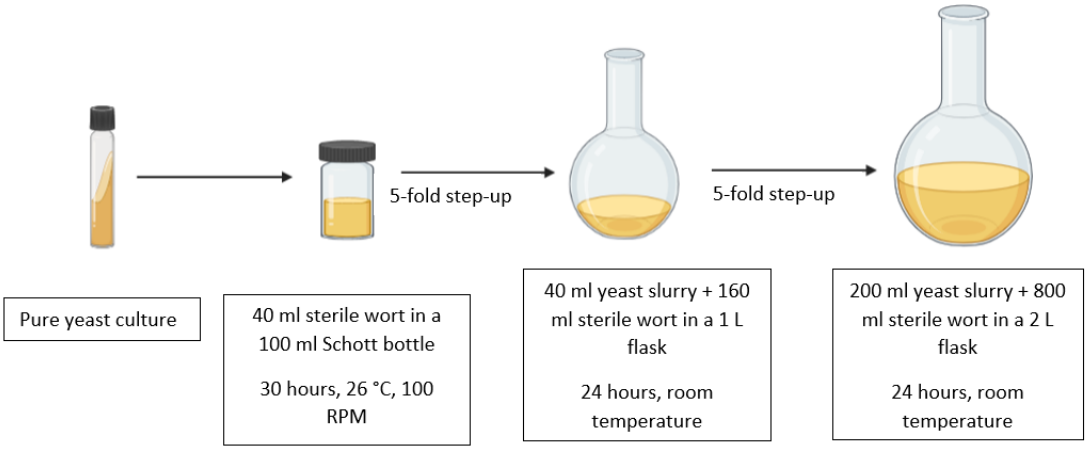

Figure 5: Laboratory yeast propagation scheme

3.3 Determination of yeast cell concentration

The propagated cultures were centrifuged (4500 RPM (4750 g), 15 minutes, 4 °C) in the Allegra X-15R series benchtop centrifuge (Beckman Coulter Inc., Brea, California, United States), to concentrate the yeast in the pellet. After centrifugation, the supernatant was discarded, and the pellet of each yeast strain was resuspended in 50 ml sterile 0,9% NaCl (saline) to ensure no carryover of sugars from the propagation wort into the media to be inoculated. To determine the cell concentrations of the propagated yeast in cells/ml, each strain was counted with an automated cellometer (Nexcelom Bioscience, LLC, Lawrence, Massachusetts, United States). Appropriate dilutions of the propagated yeast were prepared by resuspending the yeast pellet in sterile 0,9 % NaCl for conducting cell

counts. Cell counting was performed by transferring 20 µl of the diluted cell suspensions with 20 µl of a 100 µl/ml propidium iodide staining solution (1:1 dilution ratio). A volume of 20 µl of the mixture was loaded using a micropipette in two sample counting chambers of a disposable slide. The cell count results (cell/ml) were averaged over 2 counts for each strain.

3.3.1 Preparation of concentrated aqueous yeast suspensions

In preparation to inoculate the yeasts, the cell concentrations were adjusted to the pitching (inoculation) cell concentrations. Suspensions of the pure yeast strains were prepared as 10X concentrated stock solutions in sterile 0,9 % NaCl to attain the correct pitching rates listed in Table 8. Six tubes of 10X concentrated stock suspensions of yeast blends (contamination mixes) containing the negative control strain (Strain-Y) and the positive control strain (DSM 70487) adjusted to different cell concentrations per millilitre (cells/ml) were equally prepared in 0,9% NaCl, with respect to the assay performed as shown in the table below.

Table 8: Cell concentrations of the Strains in Suspension of Pure Yeast and Yeast Blends

Strain/Contamination mix	10X stock concentrations (cells/ml)	Final inoculation concentrations/ pitching rates (cells/ml)	Assay(s)
DSM 70487	1×10^9	1×10^8	Durham test, attenuation test, Agar test
Strain-Y	1×10^9	1×10^8	
TUM 1-B-8	1×10^9	1×10^8	
Mixture 1	$(1 \times 10^9)^{a} + (1 \times 10^5)^{b}$	$(1 \times 10^8)^{a} + (1 \times 10^4)^{b}$	Durham test, attenuation test
Mixture 2	$(1 \times 10^9)^{a} + (1 \times 10^4)^{b}$	$(1 \times 10^8)^{a} + (1 \times 10^3)^{b}$	
Mixture 3	$(1x10^9)^{a} + (1 \times 10^3)^{b}$	$(1x10^8)^{a} + (1 \times 10^2)^{b}$	
Mixture 4	$(5 \times 10^7)^{a} + (5 \times 10^3)^{b}$	$(5 \times 10^6)^{a} + (5 \times 10^2)^{b}$	Agar test
Mixture 5	$(5 \times 10^7)^{a} + (5 \times 10^2)^{b}$	$(5 \times 10^6)^{a} + (5 \times 10^1)^{b}$	
Mixture 6	$(5 \times 10^7)^{a} + (5 \times 10^1)^{b}$	$(5 \times 10^6)^{a} + (5 \times 1)^{b}$	

[a] = Strain-Y, [b] = DSM 70487

3.4 Preparation of Fully Attenuated Beer Medium

The ingredients used for the preparation of fully attenuated beer were strain-Y yeast slurry (biomass) and finished 'Stella Artois' beer (pale lager, 5% Alcohol by Volume). The lager yeast biomass was obtained from the Stella Artois Brewery situated in Leuven (Belgium). The yeast slurry was centrifuged at 4500 RPM for 15 minutes at 4 °C in the Allegra X-15R series benchtop centrifuge (Beckman Coulter Inc., Brea, California, United States). After centrifugation, the supernatant was discarded and the pellet containing the yeast was reserved. A volume of 330 ml of 'Stella Artois' beer was transferred aseptically to a Schott bottle and degassed by stirring at 200 RPM for 2 hours in a Top safe 1.2 laminar flow cabinet (Bioair Solutions, Voorhees, New Jersey, United States). Approximately 24.75 grams of yeast pellet was weighed and added to the degassed beer (7.5 g yeast/ 100 ml of beer) in a 500 ml Schott bottle. The Schott bottle was closed with a fermentation air lock and 10 ml of sterile bidistilled water was pipetted into the airlock. This was followed by an incubation period of 24 hours at 20±1 °C with continuous agitation at 100 RPM.

After the incubation period, the content of the Schott bottle was centrifuged in the Allegra X-15R series benchtop centrifuge (Beckman Coulter Inc., Brea, California, United States) at 4500 RPM for 15 minutes at 4 °C to remove the yeast cells. The supernatant, now called 'fully attenuated beer' was transferred to a sterile Schott bottle and reserved while the pellet containing the yeast cells was discarded. The supernatant (fully attenuated beer medium) was supplemented with zinc by adding 1 ml of a 200 ppm (200 mg/L) zinc chloride ($ZnCl_2$) stock solution per litre of the medium, for a final concentration of 200 ppb (200 µg/L). In a second trial, the fully attenuated beer was prepared as described above without zinc addition.

3.4.1 Physicochemical Analyses of Fully Attenuated Beer Medium

The densities (°P) of the prepared fully attenuated beers (with zinc and without zinc added) were measured using the DMA 35 density meter (Anton Paar GmbH, Graz, Austria). The pH values of the media were determined using a CG 840 digital (Schott Instruments GmbH, Mainz, Germany), before starting the assays**.** Sugar (dextrin) analyses of the autoclaved media (with and without zinc supplement) were conducted by

a third-party laboratory to determine the quantity and type of sugars present in the beer medium by HPLC method.

3.5 Modified Durham Test in Fully Attenuated Beer: Gas Production assay

A volume of 9 ml of each fully attenuated beer (with zinc and no zinc added) was transferred to test tubes containing an inverted Durham tube. The tubes containing the media were sterilized in a horizontal Touchclave system MP autoclave (LTE Scientific Ltd., Oldham, United Kingdom) at 105 °C for 10 minutes.

The fermentation test was performed with the pitching cell concentrations described in Table 8. One ml of the 10X stock of each pure strains or contamination mixes suspensions was transferred aseptically to a test tube containing 9 ml of sterile fully attenuated beer with an inverted Durham tube. The pure strains and yeast blends were incubated at room temperature (22 °C – 25 °C) for a period of 30 days. The gas formation in the Durham tubes was visualized every weekday during the incubation period.

The CO_2 build up in the Durham tubes as a result of fermentation activities was recorded as described by Mëier-Dornberg et al (2018) with few modifications, the Durham tubes (1.90 ml volume, 40 mm in length) were divided into four parts by volume described in intervals from >0.00 mL to 0.45 ml as G1/4, from 0.45 ml to 0.9 ml as G2/4, from 0.9 ml to 1.35 ml as G3/4, from 1.35 ml to 1.9 ml as G4/4 and tubes without gas formation were described as G0/4. At day 30, the content of each test tube was centrifuged (4500 RPM, 15 minutes, 4 °C) in the Allegra X-15R series benchtop centrifuge (Beckman Coulter Inc., Brea, California, United States) and the density (°P) was measured with the DMA 35 density meter (Anton Paar GmbH, Graz, Austria). The difference between the final density and the density of the fully attenuated beer before the start of the experiment serves as an indication of the consumption of residual sugars in the beer medium by the yeast strains. Test tubes with Durham tubes containing 9 ml of sterile fully attenuated beers with and without zinc were included in the test as blank samples. This assay was performed in duplicates.

3.6 Attenuation test in Fully Attenuated Beer

Fully attenuated beers with zinc supplement and without zinc added were prepared as described in section 3.4. The prepared fully attenuated beers were sterilized in a horizontal Touchclave system MP autoclave (LTE Scientific Ltd., Oldham, United Kingdom) at 105 °C for 10 minutes. The attenuation tests were carried out in 250 ml sterile Schott bottles, each equipped with a modified screw cap linked to an external silicon tubing with a PTFE (polytetrafluoroethylene) air vent filter (0.2 µm pore size) to allow air to pass through freely while protecting the culture vessel from contamination by keeping air particulates and microbial contaminants out of the schott bottles. Tubings were equally connected via the screw cap to enable the content of the bottle to be withdrawn for weekly determination of the apparent extract in °P. The sampling tube was tightened with a clamp to regulate the flow of fluids. An illustration of the set-up is depicted in Figure 6.

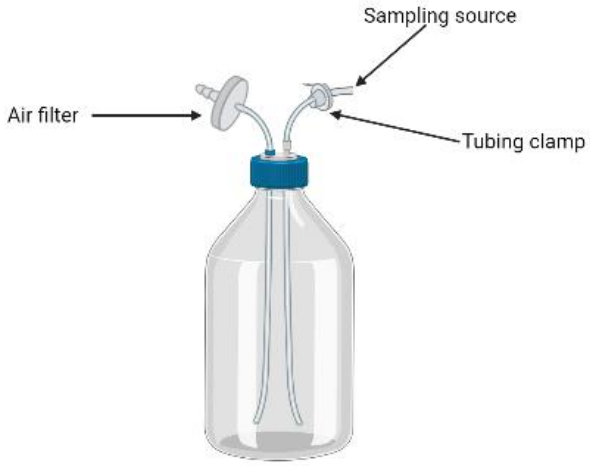

Figure 6: Representative diagram of culture bottles used for the attenuation test

The inoculation volume of the pure strains and strain blends were adjusted to obtain the appropriate final inoculation concentrations (Table 8) in a final culture volume of 100 ml. The Schott bottles were incubated at ambient temperature (22 °C to 25°C) for a period of 4 weeks on the bench. The assay was performed in duplicates. Two blank samples containing 100 ml of sterile fully attenuated beers with and without zinc were included in the test.

3.6.1 Follow-up Analyses (Attenuation test)

A 10 ml sample was withdrawn from each Schott bottle every week using a 60 ml graduated sterile syringe and transferred to a 15 ml falcon tube. Before sampling, bottles were gently shaken to homogenize the yeast at the bottle base and in suspension. Yeast was separated by centrifugation (4500 RPM, 15 minutes, 4 °C). The apparent extract (°P) of the cell-free supernatant was measured using the DMA 35 density meter (Anton Paar GmbH, Graz, Austria).

3.7 Dextrin Agar Test

3.7.1 Dextrin Agar Medium Preparation

Two dextrin agar media adjusted to two different final pH values were used for testing dextrin uptake by the yeasts of the inocula. The ingredients used included 2% agar, 1.5% dextrin type III (MP Biomedicals, Irvine, California, United States), 0.004% pH indicator dye (bromocresol purple or bromophenol blue), 0.67% YNB without amino acids and chloramphenicol (50 mg/L). The formulations of the dextrin agar media are detailed in Tables 9 and 10.

Table 9: Formulation of dextrin type III agar with bromocresol purple

Parameter/Ingredient (unit)	Quantity
Agar (g/L)	20
Dextrin type III (g/L)	15
YNB without amino acids (g/L)	6.7
Bromocresol purple (mg/L)	40
Chloramphenicol (mg/L)	50
pH	6.2

Table 10: Formulation of dextrin type III agar with bromophenol blue

Parameter/Ingredient (unit)	Quantity
Agar (g/L)	20
Dextrin type III (g/L)	15
YNB without amino acids (g/L)	6.7
Bromophenol blue(mg/L)	40
Chloramphenicol (mg/L)	50
pH	5.2

The agar, dextrin type III and the pH indicator dye (bromocresol purple or bromophenol blue) were weighed using a Sartorius Cubis MSE324P-100-DI analytical balance (Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany) and suspended in 900 ml of brewing water. The mixture was sterilized in horizontal Touchclave system MP autoclave (LTE Scientific Ltd., Oldham, United Kingdom) at 121 °C for 15 minutes. A 10X concentrated stock solution of YNB without amino acids was prepared by suspending 6.7 g of YNB without amino acids per 100 ml of brewing water, this solution was filter sterilized using a rapid-flow vacuum filtration unit with a 0.2 µm pore size nylon membrane filter (Thermo Fisher Scientific, Waltham, Massachusetts, United States). After autoclaving, 100 ml of the YNB 10X stock solution was added to autoclaved components (900 ml) of each agar medium. The two media were adjusted to the desired pH values (pH 6.2 and pH 5.2) using 2.5 M sodium hydroxide (NaOH) and 1M sulphuric acid (H_2SO_4). Once the media were adjusted to the correct pH values, 50 mg/L of the antibiotic chloramphenicol was added to each of the cooled media at 45 °C - 50 °C under sterile conditions, to suppress bacterial growth.

Agar plates were made by transferring 25 ml of agar medium into sterile disposable Petri dishes and allowed to solidify at room temperature.

3.7.2 Dextrin Utilization Assay

The dextrin utilization assay relies on pH change as an indicator of cell growth in the medium containing dextrin as the only carbon source. A pH indicator dye (either bromocresol purple or bromophenol blue), was added to the dextrin agar plates to efficiently detect cell growth. A drop in the pH from 6.2 to ≤ 5.2 will result in a colour change of the agar medium with bromocresol purple from purple to yellow, as a result of growing yeast cells that acidify the medium**.** If the pH drops from 5.2 to between 4.6 and 3.0, the colour of the agar plate containing bromophenol blue changes from blue to yellow as a result of cell metabolites (Meier-Dörnberg et al, 2018). Aliquots (100 µl) of the 10X aqueous yeast(s) suspensions (Table 8) were spread out on the dextrin agar plates using a sterile spreader. Each Inoculation was performed in duplicates. The plates were incubated at 28 °C for 1 month in a BR1E cooled incubator (Snijders Scientific, Tilburg, Netherlands). All plates were examined visually for colour change and the appearance of colonies. Sterile agar plates were equally incubated at 28 °C to check for sterility.

3.7.3 Saccharide Profiling of dextrin agar medium components

HPLC analysis of the YNB 10X stock solution (without amino acids) and a solution of 1.5% dextrin mixed with 0.67% YNB were conducted by a third-party laboratory to determine the quantity and type of sugars/dextrins present in the components of the agar medium.

3.8 Statistical Analyses

3.8.1 Attenuation Test

The assay was carried out in duplicates. The experimental values of the attenuation test were reported as mean±standard deviation. The mean values were plotted in a graph of mean apparent extract (°P) versus time (weeks) using Microsoft Excel Software version 2008. Initially, a 3-way ANOVA was performed with factors: inoculum (DSM 70487, Strain-Y, TUM 1-B-8, Mixture 1, Mixture 2 and Mixture 3), zinc (with and without zinc addition), and time (in weeks, 1 to 4), to determine if there is an interaction effect between the three independent variables (inoculum, zinc, and time) on the dependent variable (Apparent Extract in °P), at a significance of $P < 0.05$. Following a significant 3-way interaction effect of inoculum, zinc, and time, two runs of 2-way ANOVA were carried out as follows: 1) with factors inoculum and zinc, 2) with factors zinc and time. The Tukey's HSD test was used to compare the mean apparent extract values per week and per inoculated yeast(s), using a significance level of 0.05 ($P < 0.05$). The statistical tests were carried out using IBM SPSS Statistics 26 Software.

CHAPTER FOUR

4 RESULTS AND DISCUSSION

In this chapter, the results of the experimental assays and their respective discussion are presented. The media used in this study were first evaluated by HPLC to identify the possible presence of fermentable sugars that could support the growth of non-diastaticus yeast strains. Three screening assays were performed to investigate each inoculum for diastatic phenotype. Although the first two analyses (Durham test & dextrin agar test) and modifications thereof have been applied by some researchers (Abbet, 2019; Burns et al, 2020; Krogerus et al, 2019; Meier-Dörnberg et al, 2018), a third assay termed the 'attenuation test', was developed for the detection of diastaticus yeast contaminants due to the ambiguous results observed with the dextrin agar tests and Durham test.

4.1 Saccharide Profiling of Media

The saccharides quantified in the media components (1.5% dextrin solution, YNB without amino acids) and fully attenuated beer medium were fructose, glucose, sucrose, maltose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose. The saccharide compounds concentrations in g/100 ml are outlined in Table 11. According to Troilo et al (2020), fermentable sugars include fructose, glucose, sucrose (saccharose) and maltose. There were very low to negligible amounts of fermentable sugars (fructose, glucose, sucrose, and maltose) in all media types studied. The yeast nitrogen base had 0.56 g/100 ml of glucose and the fully attenuated beer had very low amounts (0.01 g/100 ml) of maltose.

Table 11: Saccharide profiles (DP 1 – 7) of the agar medium components (1.5% dextrin and YNB without amino acids) and fully attenuated beer medium. Total sugar refers to the sum of the fermentable sugars: fructose, glucose, sucrose, and maltose. DP = degree of polymerization.

Saccharide	1.5% Dextrin (g/100 ml)	YNB without amino acids (g/100 ml)	Fully attenuated beer (g/100 ml)
Fructose	0	0	0
Glucose	0	0.56	0
Sucrose	0	0	0

Maltose	0	0	0.01
Maltotriose	0	0	0.02
Maltotetraose	0	0	0.2
Maltopentaose	0	0	0.07
Maltohexaose	0	0	0.07
Maltoheptaose	0	0	0.05
Total Sugar	0	0.56	0.01

A concentration of 0.02 g/100 ml of maltotriose in fully attenuated beer was detected. Maltotriose is the second most abundant sugar of brewer's wort after maltose, but it has the lowest priority for uptake by yeast cells. The yeast cells have a slower uptake rate of maltotriose than maltose, sucrose, and glucose in that order. This may lead to incomplete uptake of maltotriose by brewer's yeast. In addition, maltotriose is not fermented but is respired by several industrial brewing yeast strains (Zastrow et al, 2001), meaning that this saccharide is not readily consumed by several brewer's yeasts. Generally, the carbohydrate uptake by yeast starts with the simplest sugars (the monosaccharides glucose and fructose), followed in increasing order of complexity by disaccharides (maltose) and trisaccharides (maltotriose) excluding maltotetraose and other dextrins which are not consumed (Lodolo et al, 2008). The results above demonstrate that the media (dextrin agar medium and fully attenuated beer) used in the present study have negligible quantities of fermentable sugars and are thus selective enough to be used for dextrin fermentation studies by *S. cerevisiae* var. *diastaticus*.

4.2 Modified Durham Test: Gas Production Assay

The detection of super-attenuating yeast was performed by static incubation of Durham tubes containing sterile fully attenuated beer medium to evaluate gas production by visual monitoring of CO_2 build-up in the inverted Durham tubes as stated by Meier-Dörnberg et al (2018), with minor modifications This test was performed at room temperature in order to mimic conditions to which packaged beer products are exposed during distribution. The gas formation in tubes containing the yeast isolates and yeast blends were tested in fully attenuated beer medium supplemented with and without $ZnCl_2$. Tables 12 and 13 show the gas formation of the investigated yeast strains during the experimental period of 30 days. Figure 7 provides examples of different levels of CO_2 build-up in Durham tubes.

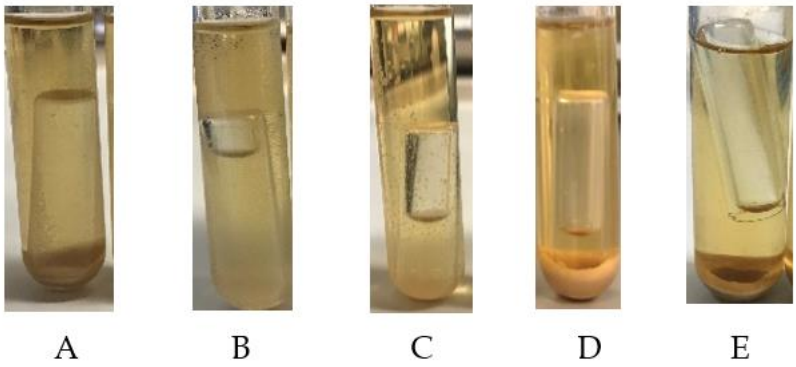

Figure 7: Illustrative pictures of different gas formation intervals in Durham tubes. A= G0/4 (no gas formation), B = G1/4 (from >0.00 mL to 0.45 ml), C = G2/4 (from 0.45 ml to 0.9 ml), D = G3/4 (from 0.9 ml to 1.35 ml) and E = G4/4 (from 1.35 ml to 1.9 ml).

No significant differences were observed in the trials performed in the presence and absence of $ZnCl_2$ in the medium. Out of the 6 inocula tested, only the positive control spoilage yeast (DSM 70487) inoculated at a concentration of 1 x 10^8 cells/ml showed gas formation in both beer media (with and without $ZnCl_2$).

Table 12: Gas Formation and days required (medium with Zinc supplement). Durham tubes volume described in intervals from >0.00 mL to 0.45 ml as G1/4, from 0.45 ml to 0.9 ml as G2/4, from 0.9 ml to 1.35 ml as G3/4, from 1.35 ml to 1.9 ml as G4/4 and tubes without gas formation were described as G0/4. [a] = Strain-Y, [b] = DSM 70487. First appearance of gas in Durham tubes is highlighted in yellow

Pitching concentration Cells/ml	**1 x 10^8**		**1 x 10^8**		**1 x 10^8**		**(1 x 10^8) [a] + (1 x 10^4) [b]**		**(1 x 10^8) [a] + (1 x 10^3) [b]**		**(1 x 10^8) [a] + (1 x 10^2) [b]**	
Strains/Blends	DSM 70487		Strain-Y		TUM 1-B-8		Mixture 1		Mixture 2		Mixture 3	
Replicates	A	B	A	B	A	B	A	B	A	B	A	B
Day(s)	Gas formation											
1	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
2	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
3	G1/4	G2/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
4	G3/4	G3/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
7	G3/4	G3/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4

8	G3/4	G3/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
9	G2/4	G2/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
10	G2/4	G2/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
11	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
14	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
15	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
16	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
17	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
18	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
21	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
22	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
23	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
24	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
25	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
28	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
29	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
30	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4

Table 13: Gas Formation by yeast strains inoculated in beer medium without zinc. Durham tubes volume described in intervals from >0.00 mL to 0.45 ml as G1/4, from 0.45 ml to 0.9 ml as G2/4, from 0.9 ml to 1.35 ml as G3/4, from 1.35 ml to 1.9 ml as G4/4 and tubes without gas formation were described as G0/4. [a] = Strain-Y, [b] = DSM 70487. First appearance of gas in Durham tubes is highlighted in yellow.

Pitching concentration Cells/ml	**1×10^8**		**1×10^8**		**1×10^8**		**(1×10^8) [a] + (1×10^4) [b]**		**(1×10^8) [a] + (1×10^3) [b]**		**(1×10^8) [a] + (1×10^2) [b]**	
Strains/Blends	DSM 70487		Strain-Y		TUM 1-B-8		Mixture 1		Mixture 2		Mixture 3	
Replicates	A	B	A	B	A	B	A	B	A	B	A	B
Day(s)	Gas formation											
0	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
1	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
2	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
3	G3/4	G3/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
4	G3/4	G4/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
7	G3/4	G3/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4

8	G3/4	G3/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
9	G2/4	G2/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
10	G2/4	G2/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
11	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
14	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
15	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
16	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
17	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
18	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
21	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
22	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
23	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
24	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
25	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
28	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
29	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4
30	G1/4	G1/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4	G0/4

The highest gas production levels were first observed at the fourth day of the experiment (Figure 8), a gas production of G3/4 was observed in the test tube of the positive control *S. cerevisiae* var. *diastaticus* (DSM 70487) inoculated in the sterilized beer medium with $ZnCl_2$ while a gas production of G4/4 was observed for the same strain in the trial without the $ZnCl_2$ supplement.

Figure 8: Gas formed in Durham tubes by DSM 70487 (duplicate samples) inoculated in fully attenuated beer with zinc adjunct (left) and without zinc adjunct (right) at day 4

Mëier-Dornberg et al (2018) assayed strains for gas-forming potential in sterilized beer medium and reported that the *S. cerevisiae* var. *diastaticus* control strain DSM 70487 had a completely filled Durham tube G4/4 after 3 days of incubation and TUM 1-B-8 exhibited the highest gas formation with a completely filled Durham tube (G4/4) after 2 days of inoculation. The observations in the present study for the *S. cerevisiae* var. *diastaticus* control strain DSM 70487 were in line with Mëier-Dornberg et al (2018). In contrast, no gas formation was observed in the tubes inoculated with the *S. cerevisiae* var. *diastaticus* (TUM 1-B-8) during static incubation for 30 days. Variation in diastatic activity may be due to differences in the concentrations of glucoamylase secreted, localization of the enzyme (complete versus partial secretion, or eventually localization to the membrane), or enzymatic functionality (Abbet, 2019).

The lower concentrations of DSM 70487 contained in the mixed cultures: mixtures 1, 2 and 3 at 1 x 10^4 cells/ml, 1 x 10^3 cells/ml, and 1 x 10^2 cells/ml respectively, failed to develop visible gas within the 30 days of incubation. Van Dijken et al (1986) examined the glucose fermentation ability of yeast strains of the genera *Saccharomyces* and *Candida*, it was observed that most yeast strains produced significant amounts of ethanol despite the absence of visible gas build-up in the Durham tubes, suggesting that the absence of gas formation is not a reliable criterion for the absence of fermentation capacity. In addition, sugar fermentation and assimilation tests using Durham tubes may lead to misidentification of strains. Some of the yeast strains give negative results because they do not release CO_2 immediately and may be termed as slow fermentative yeasts (Ruíz-Muñoz et al, 2017). In case of slow fermentations, visible gas formation may be unobserved in opened vessels like Durham tubes due to the rapid escape of CO_2 out of the tube as it is formed by the fermenting yeast. This is particularly true for yeast strains that grow at the surface of the medium during static incubation (Van Dijken et al, 1986). Meier-Dörnberg et al (2018) observed that an increase in the inoculation cell concentration leads to a faster detection in gas formation, thereby accelerating the experimental results.

From the data presented above, detecting low levels of contaminations of super-attenuating yeast strains using the modified Durham test seems challenging and time-consuming. Therefore, it is necessary to use other identification methods to confirm the presence of the super-attenuating yeast strains.

4.3 Attenuation Test

Attenuation describes the decrease in specific gravity or density of the wort during fermentation as a result of sugar consumption and ethanol production by the fermenting yeast. During fermentation, the attenuation level is altered with carbohydrate consumption (which leads to a reduction in the specific gravity) and production of ethanol (which equally lowers the specific gravity), the gravity measurement of the fermented beer results in a lower reading than would be observed from measurement of the dissolved solids in the absence of ethanol (e.g. in unfermented wort). The term apparent extract (AE) is used when referring to the uncorrected value of specific gravity or density, assuming no alcohol is present in the fermented beer (Bourque, 2013). Therefore, the density or apparent extract represents the relative amount of sugars (extract) present in the fermented medium at the given time (Forbes, 2014). In order to monitor the residual sugar consumption by the inocula, the apparent extract of the fully attenuated beer medium was determined using a portable density meter for a period of four weeks and the results were expressed in degrees Plato (°P). Figures 9 and 10 present the fermentation of the fully attenuated beers without zinc and with zinc respectively, with initial density value of 1.9 °P and initial pH of 3.3 (value measured for both beer media before sterilization by autoclaving at 105 °C for 10 minutes).

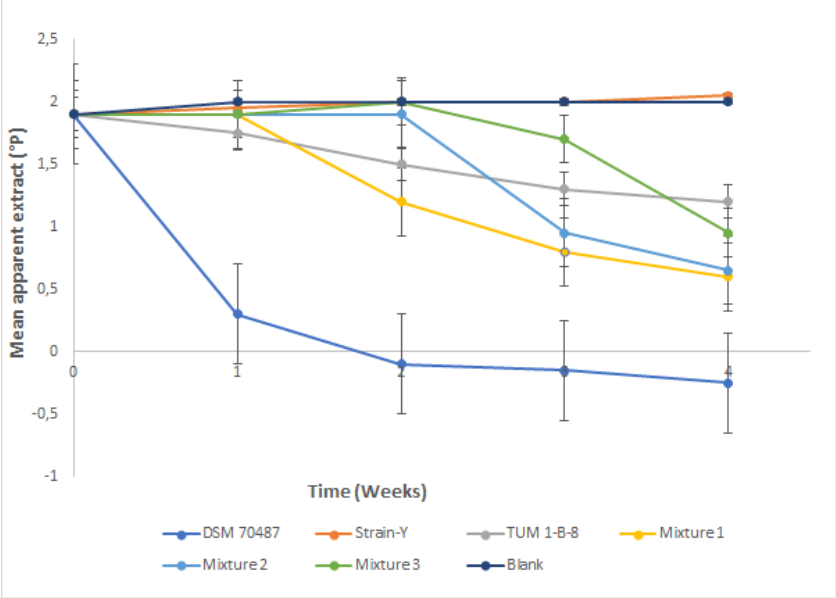

Figure 9: Evolution of the apparent extract (°P) of fully attenuated beer medium (without zinc) pitched with yeast strains for 4 weeks of incubation. Each point represents the mean

of replications; n=2. Initial density of beer medium = 1.9 °P, Initial pH = 3.3. Error bars represent the standard deviation. The weekly AE values observed between the investigated samples were significantly different ($P < 0.05$)

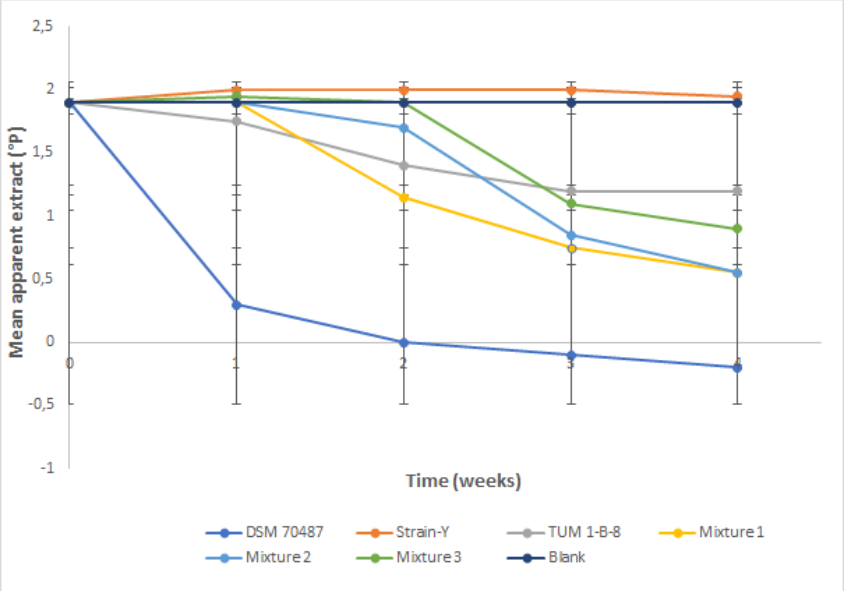

Figure 10: Evolution of the apparent extract (°P) of fully attenuated beer supplemented with 200 ppb (µg/L) zinc, pitched with yeast strains for 4 weeks of incubation. Each point is the average of two measurements. Initial density of beer medium = 1.9 °P, Initial pH = 3.3. Error bars represent the standard deviation. The weekly AE values observed between the investigated samples were significantly different ($P < 0.05$).

During fermentation, once the medium (e.g. wort) is pitched (inoculated) with yeast, the fermentable sugars are utilized by the yeast cells. The utilization of sugars triggers the decline of the apparent extract with respect to time (Forbes, 2014). A decline in the wort density over time was observed for the positive control strain (DSM 70487), the TUM 1-B-8 strains, as well as the strain blends containing the contaminant cells at concentrations 1×10^4 cells/ml (mixture 1), 1×10^3 cells/ml (mixture 2) and 1×10^2 cells/ml (mixture 3). The decline was fastest in DSM 70487, with the highest reduction (from 1.9±0.00 °P to 0.3±0.00 °P, with and without zinc supplementation) observed within one week, this was followed by a reduced rate of decline in its apparent extract. The final value of the apparent extract at week 4 was -0,25±0,07 °P (with zinc) and -0.2±0.00 °P (without zinc °P). The negative apparent extract values signify that the density of the sample was less

than the density of water (Forbes, 2014). These results show the efficiency of DSM 70487 in the consumption of residual sugars in the beer medium (super-attenuation ability).

The other spoilage yeast, TUM 1-B-8 had a slower AE reduction rate throughout the experimental period. The AE reduced from 1.9±0.00 °P (week 1) to 1.2±0.00 °P, with and without added zinc at week 4, showing a reduced diastatic activity. Likewise, Burns et al (2020) observed varying degrees of attenuation by several diastaticus strains during bottle refermentation studies in the packaged ale beer 'Lagunitas IPA' (355 ml, 2.6 °P, 6.2% ABV). A reduction of the AE from 2.6 °P to 1.6 °P was observed for the strong diastatic French saison strain while mild super-attenuators (such as Belgian saison I and Belgian saison II strains) lowered the AE by >0.6 °P after one month. This demonstrates the high variability in the spoilage risk posed by *S. cerevisiae* var. *diastaticus* strains.

Figure 11 shows the mean apparent extract value of each inoculum at different sampling times, from week 1 to 4, including the results of the significant differences at $P < 0.05$. In contrary to the positive control strain DSM 70487 (1×10^8 cells/ml inoculation rate) which showed a rapid reduction in the AE after one week incubation, a gradual decrease in the apparent extract values of the fermented beer medium containing the contaminant cells (DSM 70487) at lower concentration in the co-cultures; mixture 1, 2 and 3 was noted from week 2. This indicates that the super-attenuation rate reduces with decreasing concentrations (cells/ml) of the spoilage yeast DSM 70487. Burns et al (2020) equally observed an increased re-fermentation rate with increasing inoculation cell concentrations of diastaticus yeasts.

Thus, the higher contamination levels are detected in a shorter period of time compared to the lower contamination levels which are characterized by a slower sugar consumption rate. A contamination by DSM 70487 at a cell concentration of 1×10^8 can be more rapidly detected (one-week detection time) compared to TUM 1-B-8 at similar cell concentration, which showed a significant decline in the AE after 2 weeks.

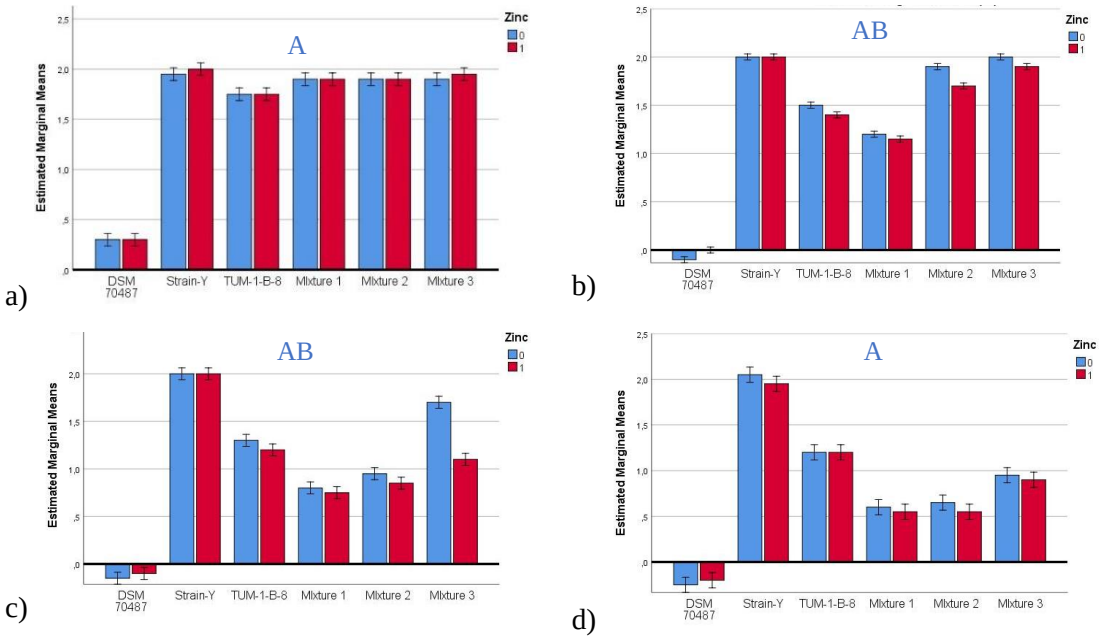

Figure 11: Mean Apparent extract values (°P) of each inoculum per sampling time (with and without zinc). a) week 1, b) week 2, c) week 3, and d) week 4. Whiskers represent 95% confidence intervals. Results of the statistical analyses (Tukey's test, $P < 0.05$) are annotated as follows: A=significant differences among inoculated yeast(s), B=significant differences between trials with and without added zinc, AB = significant differences among inocula & significant effect of zinc addition. There were significant differences in the apparent attenuation among strains and co-cultures for each sampling time, from week 1 to 4.

Figure 12 shows that there were significant differences in the apparent extract values of the inoculated yeasts during incubation. In addition, zinc addition affected the attenuation in the case of DSM 70487, TUM 1-B-8, mixture 2 and mixture 3, but not Strain-Y nor mixture 1. However, zinc supplementation did not seem to have a great impact on the super-attenuation ability of DSM 70487, TUM 1-B-8 and mixture 2. This was confirmed statistically with relatively high P-values of 0.022 (for DSM 70487 & TUM 1-B-8) and 0.04 (mixture 2) recorded for the factor, zinc (refer to Tables 6, 8 & 10 of Appendix 1). Therefore, zinc Addition slightly influenced the reduction in apparent extract for DSM 70487, TUM 1-B-8, and mixture 2 during incubation. Zinc Addition strongly influenced the reduction in apparent extract for mixture 3 during incubation ($P < 0.05$). A steep decrease in °P was noted for mixture 3 supplemented with zinc from week 2 to week 3

(1.90±0.07 °P to 1.1±0.00 °P), compared to its counterpart without zinc chloride adjunct (Figure 12f) which showed a slower reduction in the apparent extract from week 2 to week 3 (2.00±0.00 °P to 1.70±0.00 °P). Moreover, the Tukey's test revealed significant differences (P<0.05) in the apparent attenuation on the second and third week of incubation for the trials with and without zinc (Figure 11).

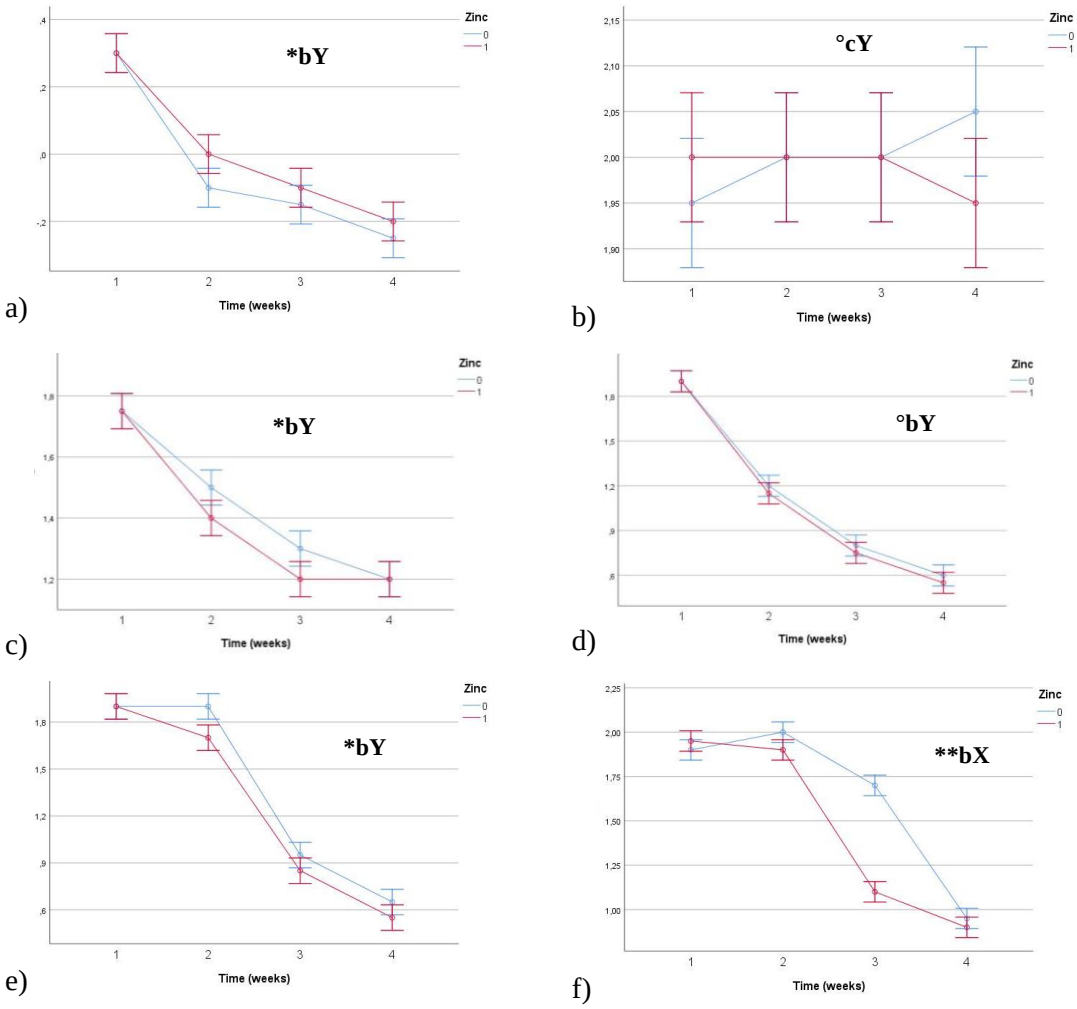

Figure 12: Apparent attenuation of fully attenuated beer medium by each inoculum during incubation. a) DSM 70487, b) Strain-Y, c) TUM 1-B-8, d) Mixture 1, e) Mixture 2, f) Mixture 3. Whiskers represent 95% confidence intervals. For significant differences (Tukey's test) among mean AE values with and without zinc addition; *p<0.05, **p<0.001, °=non-significant. For significant variations in the apparent extract values with incubation (sampling) time; a=p<0.05, b=p<0.001, c=non-significant. For existence of interaction effect between factors zinc and incubation time; X= interaction effect Y = No interaction effect.

The significant differences between the apparent extract values of the fully attenuated beer media inoculated with mixture 3 is likely related to the presence of zinc in the medium. In the production of fermented beverages, zinc is of prime importance, with levels of at least 0.3 ppm required for optimal fermentation and avoidance of stuck fermentations that may lead to reduced ethanol production. Zinc is a co-factor for numerous important biosynthetic and metabolic enzymes including various glycolytic enzymes and alcohol dehydrogenase, the terminal enzyme of the fermentation pathway. Zinc is also known to modulate yeast stress responses, mainly due to its role as a co-factor for the antioxidant enzyme superoxide dismutase. In beer fermentations, zinc is actively assimilated by yeast from the sugary wort and low zinc levels in wort may lead to slow and incomplete fermentations (De Nicola et al, 2005). Walker et al (2006) have previously shown that the zinc bioavailability significantly affects fermentation performance. Analyses of the density and ethanol concentration following inoculation of lager yeast strain LBB in malt wort media supplemented with zinc concentrations ranging from 0 to 10.8 ppm indicated a lower density and higher ethanol % in the medium with the highest zinc concentration at the end of the fermentation. Likewise, Ciosek et al (2020) showed that zinc supplementation positively influenced a brewer's ale yeast (SafAle™ US-05) performance during beer fermentation with final alcohol content of 4.2 % v/v (with zinc supplementation) and 3.9 % v/v (without zinc supplementation). De Nicola (2006) stated that optimal zinc concentrations considerably decrease the attenuation time, although this may be strain dependent. Overall, the variation in the apparent extract value was greater with incubation time compared to zinc supplementation. According to the presented findings, this assay is useful both for testing mixed cultures with diastatic and non-diastatic strains, as well as for screening pure culture isolates for diastatic activity.

4.4 Dextrin Agar Test

The growth assays on solid media were performed in two trials. In one trial, the dextrin agar medium containing bromophenol blue was set at a pH of 5.2. The second test consisted of inoculating the yeast cell suspensions in dextrin agar containing bromocresol purple with a pH of 6.2 at the beginning of the experiments. The pure strains and co-cultures were assayed for growth under aerobic conditions to study their ability to degrade and utilize dextrin. Table 14 shows the results of cell growth following a 30-day period on media containing dextrin as the sole carbon source.

Table 14: Growth of yeast cells (resuspended in 0.9% NaCl) on dextrin agar media adjusted at pH 5.2 and pH 6.2 incubated at 28 °C recorded after 30 days incubation. [a] = Strain-Y, [b] = DSM 70487

Strain Code	Comment	Inoculation concentration (cells/ml)	Bromophenol blue pH 5.2	Bromocresol purple pH 6.2
DSM 70487	Pure Strain	1×10^8	strong growth	strong growth
Strain-Y	Pure Strain	1×10^8	no growth	no growth
TUM 1-B-8	Pure Strain	1×10^8	no growth	no growth
Strain-Y + DSM 70487	Mixture 4	$(5 \times 10^6)^a + (5 \times 10^2)^b$	medium growth	medium growth
Strain-Y + DSM 70487	Mixture 5	$(5 \times 10^6)^a + (5 \times 10^1)^b$	medium growth	weak growth
Strain-Y + DSM 70487	Mixture 6	$(5 \times 10^6)^a + (5 \times 10^0)^b$	weak growth	weak growth

Three growth patterns were observed at pH 5.2 and pH 6.2 (Tables 14 and 15). At pH 5.2; strong growth (agar medium turned pale blue/greenish blue), medium growth (dense colonies on agar plate and no colour change), and weak diastatic activity (appearance of 1 to 20 colonies with no colour change). The growth behaviours exhibited at pH 6.2 included strong growth (agar medium turned completely yellow), medium growth (very low colour change to pale purple), and weak diastatic activity (appearances of colonies with no colour change). Figure 13 shows the colour change of the agar plates after 30 days of incubation. Burns et al (2020) equally noted the following behaviours during incubation at 30 °C in growth assays on agar medium containing maltodextrin and bromocresol green as pH indicator dye; strong diastatic activity (medium was turned completely yellow within 3 days), moderate diastatic activity (medium was turned completely yellow within 8 days), and weak diastatic activity (colour change was incomplete).

Figure 13: Colour change of inoculated petri plates containing dextrin agar with bromophenol blue at pH 5.2 (left) and dextrin agar medium with bromocresol purple at pH 6.2 (right) after 30 days of incubation at 28 °C.

Table 15: Days Required for observing visible growth on dextrin agar media inoculated with pure yeast strains and mixed cultures of lager yeast (Strain-Y) and spoilage yeast (*S. cerevisiae* var. *diastaticus*). [a] = Strain-Y, [b] = DSM 70487

Strain Code	Type	Inoculation concentration (cells/ml)	pH 5.2		pH 6.2	
			Days	Growth sign	Days	Growth sign
DSM 70487	Pure Strain	1×10^8	3	Blue to greenish blue colour change	4	Purple to yellow colour change
Strain-Y	Pure Strain	1×10^8	-	no growth	-	no growth
TUM 1-B-8	Pure Strain	1×10^8	-	no growth	-	no growth
Strain-Y + DSM 70487	Mixture 4	$(5 \times 10^6)^a + (5 \times 10^2)^b$	11	Colonies & no colour change	14	Colonies & colour change from purple to pale purple
Strain-Y + DSM 70487	Mixture 5	$(5 \times 10^6)^a + (5 \times 10^1)^b$	15	Colonies & no colour change	16	Colonies & no colour change
Strain-Y + DSM 70487	Mixture 6	$(5 \times 10^6)^a + (5 \times 10^0)^b$	16	Colonies & no colour change	22	Colonies & no colour change

Visible cell growth was observed in DSM 70487 (positive control) pure strain and the mixed cultures (mixtures 4, 5 and 6), showing that the DSM 70487 strain exhibits growth when co-cultivated with the non-diastatic strain (Strain-Y) on dextrin agar plates. Growth of the yeast strains at pH 5.2 was generally faster compared to the growth at pH 6.2 (Table 15). Similarly, Yalcin & Ozbas (2008) investigated the effect of pH on the growth of two *Saccharomyces cerevisiae* strains and found that the maximum specific growth rate was

reached at pH 4 and the lowest growth rate was recorded at pH 6.46. Laluce & Mattoon (1984) equally observed a higher growth rate of a *Saccharomyces diastaticus* strain inoculated in YPS medium (yeast extract, peptone and 2% Lintner starch) with an initial pH of 4.2 compared to an initial pH of 5.4. According to Narendranath & Power (2005), *Saccharomyces cerevisiae* is an acidophilic organism and, as such, grows better under acidic conditions. The optimal pH range for yeast growth can vary from pH 4 to 6, depending on temperature, the presence of oxygen, and the strain of yeast. This explains the rapid growth at pH 5.2, which is more acidic compared to the agar medium at pH 6.2. As seen in Table 15, the time required to observe cell growth increased with a decreasing cell concentration (cells/ml) of the spoilage yeast DSM 70487, suggesting that, more time will be required to detect low levels of contaminations during quality control analyses in breweries.

The reference yeast (Strain-Y) used as negative control did not grow on the dextrin agar as expected, whereas Meier-Dörnberg et al (2018) reported visible growth on dextrin agar for some investigated non-diastaticus *S. cerevisiae* and *S. pastorianus* strains incubated under aerobic and anaerobic conditions at 28 °C for 37 days. This difference may be due to the use of dextrin containing impurities such as fermentable sugars, that supported the growth of the non-diastaticus yeast strains. Another possible explanation is the presence of adherent wort (fermentable) sugars on the propagated yeasts since the yeast cells were inoculated without additional washing steps with sterile saline solution in the trial performed by Meier-Dörnberg et al (2018).

Although TUM 1-B-8 exhibited a mild super-attenuating ability by lowering the apparent extract of fully attenuated beer in the attenuation test, this strain did not grow on the dextrin agar plates. A possible explanation for this difference may be that TUM 1-B-8 performs better in a medium similar to finished beer such as the fully attenuated beer medium. Moreover, this strain may require longer period of time to express a higher super-attenuating strength and cause visible spoilage. Laluce & Mattoon (1984) demonstrated that dextrin is converted to ethanol as efficiently as a similar concentration of glucose by *S. diastaticus* strain SD2, provided that additional fermentation time is allowed. Similar observations of inconsistent results in different analyses were reported for certain *S. cerevisiae* var. *diastaticus* strains in recent studies, TUM 3-H-2 was identified as *S. cerevisiae* var. *diastaticus* by real time PCR but did not show any super-

attenuating ability when assessed for growth on solid medium containing starch as sole carbon source (Meier-Dörnberg et al, 2018) and in a study performed by Krogerus et al (2019), 15 strains were confirmed to carry the STA1 gene coding for glucoamylase in PCR but only five out of these 15 strains were able to grow on starch agar under anaerobic conditions. However, a 1162 base pairs deletion in the STA1 gene promoter region was observed in the 10 non-growing strains. Suggesting that, the presence of this deletion coincides with decreased super-attenuating ability as a result of a reduced expression of the glucoamylase-encoding gene. To conclude, the dextrin agar plate test confirmed that inoculating yeast strains in dextrin agar medium with bromophenol blue or bromocresol purple indicator dyes can be used to detect *S. cerevisiae* var. *diastaticus* yeast strains. The dextrin agar test with bromophenol blue at pH 5.2 provides faster results and enables the detection of lower contamination levels. The results above indicate that this assay is useful for testing mixed cultures (diastaticus and non-diastaticus strain blends). In addition, Meier-Dörnberg et al (2018) stated that the agar test is cheaper, faster, and easier to use in common brewing labs compared to the modified Durham test in fermented beer medium.

CHAPTER FIVE

5 CONCLUSION AND FUTURE STUDIES

5.1 Conclusion

Based on the results obtained in the present study, some conclusions/final considerations can be stated, as follows:

The modified Durham test has a high limit of detection, only the DSM 70487 inoculated at a concentration of 1 x 10^8 developed gas bubbles in the inverted Durham tube. No gas production was observed in the tubes containing DSM 70487 at lower cell concentrations, and these tubes with lower levels of the contaminant strain would be erroneously flagged as negative with the current modified Durham test.

A notable reduction in the apparent extract (AE) values were recorded for the contaminant *S. cerevisiae* var. *diastaticus* in co-cultures after two weeks in the attenuation test. The extract measurements proved to be a useful way to detect fermentation activities in slower fermentations and lower contaminant levels at 1 x 10^4 cells/ml, 1 x 10^3 cells/ml, and 1 x 10^2 cells/ml of *S. cerevisiae* var. *diastaticus*. In addition, the attenuation test may be useful to detect non-gas forming strains in the modified Durham test and avoid false negative results.

The findings of the agar test suggest that this assay is useful for testing mixed cultures (diastaticus and non-diastaticus strain blends) and to test pure yeast isolates for their super-attenuating ability. *S. cerevisiae* var. *diastaticus* demonstrated a faster growth rate at pH 5.2 compared to pH 6.2, thus the agar test at a pH value of 5.2 proved to be a reliable and more rapid test (in comparison to the agar test at pH 6.2) to detect super-attenuating yeasts.

The attenuation test has proven to be the best detection assay in this study due to its ability to detect both strong and low diastaticus activity in all contaminated samples from week 2 of incubation. In addition, the attenuation test was reproducible with consistent results obtained for all investigated duplicate samples.

This study has revealed that there is variability in diastatic activity and beer-spoilage potential in *S. cerevisiae* var. *diastaticus* strains. It was observed that TUM 1-B-8 has a

lower spoilage potential compared to DSM 70487. DSM 70487 showed strong super-attenuating ability in all the assays performed while TUM 1-B-8 showed super-attenuating ability in the attenuation test with a mild drop in the AE and did not exhibit any diastatic activity in the Durham test and agar test. This indicates that the super-attenuating strength of *S. cerevisiae* var. *diastaticus* and consequently the spoilage potential is strain dependent. Furthermore, the results show that several detection assays should be performed simultaneously for a reliable classification of investigated strains as diastaticus or non-diastaticus and for evaluating the spoilage potential of diastaticus strains.

5.2 Future Works

In order to mimic the conditions of a packaged beer, the attenuated beer medium's pH and the pH of the dextrin agar medium with bromophenol blue will be adjusted to a pH of 4.5. Lowering the pH may be useful, particularly in detecting the diastatic activity of slow fermenting super-attenuators.

Additional dextrin (commercial dextrin type III) will be incorporated in the prepared fully attenuated beer medium to enable a rapid detection and to detect lower contamination levels (improving the limit of detection) by providing additional carbohydrates for the yeast strains to metabolize.

In order to extend initial laboratory studies, the yeasts fermentative performance will be investigated under various zinc concentrations to determine the optimal zinc concentration for the rapid growth and detection of *S. cerevisiae* var. *diastaticus*.

An increase in the incubation temperature ranging from 28 °C to 30 °C will be tested in an attempt to accelerate the growth of *S. cerevisiae* var. *diastaticus* and reduce the detection time in all the assays performed.

Performing shelf-life studies by inoculating finished beer with varying concentrations of diastaticus yeast strains and incubating the samples for several weeks/months over a wide temperature interval in order to assess the time required to observe spoilage signs and to evaluate the influence of temperature on the spoilage risk.

6 BIBLIOGRAPHY

Abbet, M. (2019). *Understanding the emerging beer spoilage yeast Saccharomyces cerevisiae var. diastaticus* (Master's Dissertation), Chalmers University of Technology. https://odr.chalmers.se/bitstream/20.500.12380/300756/1/Master%20Thesis_%20Marie%20Abbet.pdf

Andriotis, V. M. E., Saalbach, G., Waugh, R., Field, R. A., & Smith, A. M. (2016). The Maltase Involved in Starch Metabolism in Barley Endosperm Is Encoded by a Single Gene. *Plos One*, *11*(3), 1-13. https://doi.org/10.1371/journal.pone.0151642

Aroh, K. (2019). Review: Beer Production. *SSRN Electronic Journal*, 1-15. https://dx.doi.org/10.2139/ssrn.3458983

Ashtavinayak, P. & Elizabeth, H. (2016). Review: Gram Negative Bacteria in Brewing. *Advances in Microbiology*, 6, 195-209. 10.4236/aim.2016.63020.

Bokulich, N.A., & Bamforth, C.W. (2013). The microbiology of malting and brewing. *Microbiology and Molecular Biology Reviews*, 77, 157–72. https://doi.org/10.1128/MMBR.00060-12

Bourque, C. (2013). *Fermentability of Canadian Two-Row Barley Malt: Wort Turbidity, Density, and Sugar Content as Measures of Fermentation Potential* (Master's Dissertation), Dalhousie University. https://dalspace.library.dal.ca/bitstream/handle/10222/35461/Bourque-Chris-MSc-FOSC-August-2013.pdf

Burns, L. T., Sislak, C. D., Gibbon, N. L., Saylor, N. R., Seymour, M. R., Shaner, L. M., & Gibney, P. A. (2020). Improved Functional Assays and Risk Assessment for *STA1+* Strains of *Saccharomyces cerevisiae*. *Journal of the American Society of Brewing Chemists*. https://doi.org/10.1080/03610470.2020.1796175

Buzrul, S. (2007). A suitable model of microbial survival curves for beer pasteurization. *LWT Food Science and Technology*, 40, 1330-1336. https://doi.org/10.1016/j.lwt.2006.10.005

Capece, A., Romaniello, R., Siesto, G., & Romano, P. (2018). Conventional and Non-Conventional Yeasts in Beer Production. *Fermentation*, *4*(2), 38. http://dx.doi.org/10.3390/fermentation4020038

Ciosek, A., Fulara, K., Hrabia, O., Satora, P., & Poreda, A. (2020). Chemical Composition of Sour Beer Resulting from Supplementation the Fermentation

Medium with Magnesium and Zinc Ions. *Biomolecules*, 10(12), 1599. https://doi.org/10.3390/biom10121599

De Keukeleire, D. (2000). Fundamentals of beer and hop chemistry. *Química Nova*, 23(1), 108-112. https://doi.org/10.1590/S0100-40422000000100019

De Nicola, R. (2006). *Interaction of zinc with the yeast Saccharomyces cerevisiae* (PhD Thesis), University of Abertay Dundee. https://rke.abertay.ac.uk/ws/portalfiles/portal/15147889/De_Nicola_2006_Interaction_of_zinc_PhD.pdf

De Nicola, R., Anthony, S, Walker, G. & Learmonth, R. (2005). Impact of zinc on yeast membranes and cell physiology during brewing fermentations. In: XXIVth International Specialised Symposium on Yeasts, 28 Sep - 02 Oct 2005, Castellon, Spain. https://eprints.usq.edu.au/798/

Deng, Y., Liu, J., Li, H., Li, L., Tu, J., Fang, H., Chen, J. and Qian, F. (2014). An improved plate culture procedure for the rapid detection of beer-spoilage lactic acid bacteria. *Journal of the Institute of Brewing*, 120, 127-132. https://doi.org/10.1002/jib.121

Esmaeili, S., Mogharrabi, M., Safi, F., Sohrabvandi, S., Mortazavian, A. M., Bagheripoor-Fallah, N. (2015). The common spoilage microorganisms of beer: occurrence, defects, and determination - a review. *Journal of Food Science and Technology*, 7, 68–73

Eßlinger, H. M. & Narziß, L. (2009). Beer. *Ullmann's Encyclopedia of Industrial Chemistry*. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany. http://dx.doi.org/10.1002/14356007.a03_421.pub2

Evans, D. E., Li C., Eglinton J. K. (2009). The Properties and Genetics of Barley Malt Starch Degrading Enzymes. In G. Zhang, C. Li (Eds.), *Genetics and Improvement of Barley Malt Quality* (pp. 143-189). Advanced Topics in Science and Technology in China. Springer, Berlin, Germany. https://doi.org/10.1007/978-3-642-01279-2_6

Farber, M. J., & Pham, K. (2019). *Microbiological media and methods of using same* (WO2019173389A1). World Intellectual Property Organization. https://patents.google.com/patent/WO2019173389A1/en

Forbes, J. (2014). *Evaluation of fermentation characteristics of six yeast strains* (Master's Dissertation), Dalhousie University. https://dalspace.library.dal.ca/bitstream/handle/10222/54059/Forbes-Jessica-MSc-FOSC-Aug-2014.pdf

Fox, G. (2018). Chapter 16 - Starch in Brewing Applications. In M. Sjöö & L. Nilsson (Eds.), *Starch in Food* (2nd ed., pp. 633–659). Woodhead Publishing, Massachusetts, USA. https://doi.org/10.1016/B978-0-08-100868-3.00016-0

Geißler, A. (2016*). Lifestyle of beer spoiling lactic acid bacteria* (PhD Thesis), Technische Universität München. https://mediatum.ub.tum.de/doc/1319485/1319485.pdf

Gibson, B., Geertman, J. A., Hittinger, C. T., Krogerus, K., Libkind, D., Louis, E. J., Magalhães, F., & Sampaio, J. P. (2017). New yeasts-new brews: modern approaches to brewing yeast design and development. *FEMS yeast research*, 17(4). https://doi.org/10.1093/femsyr/fox038

Guido, L. F., & Moreira, M. M. (2014). Malting. In R. Guine & P. Correia (Eds.), *Engineering Aspects of Cereal and Cereal-Based Products* (pp. 51-69). CRC Press, New York, USA.

Haikara, A., Helander, I. (2006). *Pectinatus*, *Megasphaera* and *Zymophilus*. In M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer, E. Stackebrandt (Eds.), *The Prokaryotes* (pp. 965-981). Springer, New York, USA. https://doi.org/10.1007/0-387-30744-3_32

He, Y., Dong, J., Yin, H., Zhao, Y., Chen, R., Wan, X., Chen, P., Hou, X., Liu, J. & Chen, L. (2014). Wort composition and its impact on the flavour-active higher alcohol and ester formation of beer – a review. *Journal of the Institute of Brewing*, 120, 157– 163. https://doi.org/10.1002/jib.145

Hodzic, Z., Banjanin, B., & Sadadinovic, J. (2008). Fermentable carbohydrates determination in different worts by HPLC-RI. *Journal of Engineering*, 1, 105–110. http://annals.fih.upt.ro/pdf-full/2008/ANNALS-2008-1-15.pdf

Hu, S., Dong, J., Fan, W., Yu, J., Yin, H., Huang, S., Liu, J., Huang, S. & Zhang, X. (2014). The influence of proteolytic and cytolytic enzymes on starch degradation during mashing. *Journal of the Institute of Brewing*, 120, 379– 384. https://doi.org/10.1002/jib.172

Hutzler, M., Riedl, R., Koob, J. & Jacob, F., (2012). Fermentation and spoilage yeasts and their relevance for the beverage industry - a review, *Brewing science*, 65, 33-52.

Jurková, M., Horák, T., Hašková, D., Čulík, J., Čejka, P. & Kellner, V. (2012). Control of antioxidant beer activity by the mashing process. *Journal of the Institute of Brewing*, 118, 230-235. https://doi.org/10.1002/jib.35

Kilgour, W. J., & Smith, P. (1985). The determination of pasteurisation regimes for

alcoholic and alcohol-free beer. *Proceedings of the European Brewing Convention Congress*, Helsinki, 20, 435-442.

Krogerus, K., Gibson, B. (2020). A re-evaluation of diastatic *Saccharomyces cerevisiae* strains and their role in brewing. *Applied Microbiology and Biotechnology*, 104, 3745–3756. https://doi.org/10.1007/s00253-020-10531-0

Krogerus, K., Magalhães, F., Kuivanen, J., & Gibson, B. (2019). A deletion in the *STA1* promoter determines maltotriose and starch utilization in *STA1+ Saccharomyces cerevisiae* strains. *Applied Microbiology and Biotechnology*, 103, 7597–7615. https://doi.org/10.1007/s00253-019-10021-y

Krogerus, K., Magalhães, F., Vidgren, V., & Gibson, B. (2015). New lager yeast strains generated by interspecific hybridization. *Journal of Industrial Microbiology & Biotechnology*, *42*(5), 769–778. https://doi.org/10.1007/s10295-015-1597-6

Laluce, C., & Mattoon, J. R. (1984). Development of Rapidly Fermenting Strains of *Saccharomyces diastaticus* for Direct Conversion of Starch and Dextrins to Ethanol. *Applied and Environmental Microbiology*, 48(1), 17–25. https://doi.org/10.1128/aem.48.1.17-25.1984

Latorre, M., Hutzler, M., Michel, M., Zarnkow, M., Jacob, F., & Libkind, D. (2020). YEAST-SPECIAL Genotypic diversity of *Saccharomyces cerevisiae* spoilers in a community of craft microbreweries. *Brewing Science*, 73, 51-57. https://doi.org/10.23763/BrSc20-06latorre

Lattici, F., Catallo, M., & Solieri, L. (2020). Designing New Yeasts for Craft Brewing: When Natural Biodiversity Meets Biotechnology. *Beverages*, *6*(1), 3. http://dx.doi.org/10.3390/beverages6010003

Laurentin, A., & Edwards, C.A. (2013). Fiber: Resistant Starch and Oligosaccharides. In B. Caballero, L.H. Allen, A. Prentice (Eds.), *Encyclopaedia of human nutrition* (pp. 246-253). Academic Press, New York, USA. https://doi.org/10.1016/B978-0-12-375083-9.00109-4

Libkind, D., Hittinger, C. T., Valério, E., Gonçalves, C., Dover, J., Johnston, M., Gonçalves, P., & Sampaio, J. P. (2011). Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proceedings of the National Academy of Sciences of the United States of America*, 108(35), 14539–14544. https://doi.org/10.1073/pnas.1105430108

Lodolo, E. J., Kock, J. L., Axcell, B. C., & Brooks, M. (2008). The yeast *Saccharomyces cerevisiae*- the main character in beer brewing. *FEMS yeast research*, 8(7), 1018–Meier-Dörnberg 1036. https://doi.org/10.1111/j.1567-1364.2008.00433.x

Meier-Dörnberg, T., Jacob, F., Michel, M., & Hutzler, M. (2017). Incidence of *Saccharomyces cerevisiae* var. *diastaticus* in the beverage industry: cases of contamination, 2008–2017. *MBAA TQ (Technical Quarterly)*, 54(4), 140 – 148. http://dx.doi.org/10.1094/TQ-54-4-1130-01

Meier-Dörnberg, T., Kory, O. I., Jacob, F., Michel, M., & Hutzler, M. (2018). *Saccharomyces cerevisiae* variety *diastaticus* friend or foe? -spoilage potential and brewing ability of different *Saccharomyces cerevisiae* variety *diastaticus* yeast isolates by genetic, phenotypic and physiological characterization. *FEMS yeast research*, 18(4). https://doi.org/10.1093/femsyr/foy023

Milani, E. A., Gardner, R. C., & Silva, F. V. (2015). Thermal resistance of *Saccharomyces* yeast ascospores in beers. *International journal of food microbiology*, 206, 75–80. https://doi.org/10.1016/j.ijfoodmicro.2015.04.002

Montanari, L., Floridi, S., Marconi, O., Tironzelli, M., & Fantozzi, P. (2005). Effect of mashing procedures on brewing. *European Food Research and Technology*, 221, 175–179. https://doi.org/10.1007/s00217-005-1166-8

Moretti, E. (2013). *Development of guidelines for microbiological control in microbrewery* (PhD Thesis), University of Perugia. http://amsdottorato.unibo.it/5872/1/moretti_elio_tesi.pdf

Narendranath, N. V., & Power, R. (2005). Relationship between pH and medium dissolved solids in terms of growth and metabolism of lactobacilli and *Saccharomyces cerevisiae* during ethanol production. *Applied and Environmental Microbiology*, 71(5), 2239–2243. https://doi.org/10.1128/AEM.71.5.2239-2243.2005

Naydenova, V. N., Kostov, G., & Popova, Z. (2012). *Comparative study of brewing yeast strains for beer production with immobilized cell*. 6th Central European Congress on Food, CEFood 2012, Belgrade, Serbia. Google Scholar

Obi, C. N. (2017). Brewery Contaminants, Challenges and Remedies-A Review. *Nigerian Journal of Microbiology*, 31(1), 3926-3940. Google Scholar

Ogata, T., Iwashita, Y., & Kawada, T. (2017). Construction of a brewing yeast expressing the glucoamylase gene *STA1* by mating. *Journal of The Institute of Brewing*, 123, 66–69. https://doi.org/10.1002/jib.394

Oser, H.H. (2015). *Producing Quality Barley for the Malting Industry* (Doctoral Document), University of Nebraska. http://digitalcommons.unl.edu/planthealthdoc/5

Pereira de Moura, F., & Rocha dos Santos Mathias, T. (2018). A Comparative Study of Dry and Wet Milling of Barley Malt and Its Influence on Granulometry and Wort Composition. *Beverages*, 4(3), 51. http://dx.doi.org/10.3390/beverages4030051

Pires, E. J., Teixeira, J. A., Brányik, T., & Vincente, A. (2014). Yeast: the soul of beer's aroma – a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology and Biotechnology*, 98, 1937–1949. https://doi.org/10.1007/s00253-013-5470-0

Reed, G., & Nagodawithana, T. W. (1991). *Yeast technology* (2nd ed). Van Nostrand Reinhold, New York, USA.

Reveron, I., Barreiro, J. and Sandoval, A. (2003), Thermal Resistance of *Saccharomyces cerevisiae* in Pilsen Beer. *Journal of the Institute of Brewing*, 109, 120-123. https://doi.org/10.1002/j.2050-0416.2003.tb00140.x

Ruíz-Muñoz, M., Bernal-Grande, M. D. C., Cordero-Bueso, G., González, M., Hughes-Herrera D., Cantoral, J. M. A. (2017). Microtiter Plate Assay as a Reliable Method to Assure the Identification and Classification of the Veil-Forming Yeasts during Sherry Wines Ageing. *Fermentation*, 3(4), 58. https://doi.org/10.3390/fermentation3040058

Saarni, A., Miller, K. V., & Block, D. E. (2020). A Multi-Parameter, Predictive Model of Starch Hydrolysis in Barley Beer Mashes. *Beverages*, *6*(4), 60. https://doi.org/10.3390/beverages6040060

Sakamoto, K., & Konings, W. N. (2003). Beer spoilage bacteria and hop resistance. *International Journal of Food Microbiology*, 89(2-3), 105–124. https://doi.org/10.1016/s0168-1605(03)00153-3

Stanley, D., Rejzek, M., Naested, H., Smedley, M., Otero, S., Fahy, B., Thorpe, F., Nash, R. J., Harwood, W., Svensson, B., Denyer, K., Field, R. A., & Smith, A. M. (2011). The role of alpha-glucosidase in germinating barley grains. *Plant physiology*, 155(2), 932–943. https://doi.org/10.1104/pp.110.168328

Stewart, G. (2016). *Saccharomyces* species in the Production of Beer, *Beverages*, *2*(4), 34. http://dx.doi.org/10.3390/beverages2040034

Suiker, M. I., Arkesteijn, G. J. A., Zeegers, P. J. & Wösten, H. A. B. (2021). Presence of *Saccharomyces cerevisiae* subsp. diastaticus in industry and nature and spoilage capacity of its vegetative cells and ascospores. *International Journal of Food Microbiology*, 347, 109173. https://doi.org/10.1016/j.ijfoodmicro.2021.109173.

Suzuki, K. (2011). 125th Anniversary Review: Microbiological Instability of Beer

caused by Spoilage Bacteria. *Journal of the Institute of Brewing*, 117(2), 131-155. https://doi.org/10.1002/j.2050-0416.2011.tb00454.x

Troilo, A., De Francesco, G., Marconi, O., Sileoni, V., Turchetti, B. & Perretti, G. (2020). Low Carbohydrate Beers Produced by a Selected Yeast Strain from an Alternative Source. *Journal of the American Society of Brewing Chemists*, 78(1), 80-88. https://doi.org/10.1080/03610470.2019.1682887

Van Dijken, J.P., Van Den Bosch, E., Hermans, J.J., De Miranda, L.R. & Scheffers, W.A. (1986). Alcoholic fermentation by 'non-fermentative' yeasts. *Yeast*, 2, 123-127. https://doi.org/10.1002/yea.320020208

Vinje, M. A., Duke, S. H., & Henson, C. A. (2015). Comparison of Factors Involved in Starch Degradation in Barley Germination under Laboratory and Malting Conditions. *Journal of the American Society of Brewing Chemists*, 73(2), 195-205. https://doi.org/10.1094/ASBCJ-2015-0318-01

Vogeser, G. J. (2019). *Saccharomyces cerevisiae* var. *diastaticus*, a guest in every brewery? Aspects of its occurrence and detection. *Master Brewers Conference 2019*, Calgary, Alberta. https://mbaa.confex.com/mbaa/2019/meetingapp.cgi/Paper/1399

Vriesekoop, F., Rathband, A., MacKinlay, J. & Bryce, J. H. (2010). The Evolution of Dextrins During the Mashing and Fermentation of All-malt Whisky Production. *Journal of the Institute of Brewing*, 116, 230-238. https://doi.org/10.1002/j.2050-0416.2010.tb00425.x

Walker, G., & Stewart, G. (2016). *Saccharomyces cerevisiae* in the Production of Fermented Beverages. *Beverages*, *2*(4), 30. http://dx.doi.org/10.3390/beverages2040030

Walker, G.M., De Nicola, R., Anthony, S., Learmonth, R. (2006). Yeast metal interactions: impact on brewing and distilling fermentations. In: Institute of Brewing and Distilling Asia Pacific Section Convention, Hobart, Australia. https://eprints.usq.edu.au/1064/1/Walker-De_Nicola__Anthony_Learmonth_aper_IBD_Hobart_2006.pdf

Willaert, R. (2007). Chapter 20 - The beer brewing process: wort production and beer fermentation. In Y. H. Hui, R. C. Chandan, S. Clark, N. A. Cross, J. C. Dobbs, W. J. Hurst, L. M. Nollet, E. Shimoni, N. Sinha, E. B. Smith, S. Surapat, F. Toldrá, ... A. Titchenal (Eds.), *Handbook of food products manufacturing* (pp. 443-506). John Wiley & Sons, New Jersey, USA.

Yalcin, S. K., & Yesim Ozbas, Z. (2008). Effects of pH and temperature on growth and glycerol production kinetics of two indigenous wine strains of *Saccharomyces*

cerevisiae from Turkey. *Brazilian Journal of Microbiology*, 39(2), 325–332. https://doi.org/10.1590/S1517-838220080002000024

Zastrow, C. R., Hollatz, C., de Araujo, P. S., & Stambuk, B. U. (2001). Maltotriose fermentation by *Saccharomyces cerevisiae*. *Journal of Industrial Microbiology & Biotechnology*, 27(1), 34–38. https://doi.org/10.1038/sj.jim.7000158

Zufall, C., & Wackerbauer, K. (2000). The biological impact of flash pasteurization over a wide temperature interval. *Journal of the Institute of Brewing*, 106(3), 163-168. https://doi.org/10.1002/j.2050-0416.2000.tb00053.x

7 APPENDIX

7.1 Appendix 1: SPSS output tables

Table 1: 3-Way ANOVA table with factors strain (inoculum), zinc and time

Tests of Between-Subjects Effects

Dependent Variable: ºP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	50,890[a]	47	1,083	649,654	,000
Intercept	143,570	1	143,570	86142,250	,000
Strain	37,023	5	7,405	4442,800	,000
Zinc	,070	1	,070	42,250	,000
Time	8,805	3	2,935	1761,083	,000
Strain * Zinc	,118	5	,024	14,200	,000
Strain * Time	4,571	15	,305	182,833	,000
Zinc * Time	,069	3	,023	13,750	,000
Strain * Zinc * Time	,233	15	,016	9,300	,000
Error	,080	48	,002		
Total	194,540	96			
Corrected Total	50,970	95			

a. R Squared = ,998 (Adjusted R Squared = ,997)

Table 2: 2-Way ANOVA table with factors strain (inoculum) and zinc at week 1

Tests of Between-Subjects Effects

Dependent Variable: ºP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8,545[a]	11	,777	466,091	,000
Intercept	63,375	1	63,375	38025,000	,000
Strain	8,540	5	1,708	1024,800	,000
Zinc	,002	1	,002	1,000	,337
Strain * Zinc	,003	5	,001	,400	,840
Error	,020	12	,002		
Total	71,940	24			
Corrected Total	8,565	23			

a. R Squared = ,998 (Adjusted R Squared = ,996)

Table 3: 2-Way ANOVA table with factors strain (inoculum) and zinc at week 2

Tests of Between-Subjects Effects

Dependent Variable: ºP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11,981[a]	11	1,089	2614,091	,000
Intercept	46,204	1	46,204	110889,000	,000
Strain	11,909	5	2,382	5716,200	,000
Zinc	,020	1	,020	49,000	,000
Strain * Zinc	,052	5	,010	25,000	,000
Error	,005	12	,000		
Total	58,190	24			
Corrected Total	11,986	23			

a. R Squared = 1,000 (Adjusted R Squared = ,999)

Table 4: 2-Way ANOVA table with factors strain (inoculum) and zinc at week 3

Tests of Between-Subjects Effects

Dependent Variable: ºP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10,553[a]	11	,959	575,636	,000
Intercept	25,627	1	25,627	15376,000	,000
Strain	10,168	5	2,034	1220,200	,000
Zinc	,107	1	,107	64,000	,000
Strain * Zinc	,278	5	,056	33,400	,000
Error	,020	12	,002		
Total	36,200	24			
Corrected Total	10,573	23			

a. R Squared = ,998 (Adjusted R Squared = ,996)

Table 5: 2-Way ANOVA table with factors strain (inoculum) and zinc at week 4

Tests of Between-Subjects Effects

Dependent Variable: °P

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11,005[a]	11	1,000	343,000	,000
Intercept	17,170	1	17,170	5887,000	,000
Strain	10,977	5	2,195	752,714	,000
Zinc	,010	1	,010	3,571	,083
Strain * Zinc	,017	5	,003	1,171	,378
Error	,035	12	,003		
Total	28,210	24			
Corrected Total	11,040	23			

a. R Squared = ,997 (Adjusted R Squared = ,994)

Table 6: 2-Way ANOVA table with factors zinc and sampling time for DSM 70487

Tests of Between-Subjects Effects

Dependent Variable: °P

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,640[a]	7	,091	73,143	,000
Intercept	,010	1	,010	8,000	,022
Zinc	,010	1	,010	8,000	,022
Time	,625	3	,208	166,667	,000
Zinc * Time	,005	3	,002	1,333	,330
Error	,010	8	,001		
Total	,660	16			
Corrected Total	,650	15			

a. R Squared = ,985 (Adjusted R Squared = ,971)

Table 7: 2-Way ANOVA table with factors zinc and sampling time for Strain-Y

Tests of Between-Subjects Effects

Dependent Variable: ºP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,014[a]	7	,002	1,095	,446
Intercept	63,601	1	63,601	33920,333	,000
Zinc	,001	1	,001	,333	,580
Time	,002	3	,001	,333	,802
Zinc * Time	,012	3	,004	2,111	,177
Error	,015	8	,002		
Total	63,630	16			
Corrected Total	,029	15			

a. R Squared = ,489 (Adjusted R Squared = ,043)

Table 8: 2-Way ANOVA table with factors zinc and sampling time for TUM 1-B-8

Tests of Between-Subjects Effects

Dependent Variable: ºP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,768[a]	7	,110	87,714	,000
Intercept	31,923	1	31,923	25538,000	,000
Zinc	,010	1	,010	8,000	,022
Time	,748	3	,249	199,333	,000
Zinc * Time	,010	3	,003	2,667	,119
Error	,010	8	,001		
Total	32,700	16			
Corrected Total	,778	15			

a. R Squared = ,987 (Adjusted R Squared = ,976)

Table 9: 2-Way ANOVA table with factors zinc and sampling time for Mixture 1

Tests of Between-Subjects Effects

Dependent Variable: °P

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4,114[a]	7	,588	313,476	,000
Intercept	19,581	1	19,581	10443,000	,000
Zinc	,006	1	,006	3,000	,122
Time	4,107	3	1,369	730,111	,000
Zinc * Time	,002	3	,001	,333	,802
Error	,015	8	,002		
Total	23,710	16			
Corrected Total	4,129	15			

a. R Squared = ,996 (Adjusted R Squared = ,993)

Table 10: 2-Way ANOVA table with factors zinc and sampling time for Mixture 2

Tests of Between-Subjects Effects

Dependent Variable: °P

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5,100[a]	7	,729	291,429	,000
Intercept	27,040	1	27,040	10816,000	,000
Zinc	,040	1	,040	16,000	,004
Time	5,040	3	1,680	672,000	,000
Zinc * Time	,020	3	,007	2,667	,119
Error	,020	8	,002		
Total	32,160	16			
Corrected Total	5,120	15			

a. R Squared = ,996 (Adjusted R Squared = ,993)

Table 11: 2-Way ANOVA table with factors zinc and sampling time for Mixture 3

Tests of Between-Subjects Effects

Dependent Variable: °P

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3,230[a]	7	,461	369,143	,000
Intercept	38,440	1	38,440	30752,000	,000
Zinc	,123	1	,123	98,000	,000
Time	2,855	3	,952	761,333	,000
Zinc * Time	,252	3	,084	67,333	,000
Error	,010	8	,001		
Total	41,680	16			
Corrected Total	3,240	15			

a. R Squared = ,997 (Adjusted R Squared = ,994)