Rita Barata Garcia

Bachelor Degree in Applied Biology

Yeast cell wall hydrolysis by enzymes for the production of value-added products

Dissertation to obtain the Degree of Master in Biotechnology

Supervisor: Dr. Dina Krüger, Project Manager in R&D department at Ohly GmbH

Jury:

President: Professor Dr. Pedro Miguel Ribeiro Viana Baptista

Examiner: Professor Dr. Maria Filomena Andrade de Freitas

Liaison: Professor Dr. Pedro Miguel Calado Simões

FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Abstract

Yeast cell walls (YCW) are side-stream products from yeast extract production and are usually lower value products. In the first part of the project, we explored the potential of enzymes in converting Ohly's YCW into high(er) added value new products. Accordingly, a better understanding of the enzymatic hydrolysis of YCW and the subsequent hydrolysates properties was obtained.

An enzymatic assay was developed in lab-scale by standardizing process conditions such as enzyme dosage, temperature, pH and reaction time. Over 56 Carbohydrases and 4 Phospholipases were tested. The hydrolysates characterization was done by visual, analytical and chemical analysis. The following Ohly starting materials were used: YCW from the *S. cerevisiae* (hydrolysis process) and from *C. utilis* a.k.a Torula (Prime process). Hydrolysates were clustered using a PCA into 6 groups based on their profile of: free reducing sugars, free glucose, and free mannose. From all enzymes the most interesting were AO3, NC5, SN7, SN8, ET4, NC1 and ST3.

In the second part of the project, the Torula YCW were used as a starting material for the production of a new yeast extract due to their high protein content. An enzymatic assay was performed by standardizing process conditions such as enzyme dosage, combination of different enzymes, temperature, pH and reaction time. The hydrolysates characterization was done by analytical and chemical analysis, and by sensory analysis. Proteolytic enzymes (9), Carbohydrases (2), Phospholipases (4) from various manufactures were tested. Results show that the optimal recipe to produce a new yeast extract from Torula YCW consisted of: 0.5% NS11 + 0.5% EN1 + 0.1% AO5 as the best combination when tested in an application (vegetable broth).

Keywords: Hydrolysed yeast cell wall; Torula yeast cell wall; Enzymatic hydrolysis; Umami; Yeast extract; Molecular weight.

Resumo

A Parede celular (PC) de levedura, fração insolúvel, é obtida como subproduto da produção de extrato de levedura de *S. cerevisae* ou *C. utilis*, sendo considerada um produto de baixo valor económico.

Na primeira parte, foi explorado o potencial de conversão da PC em hidrolisados com alto valor económico. Nesse sentido, foi realizado um ensaio enzimático a nível laboratorial. Condições como a dosagem da enzima, temperatura, pH e tempo de reação foram estabelecidos para estudar o potencial de conversão enzimática de 60 enzimas. Como material de partida foi utilizada a PC da *S. cerevisiae* obtida por um processo de hidrólise e a PC da *C. utilis* ou Torula, obtida através de um processo Prime. Posteriormente, os hidrolisados foram caraterizados por análise visual, analítica e química, tendo sido ainda categorizados em 6 grupos de acordo com uma Análise de Componentes Principais. Com este ensaio, foi possível perceber o perfil das diferentes enzimas na conversão da PC das eveduras e consequentemente as propriedades dos hidrolisados obtidos. As enzimas consideradas mais interessantes durante este estudo foram AO3, NC5, SN7, SN8, ET4, NC1 e ST3.

Na segunda parte, foi usado como material de partida para o desenvolvimento de um novo extrato de levedura a PC da Torula, devido ao alto conteúdo de proteínas ainda existente na PC. Assim, foi realizado um ensaio com diferentes enzimas, tendo sido estabelecidas as condições do processo como dosagem e combinação das mesmas, temperatura, pH, e tempo de reação. No final os hidrolisados foram caraterizados por análise analítica, química e sensorial. Após análise das possíveis combinações de enzimas para produção do novo extrato de levedura a partir da PC da Torula, verificou-se que a mais interessante seria 0.5% NS11 + 0.5% EN1 + 0.1% AO5, tendo sido testada em aplicação (caldo de vegetais).

Palavras-chave: Parede celular da levedura hidrolisada; Parede celular da Torula; Hidrólise enzimática; Umami; Extrato de levedura; Peso molecular.

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List of Abbreviations and Symbols

A - adenine

AA - amino acids

a.k.a. - also known as

Ala - alanine

AMP - adenylate (adenosine 5'-monophosphate)

Arg - arginine

ASL- alkali sensitive linkage

Asp - aspartic acid

$AspNH_2$ - asparagine

ATP - adenosine triphosphate

BLAST - basic local alignment search tool

C - cytosine

CAZyme - carbohydrate-active enzyme

CMP - cytidylate (cytidine 5' monophosphate)

CW - cell wall

CWP - cell wall proteins

Cys - cysteine

DB - degree of branching

DM - dry matter

DNA - desoxyribonucleic acid

DNS - 3,5-dinitrosalicylic acid

DP - degree of polymerization

FDA - food and drug administration

EC - enzyme commission number

e.g. - for example

G - guanine

G-6-P - glucose-6-phosphate

G6P-DH - glucose-6-phosphate dehydrogenase

GHs - glycoside hydrolases

GlcNAc - N-Acetylglucosamine

Glu - glutamic acid

Gly - glycine

GMO - genetically modified organism

GMP - guanylate (guanosine 5'-monophosphate)

GOD - glucose oxidase

GPCRs - G protein coupled receptors

GPI - glycosylphosphatidylinositol

GRAS - generally recognized as safe

GTs - glycosyltransferases

His - histidine
HK - hexokinase
HPAE - high performance anion-exchange chromatography
HPLC - high performance liquid chromatography
HYCW - hydrolysed yeast cell wall
i.e. - that is
Ile - isoleucine
IMP - inosine monophosphate
IP_3 - inositol-1,4,5-triphosphate
RI - refractive index detector
IUPAC - international union of pure and applied chemistry
LDL - low-density lipoprotein
Leu - leucine
LPHase - laminaripentaose-producing β-(1,3)-glucanase
LSD - least significant difference
Lys - lysine
M-6-P - mannose-6-phosphate
Met - methionine
mGluR - metabotropic glutamate receptor
MOS - mannanoligosaccharides
mRNA - messenger ribonucleic acid
MSG - mono sodium glutamate
MW - molecular weight
NAD - nicotinamide-adenine dinucleotide
NADP - nicotinamide adenine dinucleotide phosphate
OD - optical density
Orn - ornithine
PC - parede celular
PCA - principal component analysis
Pir - proteins with internal repeats
PGI - phosphoglucose isomerase
PLs - polysaccharide lyases
Phe - phenylalanine
PMI - phosphomannose isomerase
PO_4 - phosphate
Pro - proline
QC - quality control
QDA - quantitative descriptive analysis
RNA - ribonucleic acid
RS - reducing sugars

SCP - single cell protein
SEC - size exclusion chromatography
SEM - scanning electron microscope
Ser - serine
SN - supernatant
SPB - spindle pole bodies
SYEAA - standard yeast extract with high content in free amino acids
T - thymine
Thr - threonine
tRNA - transfer ribonucleic acid
Trp - tryptophan
TYC - Torula yeast cell
TYCW - Torula yeast cell Wall
Tyr - tyrosine
U - uracyl
UDP - uridine diphosphate
UMP - uridylate (uridine 5' monophosphate)
Val - valine
VFM - Venus flytrap module
w/v - solute’s weight per total solution’s volume
w/w - solute’s weight per total solution’s weight
YCW - yeast cell wall
YE - yeast extract
YEHP - yeast extract with high content of peptides
YEHS - yeast extract high on salt content

Ohly GmbH

Ohly GmbH is one of the world's leading suppliers of yeasts extracts, yeast based flavours and specialty powders for food, fermentation, nutrition and animal feed markets [1].

Ohly's history goes back to 1836 with "Heinrich Helbing Korn Distillery", founded by Heinrich Helbing in Hamburg (Germany). At the time the company was producing yeast and spirits for the local market. In the mid 1930's, Götz Ohly was the first person to develop yeast extracts and a few years later, in 1961, the company was named Deutsche Hefewerke GmbH [1].

In 1994, Burns Philp, started a collaboration between Provesta Corporation in Hutchinson (US) and Deutsche Hefewerke GmbH in Hamburg (Germany). In 2004, ABF Ingredients bought parts of the Burns Philp group and, in 2007 these two companies started to operate under the well-established brand Ohly to better correspond to market needs. In 2011, Ohly acquired Bakon Yeast Inc. located in Rhinelander, Wisconsin (USA), which is a manufacturer of Torula yeast based savoury ingredients for the food industry [1].

Nowadays, Ohly operates three plants, two in the United States and one in Germany. In Hamburg, Germany, Ohly produces yeast derivatives based on *S. cerevisiae* and starter cultures. The site in Hutchinson, Minnesota, is specialised in the continuous fermentation of *C. utilis*. In Boyceville, Wisconsin, US a variety of Torula, brewer's and baker's yeast extracts and specialty powders for food are manufactured [1].

1 Theoretical part

1.1 Food Yeasts

In biological terms, yeasts belong to Eukarya domain which are defined by their enclosed nucleus with a double DNA strand. They have multiple organelles specialized to that species outside of the nucleus, such as ribosomes, endoplasmic reticulum, Golgi body, etc. (Figure 1.1) [2, 3]. Yeasts are part of Fungi Kindgom which are known for non-vascular and heterotrophic[1] species [2].

Yeasts are unicellular, however some species may turn into multicellular through the formation of strings of connected budding cells (pseudohyphae or false hyphae). Yeasts can display both asexual and sexual stages in their life cycle. The asexual stage of given yeast is called the anamorph, while the sexual stage is the teleomorph. Most yeasts reproduce asexually by mitosis, and some of them do so by an asymmetric division called budding [4].

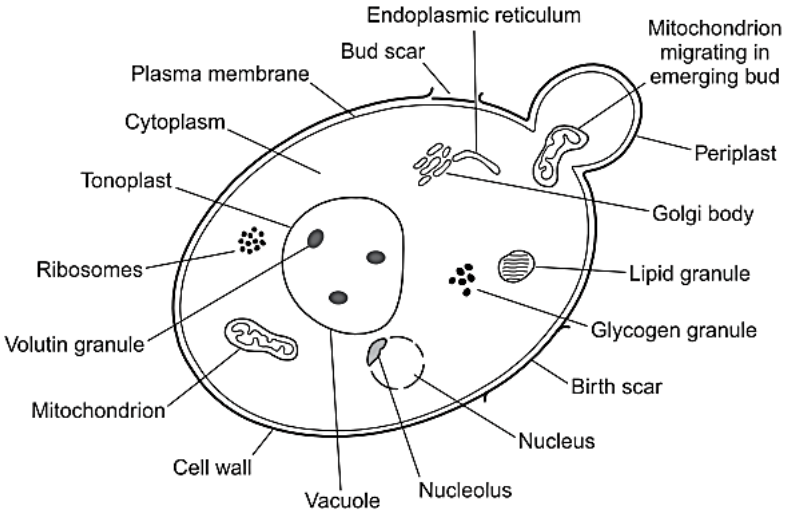


Figure 1.1 Diagrammatic representation of a section through a typical budding yeast cell [3].

Yeasts are a rich source of protein and B-complex vitamins, thus they have been used as a complementary protein source in fish diet, as supplement in animals feed to compensate for the amino acid (AA) and vitamin deficiencies of cereals, they can be used as substitute for soybean oil in diets for fowl, being considered a natural and cheaper dietary supplement [5]. According to the International Union of Pure and Applied Chemistry (IUPAC) "*Food yeast is a yeast that has been killed, and dried; it should have no diastase activity, and has not been submitted to any extraction process or received any additive*"[6].

[1] "*Heterotrophs obtain organic material by the second major mode of nutrition. Unable to make their own food, they live on compounds produced by other organisms.*"

From the industrial viewpoint, there are three main genera of interest, the *Saccharomyces*, *Candida* and *Kluyveromyces* [7, 8]. In Table 1.1 the principal industrial yeasts and their applications are represented [8].

Table 1.1 Principal industrial yeasts and their applications [8].

Yeast species	Products/applications
Saccharomyces cerevisiae	Bread-type products, beer brewing, wine making, distilled beverages, ethanol, cider, food yeast, feed yeast, yeast-derived products (autolysates, hydrolysates, protein, biochemicals), invertase
Saccharomyces uvarum	Beer brewing
Saccharomyces saké	Sake brewing
Saccharomyces bayanus	Sparkling wines
Saccharomyces lactis	Lactase
Kluyveromyces fragilis	Food yeast, feed yeast, ethanol
Candida utilis	Food yeast, nucleic acids, feed yeast
Candida tropicalis	Food yeast, feed yeast
Candida pseudotropicalis	Food yeast, feed yeast
Candida lipolytica	Feed yeast

Yeasts are quantitatively and economically the most important group of microorganisms exploited by humans [7]. The global yeast market reached US$ 5.9 billion in 2013. It is projected to reach US$ 9.2 billion by 2019.The total global production of yeasts is in excess of three million tonnes. Annual European production of yeast is now 900.000 tones, of which over 60% is consumed in the European Union. In 2012 the global market for products manufactured from yeast, such as yeast extract and yeast β-glucan, was valued at US$ 550 million [9].

1.2 Characteristics of Food Yeasts (*Candida utilis* and *Saccharomyces cerevisiae*)

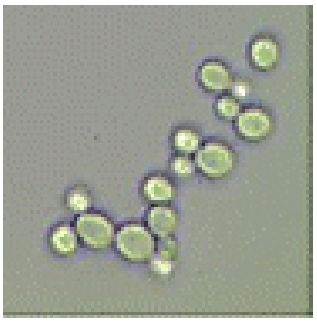


Figure 1.2 Exemplary microscopic photography of *C. utilis* cells cultured in yeast extract-peptone-dextrose (scale: 45 µm) [10].

Candida utilis (Henneberg) Lodder & Kreger-van Rij (1947), a.k.a Torula (Figure 1.2), had been proposed as the asexual state of *Lindnera jadinii* (A. & R. Sartory, Weill & J. Meyer) Kurtzman, Robnett & Basehoar-Powers (2008) because of phenotypic similarities. DNA re-association studies demonstrated that both species share ~85% common base sequences. These data, which were confirmed by Manachini (1979), show that *C. utilis* is the anamorphic state of L. *jadinii* [11, 12].

The growth of *C. utilis* is barely affected by extremes in pH, and being Crabtree-negative it does not produce ethanol in aerated cultures, which limits growth in other yeast species [13]. *C. utilis* also is known for assimilate carbohydrates, alcohols, organic acids, and hydrocarbons. This offers an opportunity for various waste materials, such as molasses and vinasse, wood hydrolysates, waste sulfite liquors, starch and lignincellulose wastes to be used as medium for growing yeasts. The use of waste materials not only reduces the cost of yeast biomass production but also empowers their economic utilization [11, 13]. Subsequently, *C. utilis* is used as a food yeast to produce single-cell protein (SCP) of high quality from cheap biomass; is also used as a source of diverse endogenous products such as glutathione, biotin, glucomannan, L-phenylacetylcarbinoll, RNA, NAD and several enzymes, including invertase [13]. *C. utilis* is characterized by high respiratory activity, high protein and also carbohydrates content (Table 1.2) and good AA profile (Table 1.3) [11, 13, 14].

Saccharomyces cerevisiae Meyen ex E. C. Hansen (1883) (Figure 1.3) is the main yeast exploited in biotechnology worldwide, due largely to its unique physiology and associated to many food fermentations and other industrial processes (e.g. foods and beverages, alcohol fermentation, vaccines, and therapeutic proteins) [12, 15, 16, 17]. Other long-standing industrial processes involving *S. cerevisiae* are production of fuel ethanol, SCP, feeds and fodder, yeast extract (YE), industrial enzymes and small molecular weight (MW) metabolites [12, 18]. It was the first eukaryote to be sequenced, which has flashed a whole new era developing genomics tools, e.g., genome sequencing, transcriptional profiling, metabolomics, carbon flux estimations, proteomics, etc. [16, 17]. In Genus, *Saccharomyces* vegetative cells may be globose, ovoid, ellipsoidal, or elongate and usually they are in pairs or in small clusters. All species have a strongly fermentative as well as a

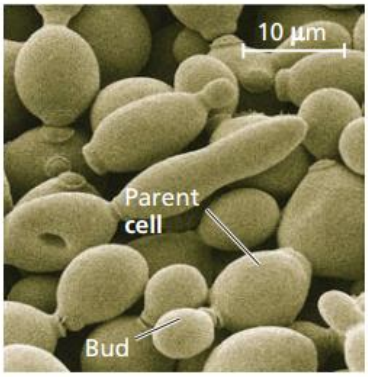


Figure 1.3 Exemplary scanning electron microscope photography of *S. cerevisiae* in several stages of budding (scale: 10 µm) [2].

respiratory metabolism and nitrate is not utilized [7, 19]. Strains of *S. cerevisiae* are unable to use lysine as a nitrogen source and they have a high tolerance to SO_2 and ethanol [20].

Table 1.2 has an average composition of *S. cerevisiae*, showing a considerable amount of proteins and carbohydrates, some lipids and ash. The essential free AA are shown on Table 1.3 [8, 15, 21].

Due to *S. cerevisiae* long history of state use and consumption, and lack of production of toxins, most strains have been considered as generally recognized as safe (GRAS) for use in foods and as a production organism of native and recombinant products [12]. *C. utilis* was also approved by FDA (Food and Drug Administration) as safe for consumption and added to the GRAS list [11, 13].

Table 1.2 Average composition (as % dry matter) of *C. utilis* and *S. cerevisiae* [15, 21].

Component (%)	*C. utilis*	*S. cerevisiae*
Kjeldahl protein (Nx6.25)	45-47	37-40
Carbohydrates	32-38	35-40
Lipids	1-2	4-5
Ash	8-10	4.5-7.5

Table 1.3 Some essential amino acids composition of different food yeast species [8, 21].

Amino acid	**Content in yeast (g/16 g N)**	
	S. cerevisiae from molasses	*C. utilis* from molasses
Lysine	8-2	10-7
Valine	5-5	5-7
Leucine	7-9	8-1
Isoleucine	5-5	7-3
Threonine	4-8	4-8
Methionine	2-5	1-4
Phenylalanine	4-5	4-1
Tryptophan	1-2	0-5
Cystine	1-6	0-3
Histidine	4-0	2-8
Tyrosine	5-0	1-4
Argiinine	5-0	4-7

Metabolic functions have been relating to the ultrastructure and chemical composition of the yeast cell. Moreover, the morphology exhibited by a particular yeast is directly associated with its asexual reproduction mechanism [7]. Therefore, *S. cerevisiae* and *C. utilis* are part of the phylum

Ascomycota, which is known for forming ascospores [12, 18, 22]. Ascospores are usually found in groups of four or eight spores within the ascus (a single mother cell) which the plural is asci (Figure 1.4 show us an overview of the stages of spore and ascus formation of *S. cerevisiae*). These spores are formed as a means of packaging postmeiotic nuclei. Accordingly, they represent the "gametic" stage of the life cycle. The conception of these specialized cells requires a cell division mechanism distinct from that used during vegetative growth of fungal cells [22]. In *L. jadinii* case, one to four hat-shaped ascospores in unconjugated deliquescent asci are formed. It can be produced an occasional pseudohyphae which may be highly branched and true hyphae are not produced. As it was mentioned before *C. utilis* is the asexual state of the *L. jadinii*. The asexual reproduction takes place by means of conidia (singular conidium), spores cut off by septa at the ends of modified hyphae called conidiophores, which in *C. utilis* case the modified structure can be the pseudohyphae [23, 12].

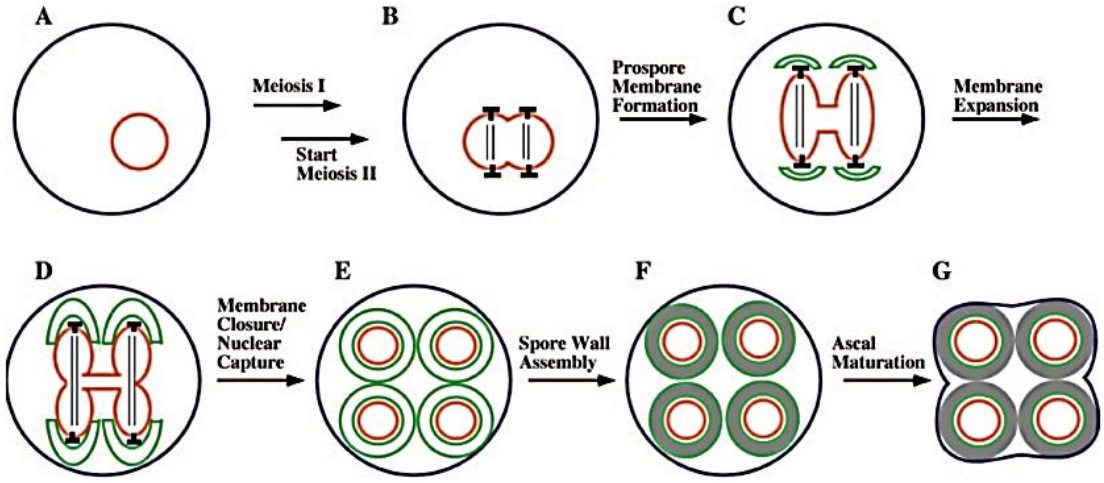


Figure 1.4 (A to G) Overview of the stages of spore and ascus formation of *S. cerevisiae*. In presence of a nonfermentable carbon source, diploid cells searching for nitrogen will undergo meiosis. During the second meiotic division, the spindle pole bodies (SPBs) (indicated as ᴛ), which are surrounded by the nuclear envelope (shown in **red**), develop sites for formation of prospore membranes (shown in **green**). As meiosis II goes on, the prospore membranes expand and immerse the forming haploid nuclei. After nuclear division, each prospore membrane closes on itself to capture a haploid nucleus in two distinct membranes. Spore wall synthesis then begins in the lumen between the two prospore membrane-derived membranes. After spore wall synthesis is complete, the mother cell breakdowns to form the ascus [22].

1.3 Yeast cell wall of Ascomycetous Yeasts

The yeast cell wall (YCW) plays an important role in structure, maintenance and in morphogenesis of yeast. The cell wall (CW) protects the cell against physical damage, degradation by foreign proteins and dehydration [12, 24]. In *S. cerevisiae* it has been estimated that about 1200 of its 6000 genes directly or indirectly affect CW formation [12].

With the increasing number of fully sequenced fungal genomes, it becomes clear that phylogenetically distinct fungi share many CW genes. For example, the *S. cerevisiae* gene FKS1 encodes a multi-spanning membrane protein that is catalytically active in the synthesis of β-(1,3)-glucan, and the gene GAS1 encodes a plasma membrane bound transglucosylase, which is can elongate β-(1,3)-glucan and may associated with connecting chains that emerge from the plasma membrane to the existing β-(1,3)-glucan network. Some studies with basic local alignment search tool (BLAST), which compares nucleotide or proteins sequences to sequence databases and calculates the statistical significance, have shown that ascomycetous yeasts such as Candida species (e.g. *C. utilis*) contain proteins homologous to Fks1p and Gas1p. This strongly suggests that the CW of ascomycetous fungi contain a similar β-(1,3)-glucan with *S. cerevisiae* [12]. The CW model of *S. cerevisiae* may well be an accurate model for most or all ascomycetous yeasts, particularly those that lack β-(1,3)-glucan in their walls [12, 24]. As the molecular architecture of CW of *S. cerevisiae* has been extensively studied, during this review we will be based on *S. cerevisiae* to explain CW dynamics, assuming that both *C. utilis* and *S. cerevisiae* have a similar CW.

The YCW represents 10 to 30 % of the dry weight of yeast cell biomass and is mostly composed of polysaccharides (~ 85 %), proteins (~15 %) and lipids. The structure of the YCW (Figure 1.5) consists of two layers, an outer layer of mannoproteins (30–55 % of the CW) and an inner layer of carbohydrate polymer β-(1,3)-glucan (30–55 %). The CW is also composed of β-(1,6)-glucan (5–10 %) and chitin (1 and 2 %) of the total composition of the CW (Table 1.4) [24-27]. The CW of *C. utilis* is also mainly composed of glucan (~50%) and proteins with various degrees of glycosylation (~40%), less than 5% of CW is composed by chitin [28].

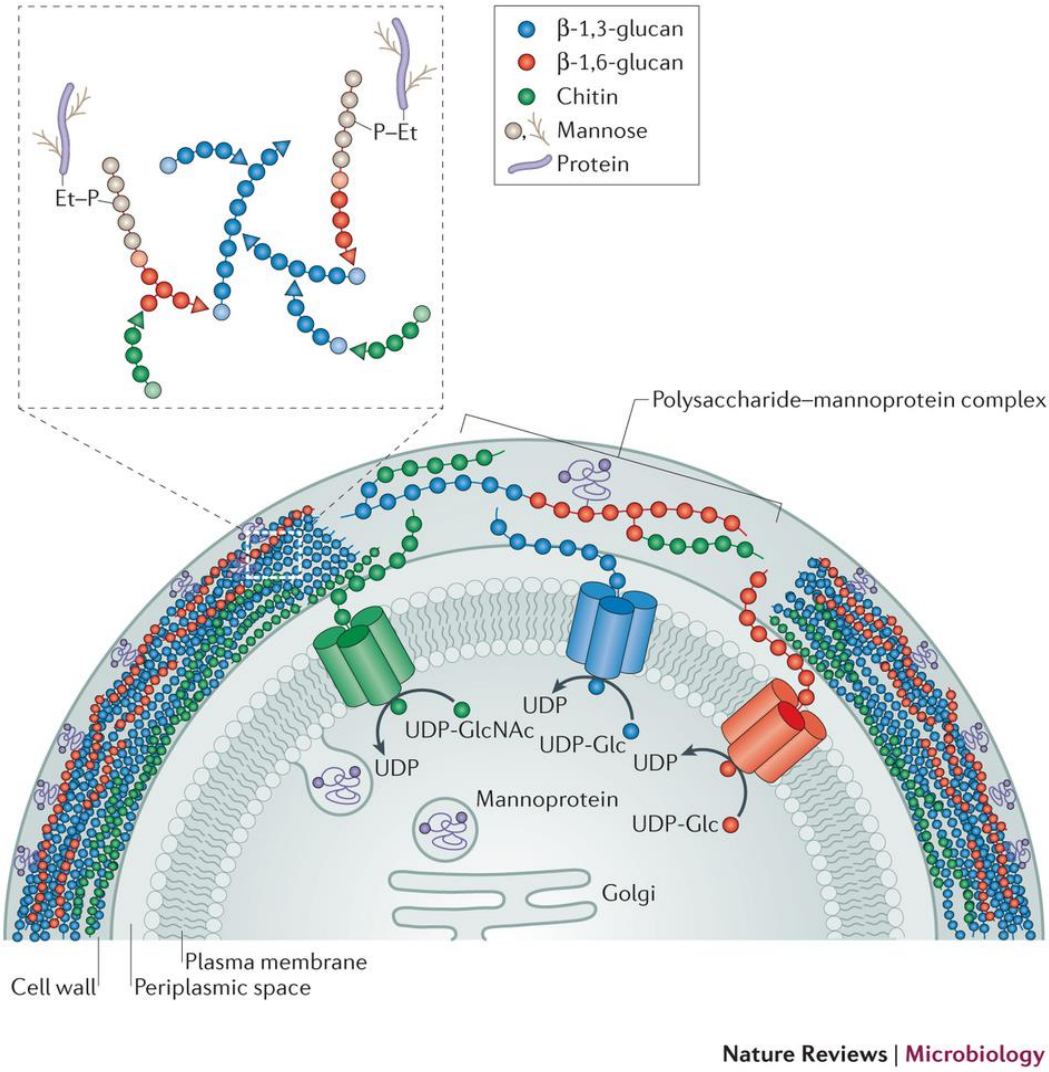


Figure 1.5 Structure of the cell wall of *S. cerevisiae* [29].

Table 1.4 Macromolecules of the cell wall of *S. cerevisiae* [12, 24, 25].

Macromolecules	Wall dry weight (%)	Mean of DP (kDa)	Branching
Mannoprotein	30-50	200 (Highly variable)	Highly branched
β-(1,6)-glucan	5-10	24 (150)	Highly branched
β-(1,3)-glucan	30-55	240 (1500)	Moderated branched
Chitin	1-2	25 (190)	Linear

The cell wall components are presented in the order in which they are found in the cell wall from the outside to the inside. DP, degree of polymerization.

This network is held together by hydrogen bonds between laterally associated β-(1,3)-glucan chains and by proteins with internal repeats (Pir) CW proteins. The β-(1,3)-glucan chains are extended at its outside with β-(1,6)-glucan chains, which are connected to a glycosylphosphatidylinositol anchor (GPI)-dependent mannoprotein. Most cell wall proteins (CWPs)

are GPI-CWPs and indirectly linked to the β-(1,3)-glucan chains. The proteins can be specifically released from the CW by using HF-pyridine, which cleaves the phosphodiester bond in the GPI remnant. Furthermore, some proteins as the Pir-CWPs are directly linked to the β-(1,3)-glucan chains through an ester linkage between specific glutamine (Gln) units and a glucosyl hydroxyl group. These proteins are termed ASL (alkali-sensitive linkage)-CWPs. The ASL-CWPs appear be uniformly distributed throughout the inner skeletal layer, while GPI-CWPs are mainly found in the outer layer of the wall, linked through covalent bonds [12, 24, 30].

1.3.1 Mannoproteins

Linked to the CW polysaccharides are a diverse set of mannoproteins that collectively form yeast mannan highly branched. Mannan can define morphology and stability of the cell and determine its ability to communicate with others cells [31]. Yeast mannan (Figure 1.6) is usually made of monosaccharides of α-D-mannose linked to the proteins by N- or O-glycosidic bonds. N-glycosylated proteins receive an oligosaccharide through an N-glycosidic bond between a GlcNAc and an asparagine (Asn) unit, forming a highly branched structure containing as many as 200 mannose residues of shorter chains of α-(1,2) residues, terminating in α-(1,3)-linked mannose residues, attached to a structure of α-(1,6)-linked mannose chain made of 50 mannose units. Whereas O-mannoslyated proteins accept short mannose chains onto the hydroxyl side chains of serine (Ser) or threonine (Thr) units through an α-mannosyl bond [24, 27, 30, 32].

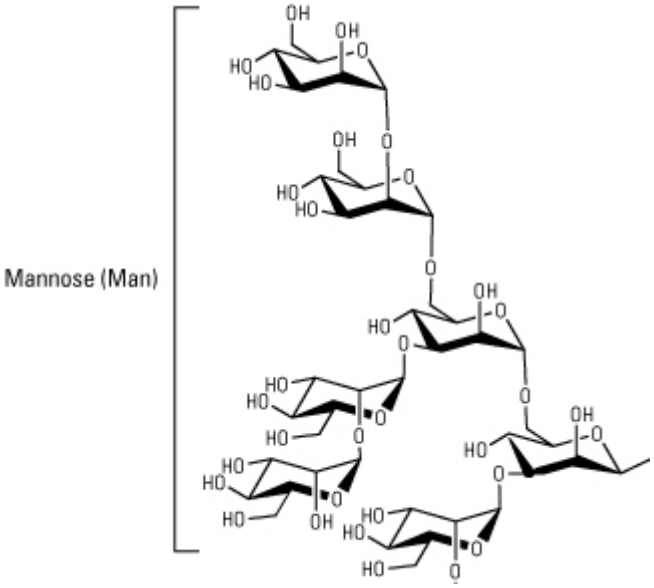


Figure 1.6 Struture of mannose units linked through α-(1,6) bonds (edited) [33].

As a prebiotic[2], mannanoligosaccharides (MOS) can bind to pathogenic bacteria in the intestinal tract, preventing them from binding to and colonizing the intestinal wall, and then carry the bacteria out of the body. Combinations of prebiotic and probiotic[3] activity are reported to have shown greater benefits than either on its own in animals [9, 34, 35]. Benefits comprise reduction in the occurrences of diarrhoea, constipation and bowel cancer, stimulation of the immune system, reduction in serum cholesterol levels, and enhanced nutrient uptake [34].

1.3.2 β-glucans

β-glucans of the YCW are composed of glucose residues held together by β-(1,3)- and β-(1,6)-bounds and covalently linked to the other wall components (Figure 1.7), forming a skeletal inner layer which is moderately branched [36].

The β-(1,3)-glucan chain has a degree of polymerization (DP) of ~1500 glucose monomers and ~40-50 glucose residues involved in branching through their C6-atom. Accordingly, β-(1,3)-glucan has several non-reducing ends allowing it to function as acceptor for β-(1,6)-glucan and chitin. When chitin is attached to β- (1,3)-glucan, β-(1,3)-glucan becomes insoluble in alkali, explaining the presence of alkali-soluble and alkali-insoluble β-(1,3)-glucan in the CW [24, 27, 37]. β-(1,3)-glucan chains belong to the so-called hollow helix family, i.e., they have a shape comparable to a flexible wire string that can exist in various states of extension. This property explains the elasticity and tensile strength of CW [24].

β-(1,6)-glucan is a highly branched, water-soluble polymer consisting on average of about ~130-140 glucose monomers. It is used in the CW to connect GPI-dependent CW proteins to the β-(1,3)-glucan network. It may also function as acceptor site for chitin, particularly in case of CW stress [24, 37].

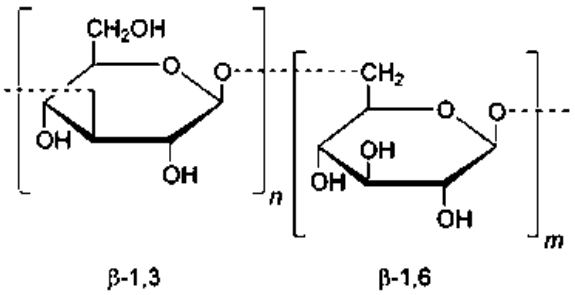


Figure 1.7 Chemical structure of the cell wall glucan [38].

[2] "*Prebiotic are food ingredients that selectively stimulate the growth and activities of specific bacteria in the gastrointestinal tract, usually bifidobacteria and lactobacilli*" [35].
[3] "*Probiotic are defined, cultured strains of lactic acid bacteria, which have been isolated from human intestinal flora, e. g., certain lactobacilli and bifidobacteria*" [39].

The molecular structure, DP, degree of branching (DB), MW, the polymer charge and/or solution conformation (triple helix, single helix or random coil conformation) and polysaccharides solubility of the yeast wall define the functional properties of β-D-(1,3)-glucans [40, 41].

β-(1,3)- and β-(1,6)-bonds are usually known for building insoluble structures. They stimulate the immune system, by activating first of all macrophages[4]. They also stimulate the skin cell response to combat free radicals and protect against the environmental pollution, significantly delaying aging process, and act as anti-inflammatory. A lower DB and lower DP are characterised by better solubility (Figure 1.8) [42].

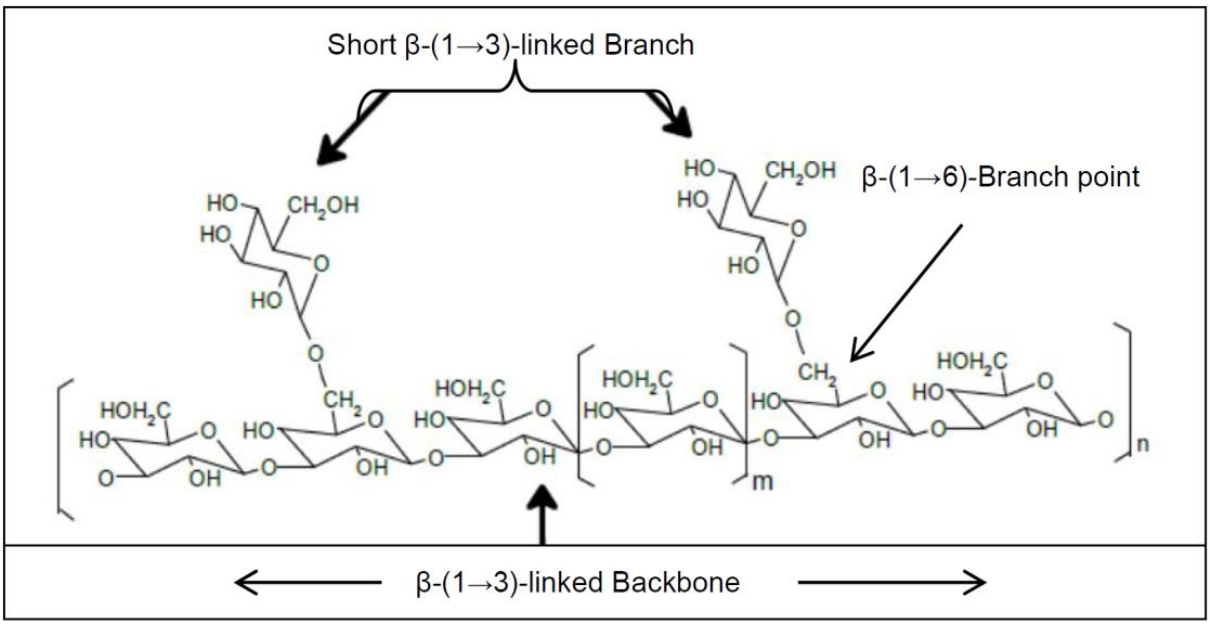


Figure 1.8 Example of a molecular structure of a soluble yeast β-glucan [42].

It is believed that insoluble β-glucans have a DP higher than 100. Generally insoluble or slightly soluble β-glucans contain very long, multi-branched side chains (Figure 1.9). MW of β-glucans obtained from several sources differs within a wide range of values from 2 kDa to 40000 kDa. From a technological point of view, β-glucans with high MW (> 3000 kDa) are characterised by high viscosity, i. e., the viscosity is directly proportional to the quantified MW. The ones that have low MW (about 9 kDa) usually constitute gels [42, 43]. Fractions with a MW between 100 to 200 kDa which exhibit a single and/or triple helix conformations have shown high biological activity, whereas a fraction from the same source with a low MW (5 to 10 kDa) have shown a limited activity. Accordingly, β-glucans which have the same MW and distinct conformation present different biological activity [41, 44].

[4] Macrophages are a type of white blood cell which play an important role in the initiation, maintenance, and resolution of an inflammation.

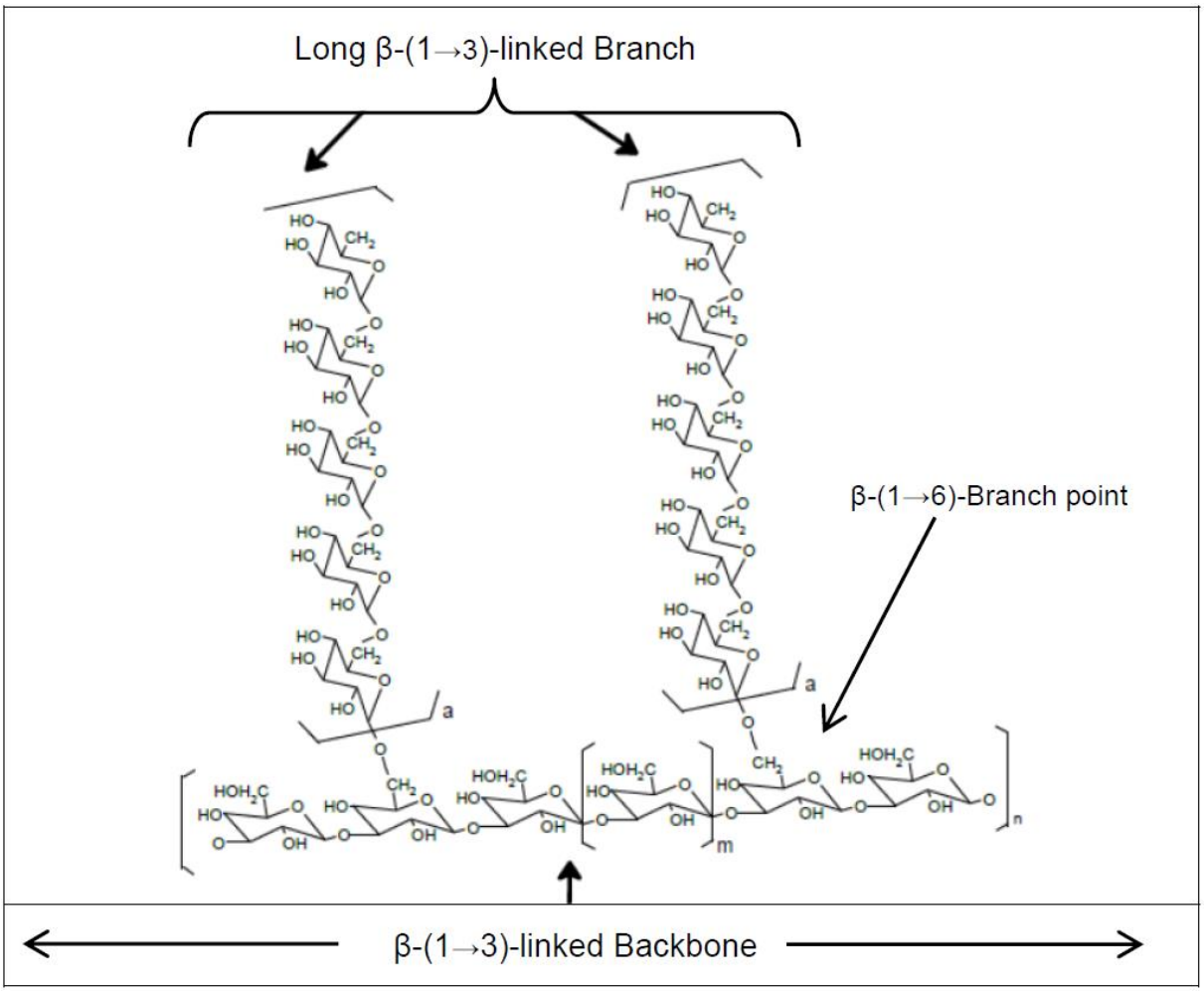


Figure 1.9 Example of a molecular structure of an insoluble yeast β-glucan [42].

Hydrolysed β-glucans are soluble, but not very viscous and normally do not constitute gels. Procedures such as enzymatic digestion or chemical methods leading to the hydrolysis of long-chain β-glucans, allow to lower the degree of depolymerisation and their particle mass in relation to native form, which simultaneously increases their solubility and lowers viscosity in liquids. There is also research published showing that viscosity of β-glucans depends on the degree of purification during their isolation [41-45].

Besides all the functional properties already mentioned, β-glucans exhibit more properties including therapeutic, pharmaceutical, cosmetic, food, and other industries. They can stimulate antimicrobial and anti-inflammatory activities, decrease the fraction of LDL (low-density lipoprotein) cholesterol, exhibit anticancer, antimutagenic, and antioxidant properties and promote wound healing. They can also act as emulsifiers, gelling agents, or as water- and fat-binding substitutes of fat, prebiotics, and film-forming substances. In wine industry, β-glucans are known for absorb mycotoxins [40, 34]

1.3.3 Chitin

Chitin (Figure 1.10) is an insoluble linear polymer made of β-1,4-linked N-acetylglucosamine (GlcNAc) [12, 24, 27]. The synthesis of chitin is mediated by chitin synthase, an integral membrane enzyme that catalyses the transfer of Nacetylglucosamine from uridine diphosphate (UDP)-N-acetylglucosamine to a developing chitin chain. Followed by an elongation of the chitin polymers via vectorial synthesis, that way the nascent chains are dismissed through the plasma membrane as they are made [47].

Stabilized hydrogen bonding between the polymers of chitin results in microfibril formation and consequent crystallization of chitin in the extracellular space immediately adjacent to the plasma membrane. This structure confers stretching resistance to the CW to resist chemical and physical challenges [30, 37, 46, 47]. If chitin synthesis is disrupted, the CW turns disordered, becoming malformed and osmotically unstable [47]. The chitin chains lengths in the CW and in bud scars are estimated to be around 100 and 190 monomers of GlcNAc, respectively. In the CW, ~40 to 50% of the chitin chains are linked to the nonreducing end of β-1,3-glucan (-GlcNAc-L1C4-Glc-) via a β-1,4 bond engaging the reducing end of the chitin polymer [27, 30, 37].

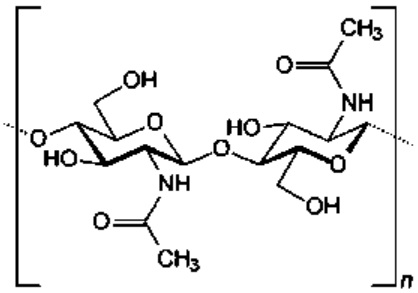


Figure 1.10 Chemical structure of the cell wall chitin [38].

1.4 Production of yeast extracts and yeast cell wall

Food yeast can be produced by different processes on the chosen substrate and yeast species [6]. In order to obtain the YE and YCW (Figure 1.11), the yeast or inoculum *S. cerevisiae* is fermented first in a medium culture with molasses (sugar, vitamins and minerals) and *C. utilis* in medium culture with ethanol, wood sugar or sulfite liquor. In both cases the medium culture is also supplied with salts, vitamins, trace elements/ supplements, water, a nitrogen source and air at ~ 30°C. When the fermenter is full and no more sugar can be added, the yeast is concentrated and washed in a centrifuge to remove the sugar residues. The result is a so-called yeast cream which will run through an extraction process (autolysis, hydrolysis or plasmolysis process sub-chapters 1.4.1; 1.4.2 and 1.4.3 respectively) [6, 8, 21, 48].

To obtain YE, the liquid must be centrifuged in order to remove the insoluble cell walls from the soluble components. Finally, the YE is concentrated in an evaporation process under vacuum conditions at ~60°C to a paste with 70-80 % dry weight or to a liquid with 45-65 % dry weight. Depending on the future use, the liquid yeast extract is then dried and pulverized. The liquid is fed

into a spray dryer and dried using hot air. During this process the water evaporates, the dried extract falls down and it is collected at the base of the tower of the spray dryer [48, 49].

There are several factors that influence such quality differences in flavour, colour, solubility and acceptability of YE. These include: type and condition of the yeast, presence of extraneous matter, extraction and processing conditions employed, level of contamination during processing, conditions applied during concentration and drying, the type of packaging and the conditions the products were exposed to, until they reach the consumer [50, 51].

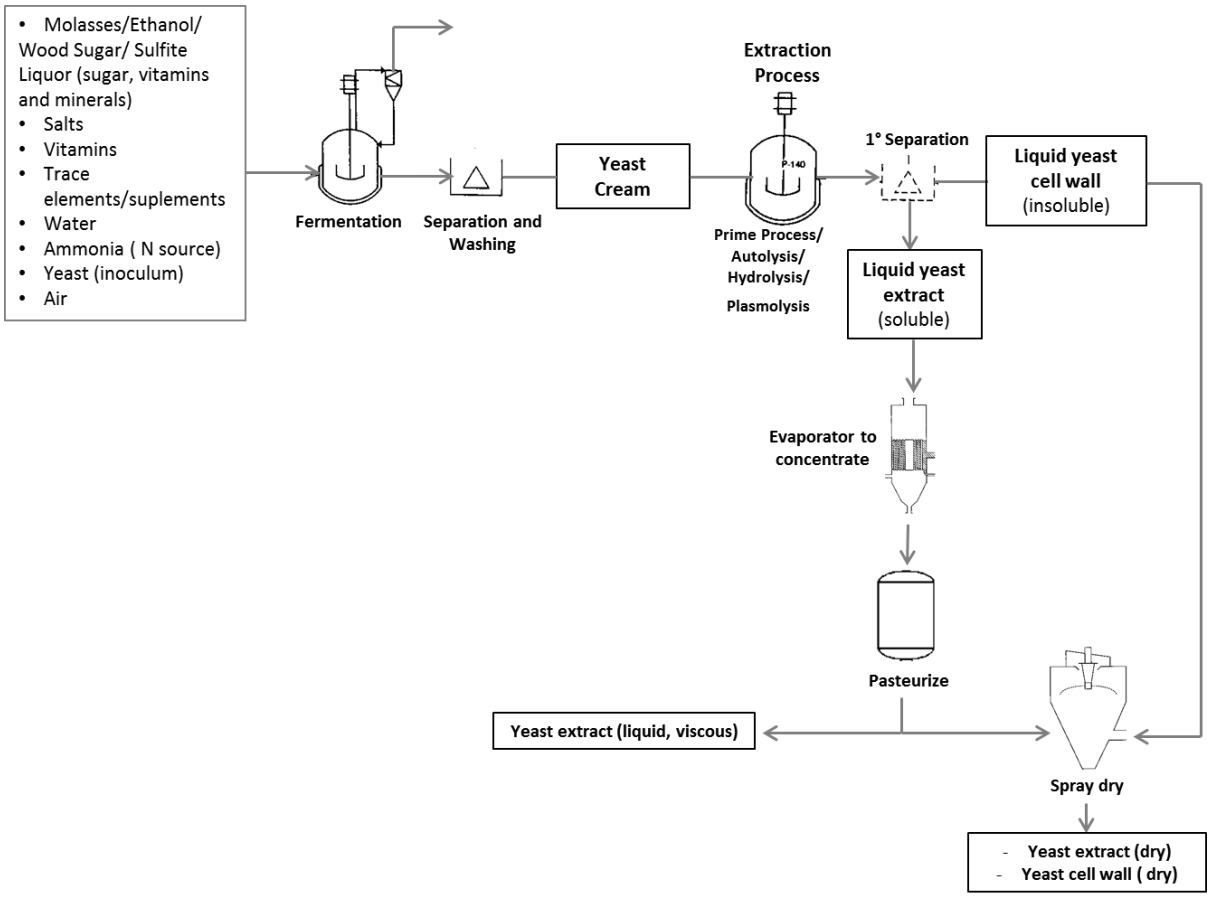


Figure 1.11 Scheme with the process to produce yeast extract and yeast cell wall (adapted) [6, 8, 21].

1.4.1 Autolysis Process

Autolysis is a process in which hydrolysis of intracellular biopolymers occur under the action of endohydrolases (e.g. nucleases, proteases and glucanases) entailed cell death and the formation of low-molecular products releasing from the intracellular space [7, 52]. Autolysis usually takes place at the end of the stationary phase of growth and is frequently associated with cell death [7, 53].

The mechanism of autolysis of yeast cell can be described in at least four stages. The first step involves a disturbance of cell endostructures, and as result the hydrolytic enzymes are released into the cytoplasm. In the second step the released hydrolytic enzymes are initially inhibited by specific cytoplasmic inhibitors, and then activated due to degradation of these inhibitors [7, 53]. In the

third step, activated enzymes interact with intracellular polymer components (substrate), resulting in accumulation of their hydrolysis products (e.g. peptides, AA, fatty acids, nucleotides, and nucleosides,) in the space restricted by CW [7, 53, 54]. In step four, as the molecular mass of hydrolysis products decreases, the said products diffuse into the extracellular medium [7, 53].

The industrial autolysis process is induced by holding an agitated slurry (15-18% yeast solids) brought slowly to 40-50°C and pH around 5. Autolysis usually requires 12 to 36h to achieve the desired degree of hydrolysis [7, 8, 21, 50].

The autolysed YEs are important food ingredients, contributing a meaty flavour and aroma to soups, sausages, meats, gravies, sauces, seafood and vegetables [8, 21].

1.4.2 Plasmolysis Process

Plasmolysis is a modified autolysis process in the presence of a so-called accelerator, such as an inorganic salt (e.g. sodium chloride) or organic solvent (e.g. toluene, ethanol, ethyl acetate) [55, 56]. Under a hypertonic or a hypotonic conditions (osmotic pressure), yeast cells are forced to undergo shrinking of protoplasm by addition of salt/ organic solvent, resulting in the release of cell water, solutes and enzymes. This event is usually called plasmolysis [39, 57, 58]. A key factor is the pH, which is generally optimum around 8.0 [57].

YE manufactured by plasmolysis may have a limited use because the searching for low-salt processed foods is increasing [21, 50].

1.4.3 Hydrolysis Process

In hydrolysis process, first an inactivation of yeast's own enzymes is done with heat treatment, followed by addition of exogenous enzymes or acid. The added enzymes (e.g. proteases) break down the yeast protein and other molecules into smaller molecules. The smaller molecules can now leave the yeast cell and mix with the aqueous solution in the tank. In this process the mixture is heated up to 100°C in a wiped film evaporator with reflux condenser and hydrolysis proceeds until the desired level of amino nitrogen is reached, generally 6-12 hours are needed for converting 50-60% of the total nitrogen to amino nitrogen. Neutralization of the hydrolysate to pH 5-6 is usually attained with sodium hydroxide [8, 50].

While hydrolysis results in the highest yields of YE, there is some loss of vitamins, protein and flavour. The high final salt content of hydrolysates may limit their use in foods [8, 21].

1.5 Proteins, peptides and free amino acids

Proteins are ubiquitous components of all living tissues, animal, plant and bacterial. They serve indispensable functions in cellular architecture, catalysis, metabolic regulation, and contractile processes and are an important weapon in the defence arsenal of many higher organisms [59]. They are synthesized from AA or activated AA present in the form of aminoacyl-transfer RNA (-tRNAs) in the process of translation on the ribosomes by peptide-bond-linking enzymes. Messenger RNAs (mRNA) which contains information on the AA sequence, is involved in the process [48]. Proteins are macromolecules/polymers constructed of linear, covalently bonded chains of AA. The lower boundary for the MW of proteins is taken to be about 5000 and substances with lower MW built from similar units are called polypeptide. There is no clear line of demarcation indicating how many monomeric units are required to make a protein [59].

Peptides

The bonds related with formation of secondary amide linkages between the α-carboxyl and α-amino functions of adjacent AA are termed peptide bonds. The structures resulting from the formation of peptide bonds are named peptides and the individual AA of peptides are termed residues [59].

Some sequence of peptides are associated with good taste properties. Aspartame (L-aspartyl-L-phenylalanine methyl ester) and also pCN-C_6H_4-NHCO-L-Asp-L-(αMe)Phe-OMe are used as a non-caloric sweetener. Asp cannot be substituted whereas Phe can be substituted by some hydrophobic residue. H-Asp-Met-OMe is nearly as sweet and H-Asp-Tyr-OMe is slightly less sweet than aspartame. All possible chiral isomers, i.e. D-L, L-D and D-D H-Asp-Phe-OMe are bitter [60]. For example, an octapeptide, whose sequence is H-Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala-OH was termed “delicious peptide” since it produced a taste like beef soup. Only few fragments of this peptide are associated with umami taste [60, 61]. The taste properties of the peptide fragments that provide an umami taste are reported in Table 1.5. These peptides (around 52), like the AA Glutamate, might have a synergy with ribonucleotides. It is possible that umami taste is a consequence of partial hydrolysis leading to sizeable concentrations of Asp or Glu [60, 61]. Some studies also relate fractions with a MW less than 500 Da with the most intense umami taste [62].

Table 1.5 Peptides reported to have umami taste [60, 61].

Peptide	Taste
KGDEESLA	Umami/ sour
AEA	Umami
GDG	Umami
VEV	Umami
EEE	Umami
KG	Salty/ umami
DE	Salty/umami
DL	Umami
EE	Salty/umami
EL	Umami
EK	Umami/sour
ED	Salty/umami
DD	Salty/umami

Many peptides hosting residues with hydrophobic side chains have a distinct bitter taste, this taste is also related with fermented foodstuff because of enzymatic hydrolysis [60].

Amino acids

The complete hydrolysis of proteins yields a mixture of about twenty AA. In a general way the structure of AA, with a few exceptions, is [39, 59]:

(1) NH_2-CHR-COOH

In the simplest case, R=H (aminoacetic acid or glycine). In other amino acids, R is an aliphatic, aromatic or heterocyclic residue and may incorporate other functional groups.

AA can be classified having in account intra- and intermolecular interactions in proteins.

- AA with nonpolar, uncharged side chains: e.g., glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan and methionine.
- AA with uncharged, polar side chains: e.g., serine, threonine, cysteine, tyrosine, asparagine and glutamine.
- AA with charged side chains: e.g., aspartic acid, glutamic acid, histidine, lysine and arginine.

Free AA can contribute to the flavour of protein-rich foods in which hydrolytic processes occur. Taste quality is influenced by the molecular configuration. Usually sweet AA are primarily found among members of the D-series, whereas bitter AA are generally acids with a cyclic side chain (1-aminocycloalkane-1-carboxylic acids) are sweet and bitter [39].

1.6 Nucleic Acids and nucleotides

The nucleic acids are macromolecules essential for many functions of a living organism, e.g. the storage and transmission of its genetic make-up and the means by which this information is utilized in the synthesis of all cellular proteins, and hence of all cellular constituents. The basic structure comprises a nitrogenous base, a five carbon (sugar) and a phosphate group [59].

The bases found in nucleic acids in DNA are either pyrimidines (thymine (T) and cytosine (C)) or purines (adenine (A) and guanine (G). Most RNAs also contain only four bases: the purines (A and G) and the pyrimidines (uracil (U) and C) [59].

Nucleoside comprises a nitrogenous pyrimidine or purine base and a sugar (D-ribose or D-2-deoxyribose) [59, 63].

Nucleotides are phosphate esters of nucleosides, the phosphoryl residue being attached to at least one hydroxyl groups of the sugar and can be obtained by chemical or enzymatic hydrolysis of the nucleic acids. Thus ribonucleotides can be phosphorylated on the 2'-, 3'- and 5'- positions. Furthermore, cyclic phosphates can be formed by the subsequent phosphorylation of a second hydroxyl group, giving rise to 2'-, 3'- and 5'- cyclic phosphates. Deoxyribonucleosides can be phosphorylated only on the 3'- and 5'- positions [59, 63].

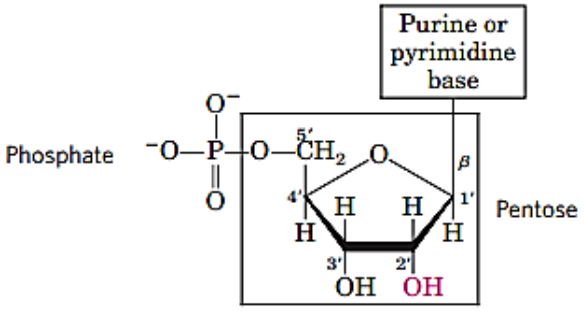


Figure 1.12 General structure of a ribonucleotide. OH at the 2´position is deoxygenated and the phosphate group is attached to the 5'position [64].

1.7 Yeast extract

YEs are the soluble portion of the cell content of yeast following an enzymatic digestion of the yeast cell constituents by autolysis, hydrolysis or plasmolysis process. Biomass for YE production is obtained primarily from brewer's yeasts and secondarily from sources of primary-grown yeasts including *S. cerevisiae* and *C. utilis* [48, 51].

YEs are a natural source for savory flavour due the presence of various free AA, peptides, nucleotides and reaction flavours generated during processing [67]. A major consideration in their use as a flavouring agent is their cost-effectiveness compared to other flavouring agents on the basis of equivalent flavour intensity [66]. They are usually rich in peptides, AA, nucleotides and vitamins, therefore are good for use as supplement in culture media [65]. The choice of the starting material and enzymes for the production of YEs is related with the cost and availability of the yeast and the quality expected in the final product [65].

1.7.1 Yeast Extracts Containing 5’-Nucleotides

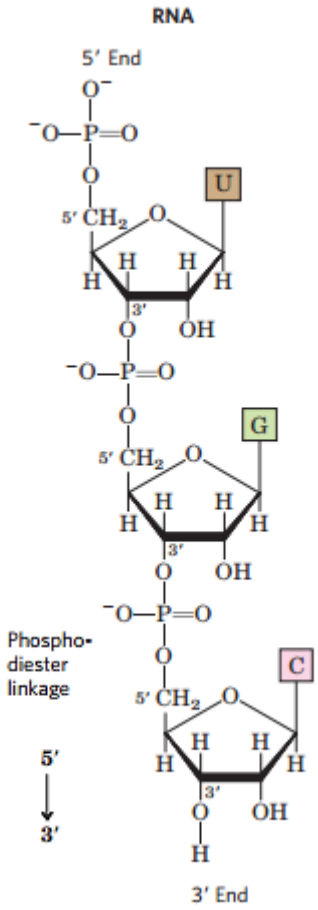


Figure 1.13 Backbone of RNA [54].

Strains of yeast are used as source of RNA (Figure 1.13) to produce YEs containing natural flavour enhancers. Among different commercial strains of yeast, *C. utilis* has been found to contain the highest level of RNA (10-15%) per dry matter as compared to 5-8% RNA in *S. cerevisiae* [8, 67].

It is essential to choose the right enzymes for the hydrolysis of the RNA to make possible produce YEs which are able to enhance some organoleptic properties in food application because of presence of 5’nucleotides (Figure 1.14). These enhancers can work as substitute of monosodium glutamate (MSG) which is the sodium salt of glutamic acid (non-essential AA) and it is associated to adverse effects [50, 67].

Figure 1.15 shows a possible explanation to YEs containing 5'-IMP and 5'-GMP, which are the most important compounds in terms of taste-enhancing properties.

First the RNA is extracted, using hot alkali treatment to breakdown the yeast cells and afterwards is hydrolysed by a fungal nuclease (a.k.a as 5'-phosphodiesterase) which are able to hydrolyse the RNA into 5´-nucleotides. 5'-AMP and 5'-GMP are removed from the mixture by adsorption, on activated charcoal, and 5'-CMP and 5'-UMP pass through the column to be discarded. Then 5'-AMP and 5'-GMP are adsorbed and eluted in a methanol/ammonia mixture. 5'-IMP can be obtained through the conversion of adenosine 5'-monophosphate (5'-AMP) by adenosine deaminase [50, 66, 67, 68].

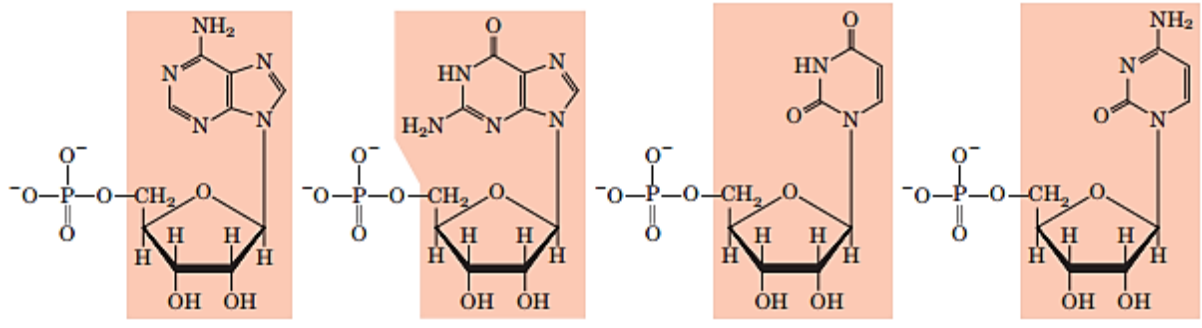


Figure 1.14 The ribonucleoties, AMP, GMP, UMP and CMP. All abbreviations assume that the phosphate group is at the 5' position [64].

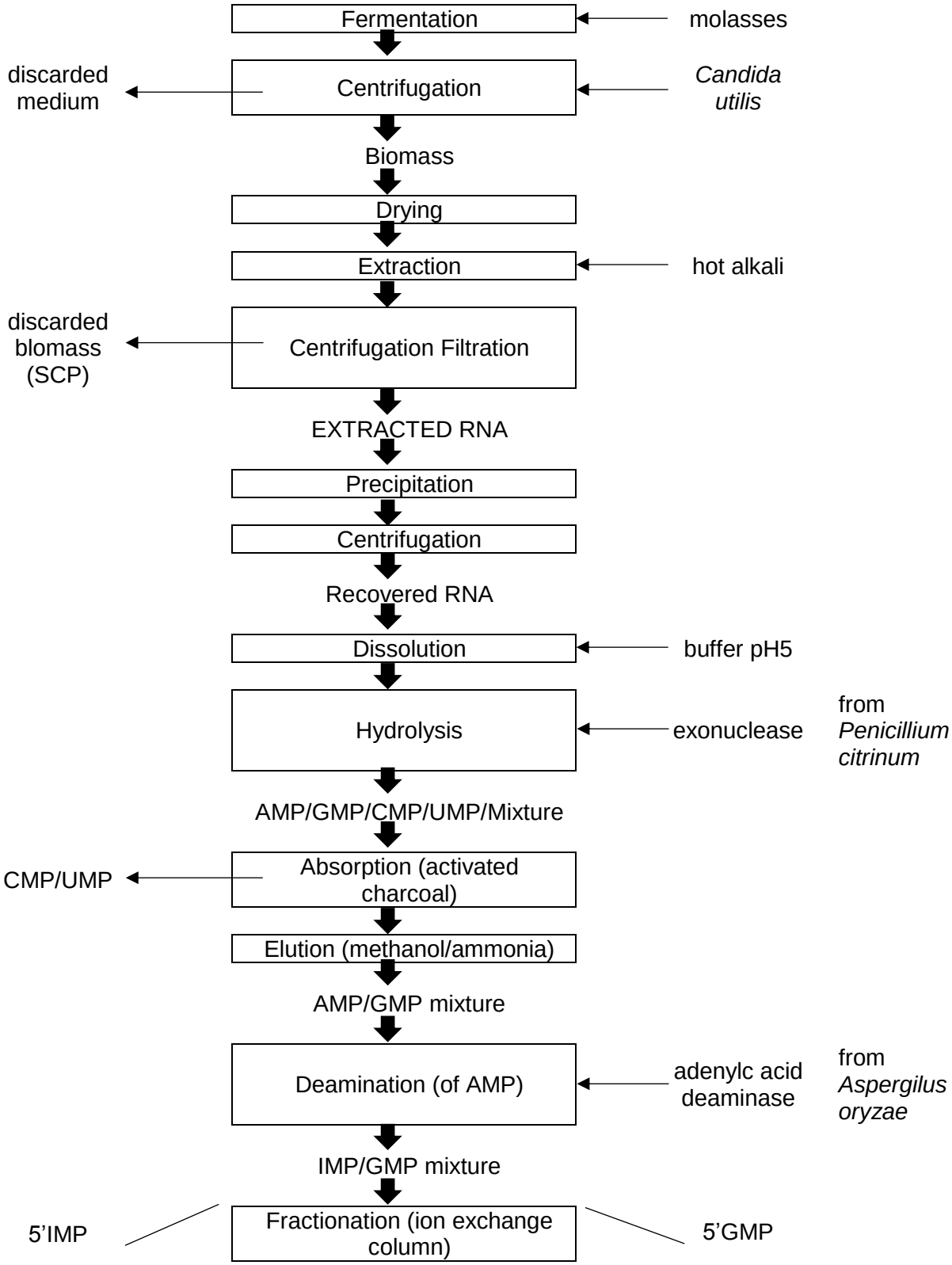


Figure 1.15 The production of 5'-IMP and 5'GMP by RNA hydrolysis (simplified) [68].

These extracts are commonly used in soups, gravies, broths, sauces, etc. The natural 5'-nucleotides of some of these yeasts extracts have been demonstrate synergistic potentiating effects by interacting with the low levels of natural glutamate present in the YE as well as in the food substrates to provide the overall delicious character and enhanced savoury attributes of processed foods [50, 56, 67].

1.7.2 Flavour enhancing 5'-Nucleotides

The best known flavour enhancers, that are in commercial use, are inosine 5'-monophosphate (5'-IMP) and guanosine 5'-monophosphate (5'-GMP). They enhance flavours have been reported as meaty, brothy, mouthfiling, well-rounded, drying or astringent. Although, 5'-GMP seems to provide a smoother "fullness" and "thickness" to the palate than 5'-IMP [50].

With 50:50 blends of the two flavour enhancers (5'-GMP and 5'-IMP), bitterness and sour are suppressed and sweet and salt perceptions enhanced. In the presence of glutamate, these compounds are known to enhance umami taste and suppress metallic and other undesirable flavours common in processed foods. The taste threshold ranges for 5'-GMP and 5'-IMP were 0.0035-0.02% and 0.01-0.025%, respectively, implying that 5'-GMP is rather more functional than 5'-IMP [50].

Synergism between glutamate and 5' nucleotides in human is extremely large. IMP or GMP alone and in presence with a low concentration of glutamate do not elicit the response. But an increase of glutamate concentration induces a large response even at concentration where glutamate alone does not elicit the response [69]. T1R1-T1R3 was established to be umami receptor. T1R1 and T1R3 belong to family C of GPCRs and have three regions (Figure 1.16): the large extracellular region, the seven-spanning transmembrane region, and the cytoplasmic region. In addition, the extracellular region can be divided into the ligand-binding region, a.k.a Venus flytrap module (VFM), and the cysteine-rich domain, which intervenes between the ligand-binding and transmembrane regions. Biding site for glutamate and 5'-IMP or 5'-GMP exist in Venus flytrap of T1R1 subunit. Here glutamate binds close to the VFM along the hinge-bending motion, which leads to stabilization of the active conformation. The 5'-nucleotide binds to an adjacent site close to the binding site for glutamate. This leads to further stabilization of the active conformation. The structure of T1R1 is in dynamic equilibrium, where the ratio between the closed (active) and the open (inactive) conformations is modulated by the presence/absence of ligand. Thus the synergism is produced by an allosteric regulation [69, 70].

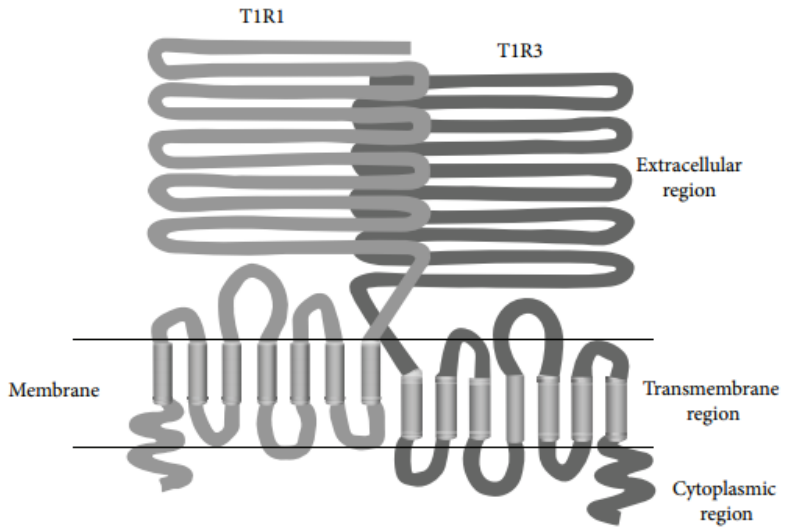


Figure 1.16 Schematic structure of T1R1-T1R3 heterodimer [69].

Umami reception is done in type II and type III cells which are able to transmit their signals to gustatory nerve fibbers. Stimulation of umami receptor T1R1-T1R3 by umami stimuli activates G-protein and leads to activation of phospholipase Cβ2 (PLCβ2). Thus, inositol-1,4,5-triphosphate (IP_3) is produced leading to inositol 1,4,5-triphosphate receptor type 3 (IP_3R3) activation which induces Ca^{2+} release from the Ca^{2+} stores. The increase in $[Ca^{2+}]i$ activates transient receptor potential of TRPM5 (transient receptor potential cation channel subfamily M member 5), inducing the depolarization of the taste cell. Finally, the taste cell induces potentials via voltage-gated Na^+ channels and releases a transmitter (seems to be ATP) to activate taste nerve fibers [69].

1.8 Sensory perception

"*Flavour, as an attribute of foods, beverages, and seasoning, has been defined as the sum of perceptions resulting from stimulation of the sense ends that are grouped together at the entrance of the alimentary and respiratory tracts*". Flavour includes [39, 71]:

- Olfactory perceptions caused by volatile substances released from a product in the mouth via the posterior nares which are usually called the aromatics;
- Gustatory perceptions (salty, sweet, sour, bitter) caused by soluble substances in the mouth, named tastes;
- The chemical feeling factors, which stimulate nerve ends in the soft membranes of the buccal and nasal cavities (astringency, spice heat, cooling, bite, metallic flavour, umami taste).

The five important basic taste perceptions are provided by [60, 71]:

- Sour taste is stimulated by acids, such as citric, malic, phosphoric, etc;
- Sweet taste is stimulated by sucrose and others sugars, such as fructose, glucose, etc., and by other sweet substances such as saccharin, aspartame, and Acesulfam K.

The T1R2-T1R3 receptor can bind all sweet molecules, sugars, some D-amino acids, sweet proteins and several synthetic sweeteners;

- Bitter taste is stimulated by substances such as quinine, caffeine, and hop bitters. Compounds that elicit bitter taste are recognized by a family of class A GPCRs, collectively known as T2Rs;
- Salty taste is stimulated by sodium salts, such as sodium chloride and sodium glutamate, and in part by others salts, such as potassium chloride;
- Umami taste is derived from the Japanese word meaning savoury or delicious. Three different receptors have been indicated as umami receptors: a truncated form of mGluR4 and also mGluR1 and the T1R1-T1R3 heterodimer [60, 71].

Until now, researchers were unsure whether the sour and salty taste had specialized receptors. Salty and sour tastes are related with hydrogen ions (H^+) for acidity and, mainly, sodium ions (Na^+) for salt. Some studies have related that many cells in the tongue might be able to pick up these signals, relaying the information in a complex pattern of nerve signals to the brain. However some studies have shown results that suggest that PKD1L3 and PKD2L1 heteromers may have function as sour taste receptors [72, 73]. The “receptor” for salt (NaCl) is apparently an epithelial-type Na^+ channel on the apical membrane of some taste cells [74].

1.9 Enzymes

Proteins molecules with the ability to affect, in a specific and efficient manner, the rates of a wide spectrum of reactions and to be themselves susceptible to a variety of controls, in other words proteins with catalytic activity, are called enzymes. Enzymes are considered exceptional catalysts because under optimal conditions, most enzymatic reactions proceed 10^8 to 10^{11} times more quickly than the corresponding no enzymatic reactions [59].

1.9.1 Carbohydrate-active enzymes (CAZymes)

Carbohydrate-active enzymes (CAZymes) contain the families of structurally-related catalytic and carbohydrate-binding modules (or functional domains) of enzymes that degrade, modify, or create glycosidic bonds, in others words, these enzymes are important for the synthesis, degradation, and modification of carbohydrate-based structures [75, 76]. CAZymes can be divided into glycoside hydrolases (GHs) [EC 3.2.1.-], glycosyltransferases (GTs) [EC 2.4.x.y] or polysaccharide lyases (PLs) [EC 4.2.2.-] [75-77].

Glycoside hydrolases (GHs) are responsible for the hydrolysis and/or transglycosylation of a glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety [76, 77].

Glycosyltransferases (GTs) are responsible for the biosynthesis of disaccharides, oligosaccharides and polysaccharides. These enzymes form glycosidic bonds when catalyze the transfer of sugar moieties from activated molecules to specific acceptor molecules [76, 77].

Polysaccharide lyases (PLs) cleave the glycosidic bonds of uronic acid-containing polysaccharides by a β-elimination mechanism to produce an unsaturated hexenuronic acid residue and a new reducing end [76, 77].

1.9.2 Phospholipases

Phospholipases can be classified in, phospholipase D (PLD), phospholipase C (PLC, phospholipase A1 (PLA1), and phospholipase A2 (PLA2). PLC and PLD cleave the first and the second phosphodieteric bond, respectively, while PLAs release fatty acids from membrane lipids (Figure 1.17) [78].

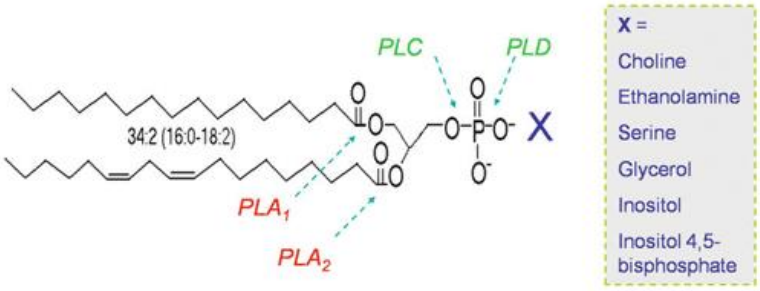


Figure 1.17 Sites of phospholipid hydrolysis by PLD, PLC, PLA2 and PLA1. The two fatty acyl chains may vary. X represents the common head group of phospholipids [78].

1.9.3 Proteases

The proteolytic enzymes play an important role in the production of yeast autolysates [7].Proteases are classified according to their source (animal, plant, microbial), their catalytic action (endopeptidase or exopeptidase), and the nature of the catalytic site. In the EC system for enzyme nomenclature, all proteases (or peptide hydrolyases) are in subclass [3.4], which is divided into [3.4.11-19], the exopeptidases, and [3.4.21-24], the endopeptidases or proteinases. Endopeptidases are the proteases most commonly used in food processing, but in some cases their action is supplemented with exopeptidases. Endopeptidases cleave the polypeptide chain at particularly susceptible peptide bonds distributed along the chain, whereas exopeptidases hydrolyze one AA (or dipeptide, in the case of [3.4.14 and 15]) at a time from either the N-terminal (aminopeptidases) or the C-terminal (carboxypeptidases) [65, 66].

1.9.4 Nucleolytic enzymes

Nucleolytic enzymes are capable of digesting the nucleic acid chain, resulting in the formation of fragments of nucleotides, nucleosides, and free bases. Those enzymes, outlined in Figure 1.18, can be divided in nucleases, phosphatases, nucleosidases, and nucleodeaminases [66].

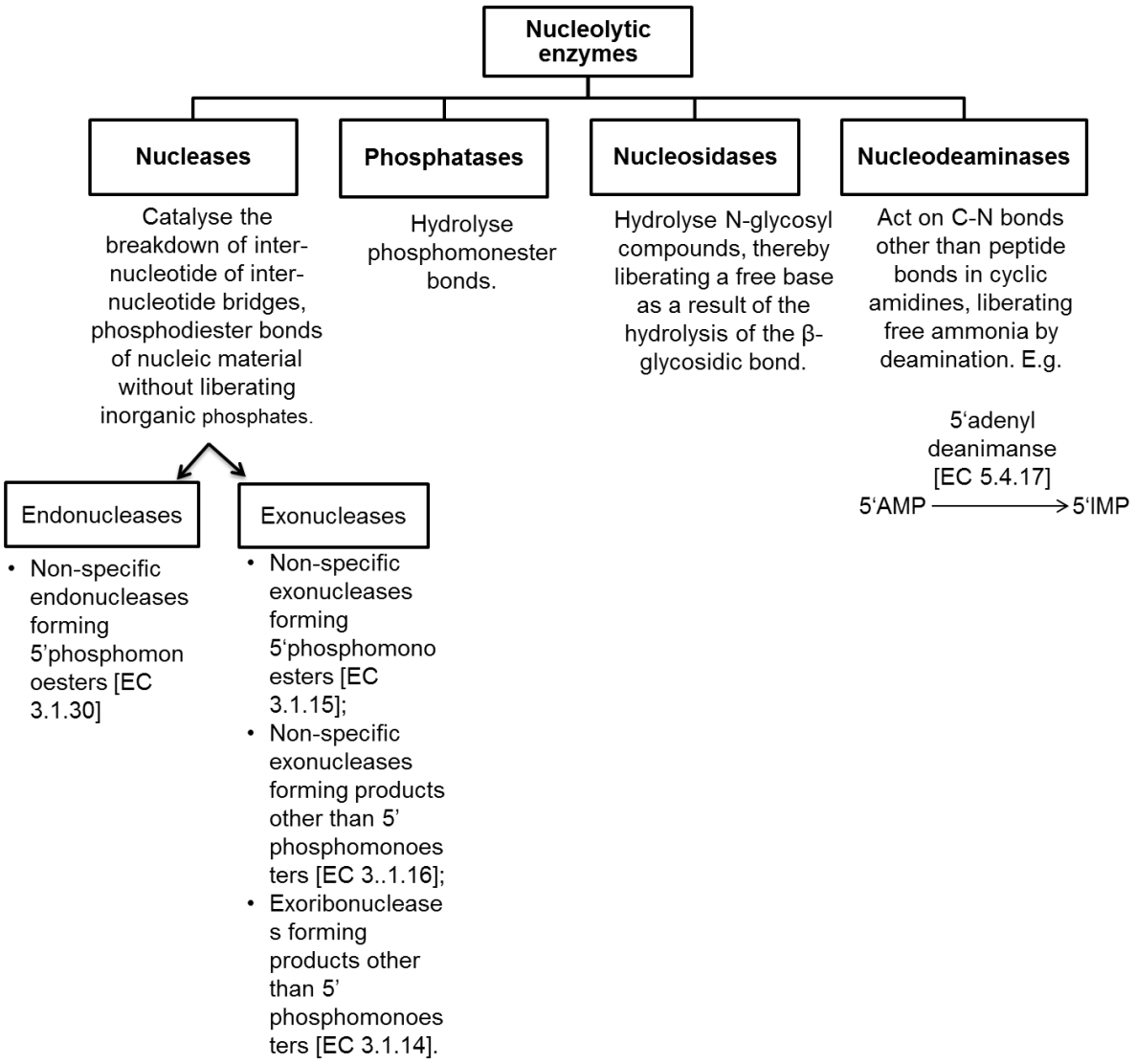


Figure 1.18 Classification and characterization of nucleolytic enzymes [adapted 66].

1.10 Ohly's Products

1.10.1 SYEAA

One of Ohly's standard YE is standard yeast extract with high content in free amino acids (SYEAA), a low salt yeast autolysate with a high content of free AA and affords a good basic flavour rounding and a mild savoury character [1].

1.10.2 TYC

Torula yeast cell (TYC) is a high quality, low sodium autolyzed YE, derived from *C. utilis* and contains high levels of 5'-IMP and 5'-GMP and free glutamic acid. TYC provides a strong first impact which passes into a long lasting flavour of the product and umami taste. The naturally occurring free glutamic acid in TYC works synergistically with the nucleotides to provide a greater flavour potentiating effect than either alone would offer [1].

1.10.3 TYCW

Torula yeast cell wall (TYCW) is low sodium yeast, produced from primary grown *C. utilis* yeast in continuous fermentation and mimics autolyzed yeast in flavour profile. TYCW may be used in any food system that requires heightened flavour impact and because is low sodium yeast can be used in sauces and gravies results in a fuller body flavour and smoother mouthfeel [1].

1.10.4 YEHP

Yeast extract with high content of peptides (YEHP), brings out creamy and herb notes, can balance undesired flavour notes to a more pleasant overall flavour [1].

2 Enzyme screening of YCW

2.1 Introduction

Currently, Ohly produces yeast cell walls (YCWs) as side-stream products during YE production. YCW are sold for a very low profit margin as additives for feed. Accordingly, this first part of this master thesis involved the screening of various enzymes that have different carbohydrase and phospholipase activities on YCWs.

During this master thesis, YCW obtained from hydrolysed *S. cerevisiae* will be named hydrolysed yeast cell wall (HYCW). This YCW was obtained using a heat treatment to inactivate yeast‘s own enzymes and once the temperature was cooled to certain degree, exogenous enzymes were added to break down the yeast cell. The autolysed yeast cell wall obtained from *C. utilis* in a Prime process that mimics autolysed yeast in flavour will be termed Torula yeast cell wall (TYCW).

With this thesis, we aim to have a fundamental understanding on the effect that various enzymes have on yeast cell wall conversion. On the long-term, enzymatic hydrolysis of YCWs should result in value-added products that can generate a higher profit margin than at the moment and will be useful to find new applications for the by-products in food or fermentation application.

2.2 Material and methods

2.2.1 Material

Table 2.1 List of equipment for analytical methods.

Equipment	**Producer**
Scale Quintix 5102-1Ceu (d=0.01g)	Sartorius, GmbH
Scale ABT 320-4M (d=0.1mg)	Kern, GmbH
Scale AX224 (d=0.1mg)	Sartorius, GmbH
Rapid dryer HB43-S Halogen	Mettler Toledo, GmbH
pH-meter 766 Calimatic	Knick, UK
Research® plus Pipettes (2-20µL, 10-100µL, 20-200µL, 100-1000µL and 0.25- 2.5mL)	Eppendorf, GmbH
Photospectrometer DR 600	Hach Lange, UK
Photospectrometer D3 2800	Hach Lange, UK
Waterbath	GFL, GmbH
HAAKE ViscoTester VT5R	Thermo Fisher Scientific, USA
Heraeus Multifuge X3R centrifuge	Thermo Fisher Scientific, USA
Thermometer	Julabo, GmbH
Analog vortex mixer, 230V	VWR, GmbH
Magnetic stirrer, 220V-50/60Hz	IKA®-Werke GmbH & Co. KG. Janke
Incubator shaking INCN-Line ILS4	VWR, GmbH
Epoch 2 Microplate reader (Gens version 2.09)	Biotek, USA

Table 2.2 List of material for analytical methods.

Material	**Producer**
1.5mL and 50mL falcon tubes	Nerbe plus, GmbH
250mL Erlenmeyer	Duran, GmbH
250mL beakers	Duran, GmbH
10mL, 500mL volumetric flask	Duran, GmbH
1.5mL and 2.5mL Eppendorf tube	Nerbe plus, GmbH
Disposable pipettes, 3mL	Nerbe plus, GmbH
1.5mL and 2.5mL Cuvettes	BRAND, GmbH
1.5mL vials,	WICOM GmbH
0.45µm filter SEC (PES),	WICOM GmbH
5mL Disposable syringe	BRAUN-Omnifix GmbH
8mm PP-screw caps	WICOM GmbH
8mm PTFE/Silicon septum for screw caps (blue/white)	WICOM GmbH
MN 85/70 BF Ø 90mm Filter papers	Macherey-Nagel, GmbH
Aluminum Sample Pans for Moisture Analyzer	Mettler Toledo, GmbH
Tissue Culture Plates with 96-well	VWR, GmbH

Table 2.3 List of reagents for analytical methods.

Reagent	**Producer**
5N H_2SO_4	Carl ROTH, GmbH
D-Glucose	Carl ROTH, GmbH
α-D-glucose	Merck KGaA, GmbH
3,5-dinitrosalicyliy acid	Sigma Aldrich, USA
Potassium sodium tartrate tetrahydrate 60%	Sigma Aldrich, USA
2M NaOH	Avantor performance materials, Poland
D-glucose Kit	R-Biopharm AG, GmbH
D-mannose Kit	Megazyme, Ireland
$Na_2HPO_4.2H_2O$	Merck KGaA, GmbH
$MgCl_2$	Carl ROTH, GmbH
1M NaOH	Carl ROTH, GmbH
Glucose oxidase (12.000U) plus catalase (300.000U)	Megazyme cat. No. E-GOXCA, Ireland
Pullulan, β-(1,3)-laminaripentaose and β-(1,3)-laminarbiose standards.	Shodex (Separation HPLC) Group, Grand Island

2.2.2 Methods

2.2.2.1 Enzymatic Reaction

Carbohydrases (56) and phospholipases (4) (Appendix 15) were tested on YCW products from *S. cerevisiae* (HYCW) and *C. utilis* (TYCW). The YCWs were mixed with ultrapure water to reach a DM content of ~12% (w/w) and ~21% (w/w), respectively, and were stirred for around 60min at room temperature. The yeast mixture with a volume of 200mL was adjusted to pH 4.5 using 5N H_2SO_4 and 1% (w/w) of each enzyme was added. No enzyme was added to the sample termed control. Hydrolysis was performed for 3h and 24h in duplicates, at 50°C with a shaking of 120rpm. The samples were inactivated at 80°C for 30min.

The hydrolysates characterization was done by visual, analytical and chemical analysis, such as measurement of viscosity, turbidity, solubilisation and phase separation, RS and free sugars release over time.

2.2.2.2 Hydrolysates Characterization

2.2.2.2.1 Viscosity Measurement

The viscosity measurement was done with a viscometer using the samples which the enzymatic reaction was performed for 24h before being centrifuged. A R2 and R3 spindle were used depending on viscosity of the mixture, at 25°C, with a rotation speed between 60rpm and 200rpm.

2.2.2.2.2 Solubilization and phase separation

The determination of biomass dry weight was performed in duplicates. The volumes of Pellet and supernatant (SN) were measured after the phase separation done for a total volume of 20g, for 20min at 4700rpm. The pellet form (solid or loose) was verified and the DM of SN (collected into 15mL falcon tubes) was measured. The DM of SN was measured with a NR151 Digital Refractometer which measures the Brix % and refractive index in order to measure soluble compounds [79].

2.2.2.2.3 Reducing sugars measurement (DNS)

The examination of reducing sugars (RS) was carried out by DNS method. An alkaline solution of 3,5-dinitrosalicylic acid (DNS) was used as reduced agent. This substance builds an orange/red colourant with a high level of RS which can be detected at 530nm. The evaluation was carried out having in account the calibration curve (Appendix 1).

Solution of DNS

The alkaline solution of 500mL of 3,5-dinitrosalicylic acid (with 1% (w/v) of 1,3-dinitrosalicylic acid and 30% (w/v) of Potassium sodium tartrate) was prepared adding 5g of 3,5-dinitrosalicylic, 100mL of 2M NaOH and 250mL of Potassium sodium tartrate tetrahydrate 60% into a 500mL volumetric flask and afterwards it was filled up with ultrapure water. The volumetric flask was covered with Aluminium foil and the solution was stirred overnight.

Calibration curve

For the calibration curve (Appendix 1) was used 1% (w/v) of a D-Glucose stock solution and it was followed the same procedure that was used during DNS assay to evaluate RS content in YCW samples.

Table 2.4 Table with the volumes needed to prepare the calibration curve from 1% (w/v) of a D-Glucose stock solution.

Reagents	Blank	Calibration solutions				
1% of D-glucose [mL]	0.0	0.05	0.10	0.15	0.20	0.25
ultrapure water [mL]	1.0	0.95	0.90	0.85	0.80	0.75
D-glucose final solution [mg/mL]	0.0	0.5	1.0	1.5	2.0	2.5

Preparation of samples

DNS assay must be done with gloves under the hood. The SN of the YCW samples was diluted 1:5 (3h of reaction time) and 1:10 (24h of reaction time). In some cases the dilution was adjusted because the DM of the samples was higher, but in a general way those were the dilutions used during the assay.

Afterwards 250 µL of each YCW sample was pipetted into 2 mL eppendorf tubes with 500µL of DNS. A blank eppendorf tube was performed with 250µL of ultrapure water and 500µL of DNS. Then the eppendorf tubes were heated up for 5min at 100°C, cooled at room temperature and centrifuged for 5min at 3000rpm. References (before reaction) and samples with a red/orange/yellow colour (after reaction) were prepared into 2.5mL cuvettes with 200µL of each YCW sample and 1800µL of ultrapure water. Duplicate determinations were made.

RS calculation

The RS concentration was calculated by the difference between the absorbance of the YCW sample and respective reference, having in account the dilution factor. The dividend was divided by the slope calculated in the calibration curve (1):

(1) $\text{Reducing sugars concentration (g/L)} = \frac{(\text{Abs}-\text{Ref})\times\text{DilutionFactor}}{\text{slope}}$

2.2.2.2.4 D-Glucose Measurement

The UV-method for determination of D-glucose was carried out with R-Biopharm Enzymatic BioAnalysis protocol [80].

Preparation of samples and assay

- The bottle with solution 1 was a mixture with 72g of powder, consisting in: triethanolamine buffer with a pH ~7.6; ~110mg NADP; ~260mg ATP and magnesium sulphate. This bottle was dissolved in 45mL of ultrapure water.
- The bottle with solution 2 was a ~1.1mL suspension, consisting of: ~320U hexokinase and ~160U glucose-6-phosphate dehydrogenase.
- The SN of the YCW samples was diluted 1:5 or 1:10 (3h of reaction time) and 1:10 or 1:20 (24h of reaction time). In some cases the dilution was adjusted because the DM of the samples was higher, but in a general way those were the dilutions used during the assay. Duplicate determinations were made.

Experimental details

For the determination of D-glucose was followed the procedure explained in Table 2.6. The samples were detected at 340nm, using a 96- well plate and a microplate reader which already had a pre-set measurement program. The evaluation was carried out having in account the calibration curve of a standard stock solution of 2.3g/L α-D-glucose, previously prepared (Table 2.5).

Table 2.5 Table with the volumes needed to prepare the calibration curve from a standard stock solution of 2.3g/L α-D-glucose.

Standard	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Ultrapure water (µL)	800	825	870	910	955	980	990	1000
Vol. Stock solution (µL)	200	175	130	90	45	20	10	0
Approx. con. (g/L)	0.5	0.4	0.3	0.2	0.1	0.05	0.02	0

Vol., volume. Approx., approximately. Conc., concentration.

Table 2.6 Procedure for D-glucose measurement.

Pipette into each well	Volume (µL)
Solution 1 (buffer)	70
Standard/sample	10
Ultrapure water	110
The solutions (A0) were measured during 5min (six kinetic cycles) with shaking in the beginning for 120s and between cycles for 10s. The reaction was started by addition of:	
1/5 diluted enzyme suspension 2	10
The solutions (A1) were measured during 30min (31 kinetic cycles) with shaking in the beginning for 120s and between cycles for 10s	

D-glucose calculation

The data obtained from the program Gens version 2.09 was exported to Excel. D-glucose concentration was calculated by the average of 5 stable cycles in each well, for the A0 and A1 of each standard and sample, having in account the Lambert-Beer's Law which indicates that A1-A0 is proportional to concentration.

2.2.2.2.5 Turbidity

Turbidity is the degree to which the water loses its transparency due to the presence of suspended particles [81]. The turbidity measurement was carried out for 1% of DM of SN for a total volume of 2mL in a spectrophotometer with and OD=660nm.

In some cases the procedure was applied for 0.8%, 0.5%, and 0.2% of DM of SN.

2.2.2.2.6 D-Glucose Oxidation and D-Mannose Measurement

D-glucose oxidation and D-mannose measurement were based on Megazyme method [82, 83] D-Glucose oxidation was performed before D-Mannose assay to remove D-Glucose excess.

2.2.2.2.7 D-Glucose oxidation

Preparation of reagents used for D-Glucose oxidation

- Sodium phosphate (300mM, pH 7.6) plus 5mM $MgCl_2$:

This buffer was prepared adding 13.35g of disodium hydrogen phosphate dehydrate ($Na_2HPO_4.2H_2O$) to 225mL of ultrapure water water and dissolved by stirring. Then 0.2775g of $MgCl_2$ was added and dissolved. The pH was adjusted to 7.6 with 1M NaOH and the volume was adjusted to 250mL with ultrapure water water. Afterwards the solution was stored in a well-sealed Duran bottle.

- One vial with Glucose oxidase (12,000U) plus catalase (300,000U):

The contents of the vial were dissolved in 20mL of the buffer with Sodium phosphate (300mM, pH 7.6) plus 5mM $MgCl_2$. The solution was storage into 2.0mL aliquots.

Experimental details

The reaction was prepared, pipetting 0.5mL of 300mM Sodium phosphate buffer (pH7.6+$MgCL_2$), 0.5mL of sample solution (YCW) and 0.02mL of enzyme solution (up to approx. 5mg/mL D-glucose). Then the Eppendorf tubes were incubated at 25°C, with a shaking of 160rpm overnight for the samples with a concentration of reducing sugars bigger than 10mg/mL and for those values below than 10 mg/mL, the incubation was done for 2h.

After the reaction, to inactivate the glucose oxidase and catalase, the 2mL Eppendorf tubes were incubated at 98°C for 10min.

2.2.2.2.8 D-Mannose measurement

Preparation of samples

- The bottle with solution 1 had a buffer (12mL, pH7.6) plus sodium azide (0.02% (w/v)) as a preservative.
- The bottle with solution 2 had $NADP^+$ plus ATP.
- The bottle with suspension 3 had 1.2mL of hexokinase plus glucose-6-phosphate dehydrogenase suspension.
- The bottle with suspension 4 had 2.4mL of phosphoglucose isomerase plus phosphomannose isomerase suspension.

Experimental details

For the determination of D-mannose was followed the procedure explained bellow in Table 2.7. The samples were detected at 340nm and null was read against air (without a cuvette in the light path) at room temperature, using a glass cuvette with 1.00cm light path.

Table 2.7 Procedure for D-mannose measurement.

Pipette into cuvettes	**Blank**	**Sample**
distilled water	1.05mL	1.00mL
sample solution	-	0.05mL
solution 1 (buffer)	0.10mL	0.10mL
solution 2 ($NADP^+$/ATP)	0.10mL	0.10mL
The solutions (A1) were mixed and the absorbances read after ~ 3min. The reaction was started by addition of:		
suspension 3 (HK/G-6-PDH)	0.01mL	0.01mL
The solutions (A2) were mixed and the absorbances read after ~ 5min. Then was added:		
suspension 4 (PGI/PMI)	0.02mL	0.02mL
The solutions (A3) were mixed and the absorbances read after ~ 20min.		

D-mannose calculation

The absorbance differences (A_2-A_1) were determined for both, blank and sample. The absorbance difference of the blank was subtracted from the absorbance difference of the sample in order to achieve $\Delta A_{D\text{-glucose}}$:

(2) $\Delta A_{D\text{-glucose}} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$

The absorbance differences (A_3-A_2) were determined for both, blank and sample. The absorbance difference of the blank was subtracted from the absorbance difference of the sample in order to achieve $\Delta A_{D\text{-mannose}}$:

(3) $\Delta A_{D\text{-mannose}} = (A_3\text{-}A_2)_{sample} - (A_3\text{-}A_2)_{blank}$

The measured absorbance differences were at least 0.100 absorbance units to achieve sufficiently precise results.

D-mannose was determinate according to the general equation for calculating the concentration:

(4) D-mannose concentration (g/L) $= \left(\frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{D-mannose}\right) \times F$

- V (final volume) = 1.28mL
- MW (molecular weight) = 180.16g/mol
- ε (extinction coefficient of NADPH at 340nm) = 6300 (L. $mol^{-1}.cm^{-1}$)
- d (light path) = 1cm
- v (sample volume) = 0.05mL
- F (dilution factor)

2.2.2.2.9 Molecular weight analysis of carbohydrates

The MW profiles were carried out with HPLC with size exclusion chromatography (SEC) and a refractive index detector (RI) used to detect carbohydrates, according to an Ohly GmbH standard procedure (Table 2.8).

Size exclusion chromatography (SEC), also known as gel permeation chromatography (GPC) allows the separation of molecules depending on their hydrodynamic size and shape, using a column containing different porous. Large molecules (with a diameter greater than that of the pores) are excluded and eluted first. Small and medium sized molecules are eluted later; their migration is retarded by their inclusion in the gel. There is a linear relationship between the elution volume and the MW logarithm [84].

Fractionated pullulan standards (200kDa, 100kDa, 50kDa, 20kDa, 10kDa, 5kDa), β-(1,3)-laminaripentose (0.0059kDa) and β-(1,3)-laminarbiose (0.0022kDa), were used to get a rough estimate on the molecular mass range covered. Pullulan or polymaltotriose is a linear macromolecular polysaccharide that consists of links of maltotriose; β-(1,3)-laminaripentaose and β-(1,3)-laminarbiose are linear oligosaccharides [36, 84].

Table 2.8 Conditions for MW analysis.

Eluent	ultrapure water
Flow rate	1 mL/min
Sample concentrations	Dilution 1/10, resulting in approx. 5 g/L (polysaccharide)
Injection volume	20µL
Column	PROTEEMA SEC-column
Temperature	~ 23°C
Pressure:	58 bar
Running time:	~20min
RI-Detection:	RI Detector L-2490 Hitachi, LaChrome, Elite-2490i
Wavelength Detection	Dionex Ultimate 3000 Variable Wavelength Detector Thermo Scientific, λ= 210nm
Data System:	Chromeleon 6.8 version (Dionex, GmbH)

Samples preparation

The sample SN was diluted by a factor of 1:10 using ultrapure water. Afterwards the solutions were filtered through a 1.5mL samples vials, using 0.45µm PES filters and 5mL syringe.

2.2.3 Principal Component Analysis

Principal component analysis (PCA) was kindly performed by Dr. Thibault Godard, using R program version 3.4.0 and analysed by myself. This kind of analysis is used to identify groups of attributes based on their degree of correlated behaviour and for an understanding of variance-covariance structure of the data. PCA afford a roadmap to reduce a complex data set to a smaller number of principal components and can show relationships that were not previously suspected [85, 86]. The first principal component analysis the correlation structure of a group of multivariate observations and identifies the axis along which the maximum variability in the data occurs. The second principal component is the axis along which the greatest amount of remaining variability lays subject to the constraint that the axes must be perpendicular (at right angles) to each other (i.e., orthogonal or uncorrelated). The number of principal components can never be larger than the number of observed variables [71].

2.3 Results

The present thesis study the influence of various enzymes on conversion of HYCW from *S. cerevisiae* and TYCW from *C. utilis*. As a first step those two starting materials were compared in regards to YCW composition, turbidity, viscosity, solubility, RS, free glucose and free mannose content as well as MW distribution.

2.3.1 Yeast cell wall composition

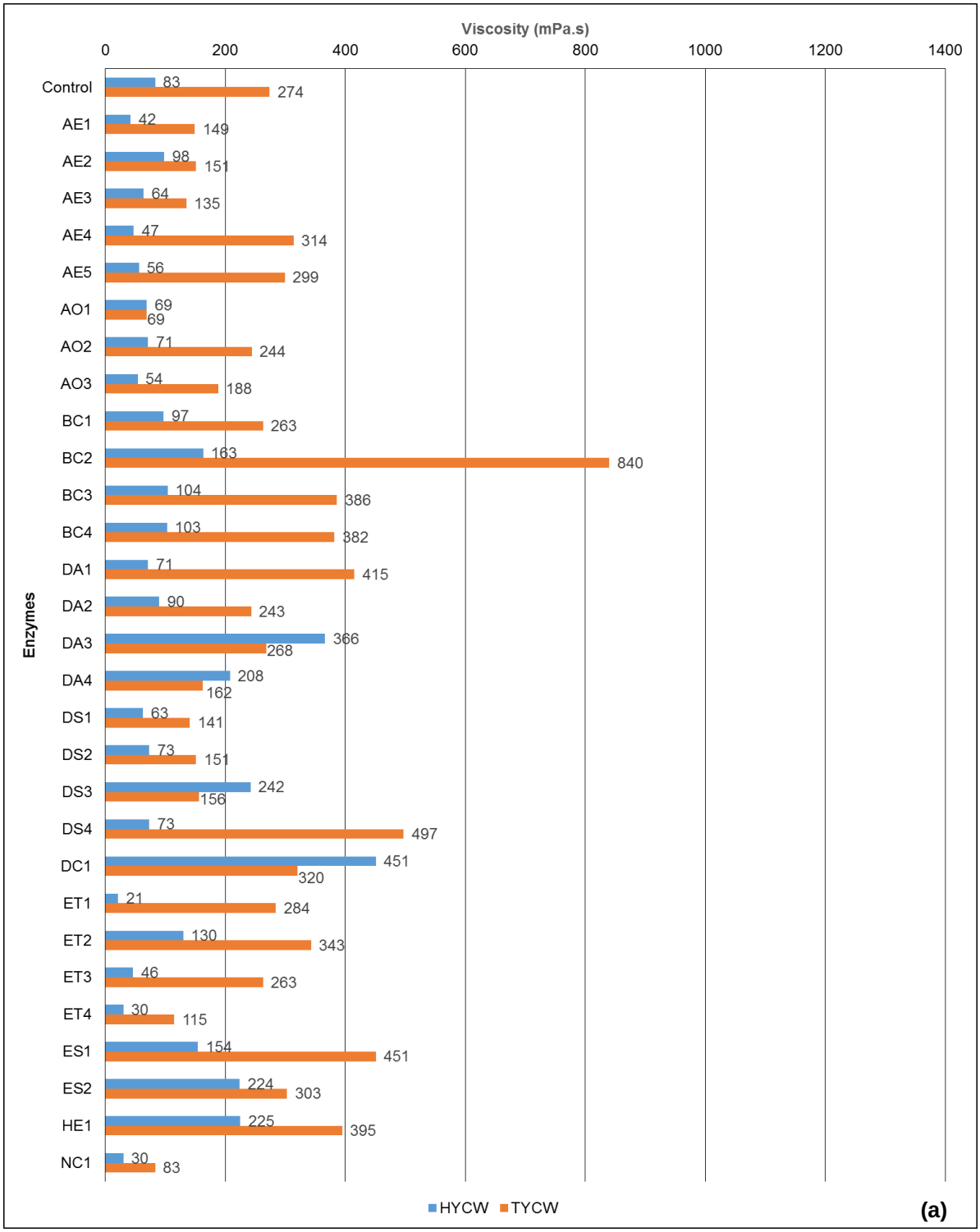
The measurement of YCW components in Table 2.9, were kindly carried out by the Quality Control Team (QC) and Dr. Mazen Rizk, from Ohly GmbH.

Table 2.9 YCW components as % (w/w).

YCW components	HYCW % (w/w)	TYCW % (w/w)
Glucan	29	9
Mannan	22	11
Protein	27	56.7
Lipid	7	10
Dry matter	97.5	97.9

Mainly TYCW has much higher protein content and lower carbohydrate content (~60% and ~20% respectively) than the HYCW (~30% and ~50% respectively). Lipids content was nearly the same for both YCW.

2.3.2 Viscosity analysis



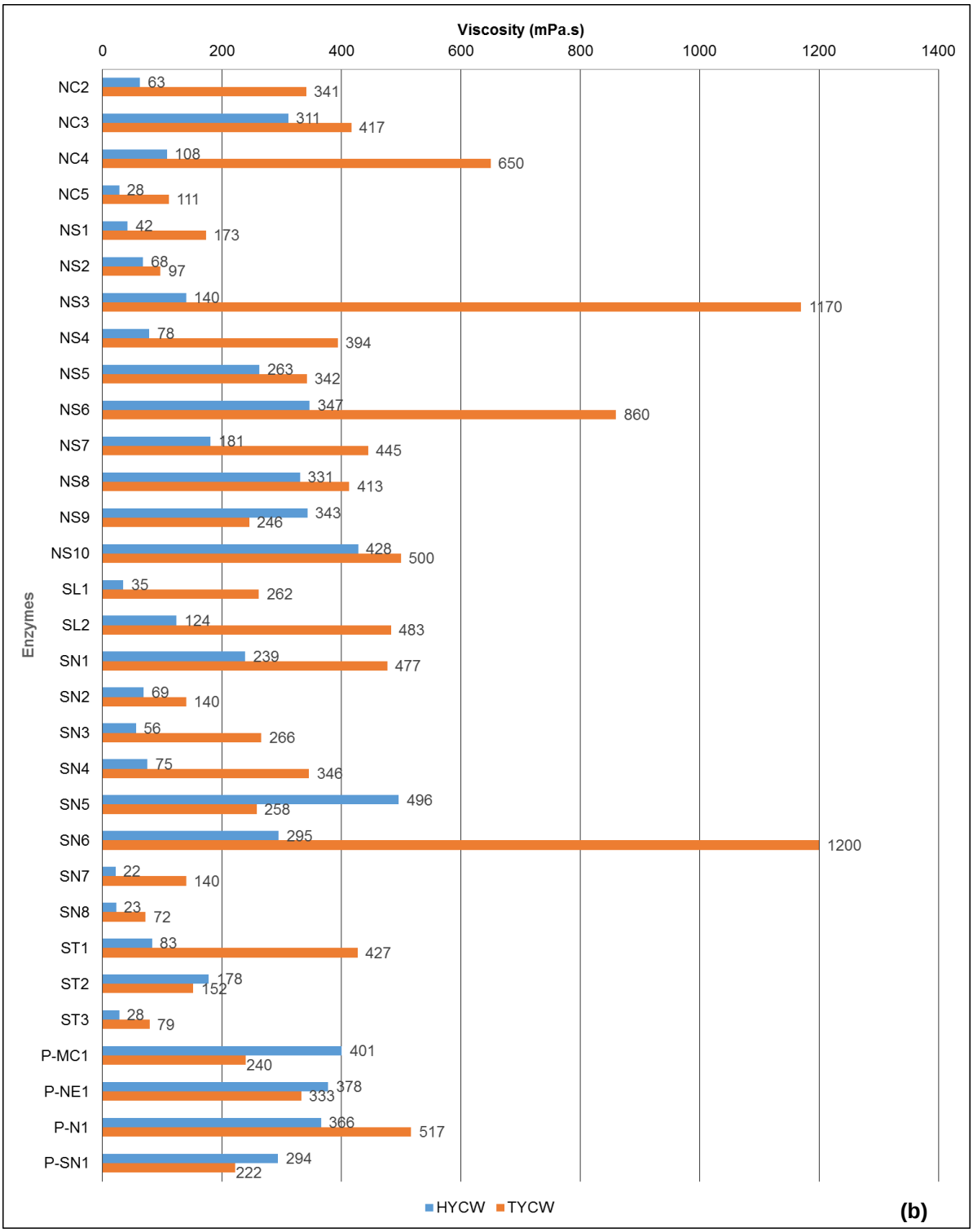


Figure 2.1 (a) and **(b)** change in viscosity (μ, [mPa.s]) of hydrolysates from enzymatic screening with HYCW and TYCW, at 25°C for 24h of reaction time. The viscosity of HYCW hydrolysates ranges from 21 to 496 mPa.s and of TYCW hydrolysates ranges from 72 to 1200mPa.s.

Viscosity measurement is used as an indicator of the biological activity of enzymatic hydrolysates (EH) and usually depends on pH, temperature, pressure, concentration and MW. It is

known that viscosity is directly proportional to the quantified MW [41, 44]. In Figure 2.1 the hydrolysate are compared in regards to their viscosity over 24h of reaction time with HYCW (starting DM 12% (w/w)) and TYCW (starting DM 21% (w/w)) as starting materials. Details about viscosity measurements are shown in Appendix 2.

Comparing with control (sample without enzymatic treatment) of HYCW with a viscosity of 83mPa.s, it was verified that some hydrolysates have a lower viscosity with a range of values from 21 (ET1) to 78 (NS4) mPa.s. Moreover, the higher results range from 90 (DA2) to 496 (SN5) mPa.s.

Comparing TYCW hydrolysates with control with a viscosity of 274mPa.s the lowers values range from 72 (SN8) to 268 (DA3) mPa.s and the highest values from 284 (ET1) to1200 (SN6) mPa.s.

In essence the results of viscosity from HYCW hydrolysates ranges from 21 (ET1) to 496 (SN5) mPa.s and for TYCW hysdrolysaes ranges from 72 (SN8) to 1200 (SN6) mPa.s. The results show that we have a higher viscosity when we use TYCW as starting material which is an expectable result because it was used a higher starting DM. The results suggest that mostly all enzymes used during the assay have a similar viscosity profile when reacting with different YCW, e.g. SN8, ST3, SN2, NS2, NC5, NC1 or AO1 have lowers values of viscosity and hydrolysates as DC1, DA3, NC3, NS5, NS6, NS8 or P-N1 exhibit high viscosity.

2.3.2 Turbidity

The more total suspended solids in the samples, the murkier it seems and the higher the turbidity, which is associated a higher absorbance.

Table 2.10 Turbidity range values of hydrolysates from enzymatic screening with HYCW and TYCW for 3h and 24h of reaction time at λ= 660nm.

	HYCW		**TYCW**	
	3h	**24h**	**3h**	**24h**
Lowest values of turbidity	0.041 (NC5)	0.041 (P-N1/NS10)	0.056 (NC5)	0.057 (P-MC1)
Control	0.268	0.262	0.194	0.313
Highest values of turbidity	0.968 (NC3)	0.677 (SN8)	0.380 (BC4)	0.713 (NS6)

() enzyme code

Table 2.10 shows the range of values for turbidity. Comparing HYCW hydrolysates with control, the turbidity values were in the range of 0.041 (NC5) - 0.968 (NC3) for 3h and 0.041 (P-N1/NS10) - 0.677 (SN8) for 24h. In TYCW case, hydrolysates were also compared with a control, the turbidity values were in the range of 0.056 (NC5) - 0.380 (BC4) for 3h and 0.057 (P-MC1) - 0.713 (NS6) for 24h.

Appendix 3 and 4 shows all the turbidity measurements performed for hydrolysates for both starting materials. The samples with a higher turbidity for HYCW were NC3 (0.968), DS3 (0.892), P-

SN1 (0.723) for 3h and SN8 (0.677), AO2 (0.643), NS8 (0.478) for 24h. The ones with a lower turbidity were NC5 (0.041), ST2 (0.050) for 3h and P-N1/ NS10 (0.041) for 24h. After 24h of reaction HYCW hydrolysates seem to be less turbid than at 3h.

In TYCW case, the samples with a higher turbidity were BC4 (0.380), ET1 (0.362), NS4 (0.303) for 3h and NS6 (0.713), ST3 (0.598) for 24h. The ones with a lower turbidity were NC5 (0.056), AO1 (0.058) for 3h and P-MC1 (0.057), NC5 (0.064) for 24h. TYCW hydrolysates seem to be a bit more turbid at 24h than at 3h. The turbidity profile for both materials is similar for 24h of reaction time, having some differences at 3h.

2.3.3 Free sugar release and molecular weight analysis

For a better understanding of YCW composition and enzymatic conversion, the hydrolysates were characterized for 3h and 24h over reaction time, the results were plotted in Appendix 5 and 6, in percentage of amount of total sugar in the SN. The total glucose and mannose contents in the supernatant was previously determined through acid hydrolysis. The total glucose and mannose contents of HYCW is ~29% (w/w) and ~22% (w/w) respectively and of TYCW is ~9% (w/w) and ~11% (w/w), respectively.

The results suggest a resemble profile for the both reaction times used during the assay, having only a highest release of sugars over 24h of reaction time. The total sugar released at 3h of HYCW hydrolysates range from ~0.3% ~to ~27.5% (w/w) and of TYCW hydrolysates from ~0.3% to ~25.5% (w/w). At 24h of reaction time the profile of HYCW range from ~0.5% to ~54.5% (w/w) and of TYCW from ~0.3% to ~37.5% (w/w).

HYCW and TYCW have a resembling profile when reacting with the same enzymes.

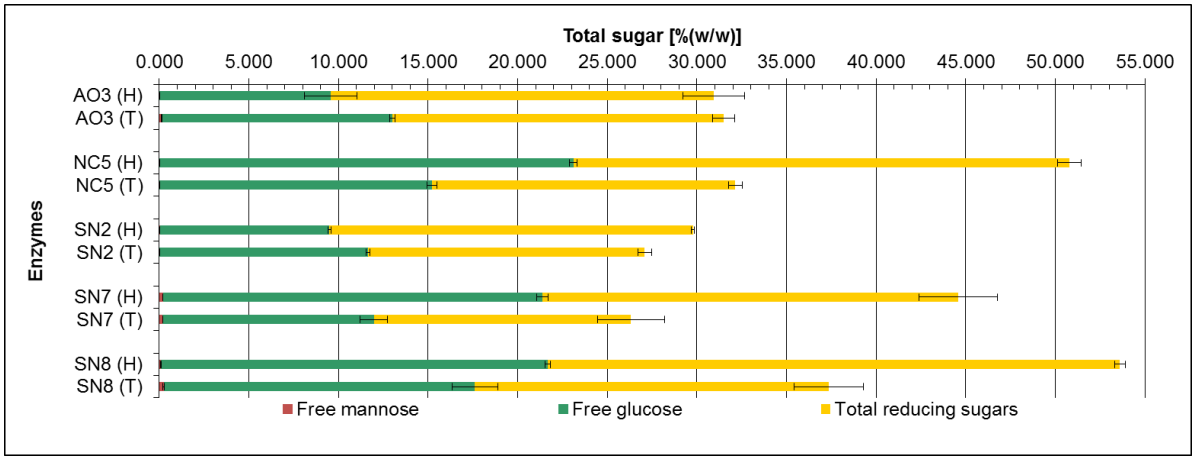


Figure 2.2 Action of various enzymes that have a high sugar and glucose release, using HYCW (H) and TYCW (T) as starting materials for 24h over reaction time.

Various enzymes as AO3, NC5, SN2, SN7 and SN8 exhibited a very high activity, in terms of RS and free glucose release. Figure 2.2 illustrates a couple of examples. With this group of enzymes, glucose represents around half of the total amount of the free RS. The other half of the RS is larger oligosaccharides.

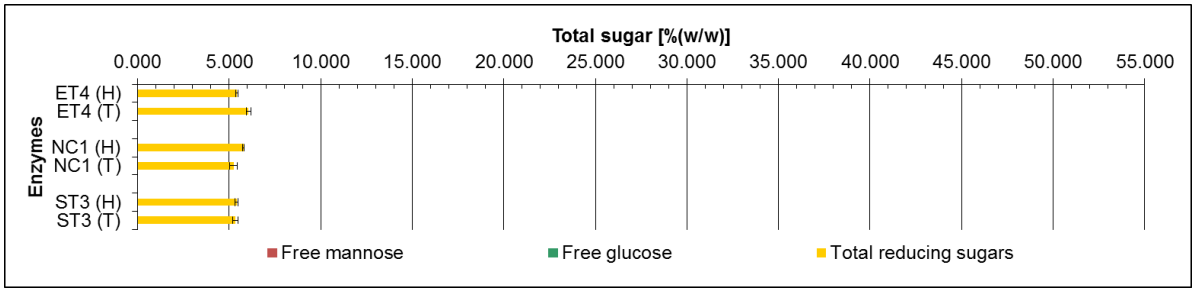


Figure 2.3 Action of 3 laminaripentaose hydrolases, that have high reducing sugar release, but no glucose release, using HYCW (H) and TYCW (T) as starting materials over 24h of reaction time.

No free glucose is released with NC1, ET4 and ST3 (Figure 2.3). Those enzymes always release a β-laminaripentaose and have similar kinetics under the same conditions, therefore it is assumed that all three of them have the same enzymatic activity. The majority of the RS is either β-laminaripentaose or mannan oligosaccharides released as mannoproteins into the SN.

The tested enzymes almost do not release free mannose. The reason for that is that commercial mannanases used in this screening are only able to break down β-bonds. YCW however is composed of mannan linked by α-bonds.

The standard deviation (represented by the error bars) has an acceptable error which means that the assays performed to measure free glucose, mannose and RS content are consistent and the values represented are very close to the real value.

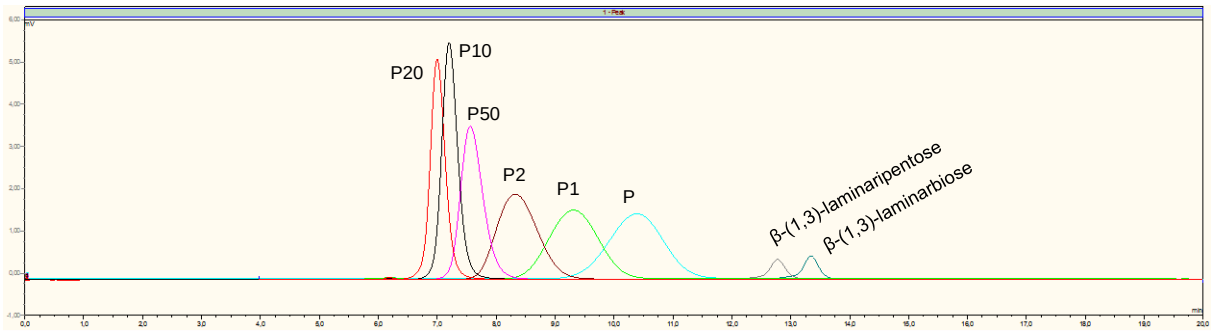


Figure 2.4 Separation of 8 different molar mass reference standards: (red) Pullulan 200kDa ; (black) Pullulan 100kDa; (pink) Pullulan 50kDa; (brown) Pullulan 20kDa, (green) Pullulan 10kDa (cyan) Pullulan 5kDa; following the β-(1,3)-laminaripentose and β-(1,3)-laminarbiose (0.99-0.0022kDa).

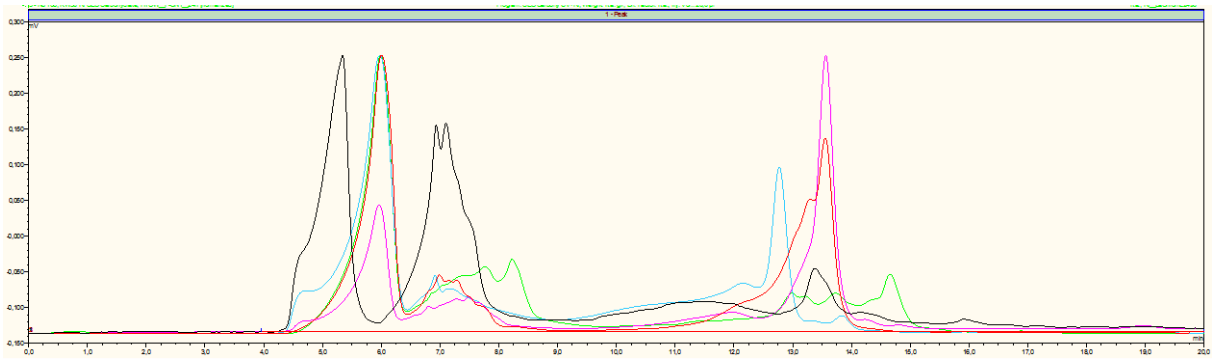


Figure 2.5 MW distribution of different hydrolysates using HYCW as starting material. The different substances in the standard solution have different retention times. Most of the detected peaks of the sample are recognized, only a few minor peaks are not identified. These are secondary components and impurities which are a part of the sample matrix. MW distribution of different hydrolysates (black) which is the Control; (red) AE3 resulted in a mid-yield of RS and glucose; (pink) SN7 resulted in a high yield of RS and free glucose; (cyan) NC1 resulted in a high yield of RS and no free glucose; (green) P-SN1 resulted in a low yield of RS and free glucose.

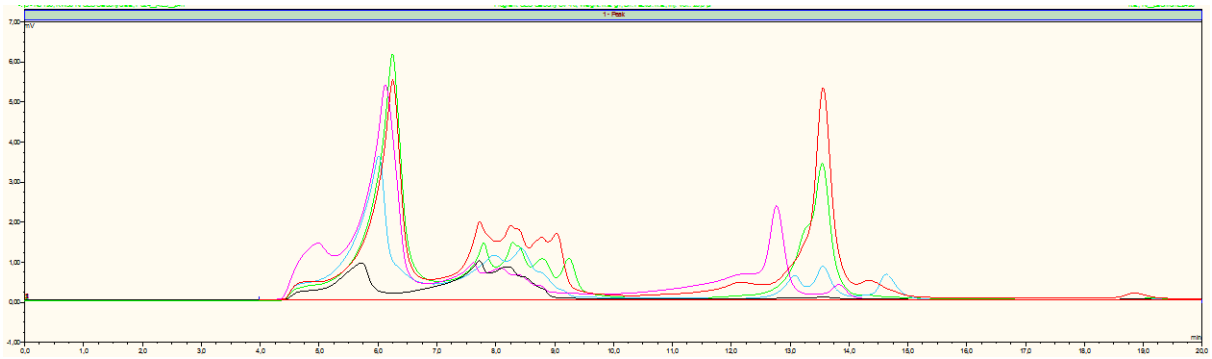


Figure 2.6 MW distribution of different hydrolysates using TYCW as starting material. The different substances in the standard solution have different retention times. Most of the detected peaks of the sample are recognized, only a few minor peaks are not identified. These are secondary components and impurities which are a part of the sample matrix. MW distribution of different hydrolysates (black) which is the Control; (red) SN7 resulted in a high yield of RS and free glucose; (pink) NC1 resulted in a high yield of RS and no free glucose; (cyan) P-N1 resulted in a low yield of RS and free glucose; (green) AE3 resulted in a mid yield of RS and glucose release.

Regarding the MW distribution, the SN from the various hydrolysates was analysed per HPLC-SEC. Figure 2.4 present the resulting chromatograms for pullulan (5-200kDa), β-laminaripentose and β-laminarbiose standards (0.99-0.0022kDa), used to compare with the enzymatic hydrolysates. An overlay of the samples chromatograms is given in Figure 2.5 for HYCW and in Figure 2.6 for TYCW, 4 samples were compared to one another that have different profile: (i) AE3 resulted in a mid-yield of RS and glucose; (ii) SN7 resulted in a high yield of RS and free glucose; (iii) NC1 resulted in a high yield of RS and no free glucose; (iv) P-SN1 and P-N1 resulted in a low yield of RS and free glucose.

SN7 has a peak around 13.5 minutes which corresponds to when glucose is detected. NC1 does not release any free glucose, instead a molecule slightly bigger, which corresponds to a glucose-pentaose (β-laminaripentose). Both SN7 and NC1 have higher peak areas throughout the

chromatogram than P-SN1or P-N1. This shows also that the former have a much higher content of soluble material than the latter.

2.3.4 Solubilisation and phase separation

Usually mono- and disaccharides have high degrees of solubility. Solubilisation represents the amount of material from the insoluble starting material that was solubilized into the supernatant at a given temperature [87, 88]. The DM of the SN from HYCW control is ~1.2% and from TYCW control is ~2.4%. After enzymatic reaction, the DM of the SN of the various hydrolysates increases in direct correlation with the amount of material solubilized as it is possible confirm in Appendix 7. In some cases, the volume of the insoluble pellet also significantly decreases and that of the supernatant consequently increases. The solubilisation profile is similar for both starting materials when using the same enzyme.

Table 2.11 Sugar release profile of selected few samples that exhibit different solubilisation rates.

Enzymes	Sugar release profile in hydrolysate	Solubilisation
SN7	High reducing sugar / high free glucose yields	High
NC5	High reducing sugar / high free glucose yields	High
SN8	High reducing sugar / high free glucose yields	High
ST3	High reducing sugar / no free glucose yields	High
ET4	High reducing sugar / no free glucose yields	High
NC1	High reducing sugar / no free glucose yields	High
NS1	Low reducing sugar / low free glucose yields	Low
ET2	Low reducing sugar / low free glucose yields	Low
P-NE1	No reducing sugar / no free glucose yields	None

NC1, ET4 and ST3 have the highest solubilisation rate. With those 3 enzymes it seems like the pellet was completely ‘liquefied’. What is intriguing about this cluster of enzymes is that they are able to completely solubilize the pellet resulting in almost no insoluble matter at the end of the hydrolysis time. This was not observed with any of the other enzymes.

Table 2.11 shows a list of selected enzymes that have various solubilisation rates (either high or low). Enzymes that have very high RS releases also have a high solubilisation rate. On the other hand, enzymes that did not have a high RS release did not result in a very high solubilisation rate. This means, that those enzymes mixtures were not able to hydrolyse the YCW. It is not expected for the hydrolysates SN to be rich in larger non-RS or proteins.

2.3.5 Principal Component Analysis (PCA)

Over 60 enzymes were used to hydrolyse the HYCW and TYCW. Based on the aforementioned results, a PCA analysis was performed on the autoscalled data and K-means clustering with 95 % confidence ellipses was performed on those results. Combining PCA with clustering allows improve data visualization. Principal component methods can be used as pre-processing step for noise reduction of the data, to transform categorical variables in continuous variables [89].

Hierarchical trees, represented by a dendrogram, are often used to illustrate the arrangement of the clusters produced by decomposition of total inertia (total variance) in between and within-group variance. The within inertia characterizes the homogeneity of a cluster. The number of clusters can then be chosen looking at the overall appearance (or the shape) of the tree and the bar plot of the gain in within inertia [89].

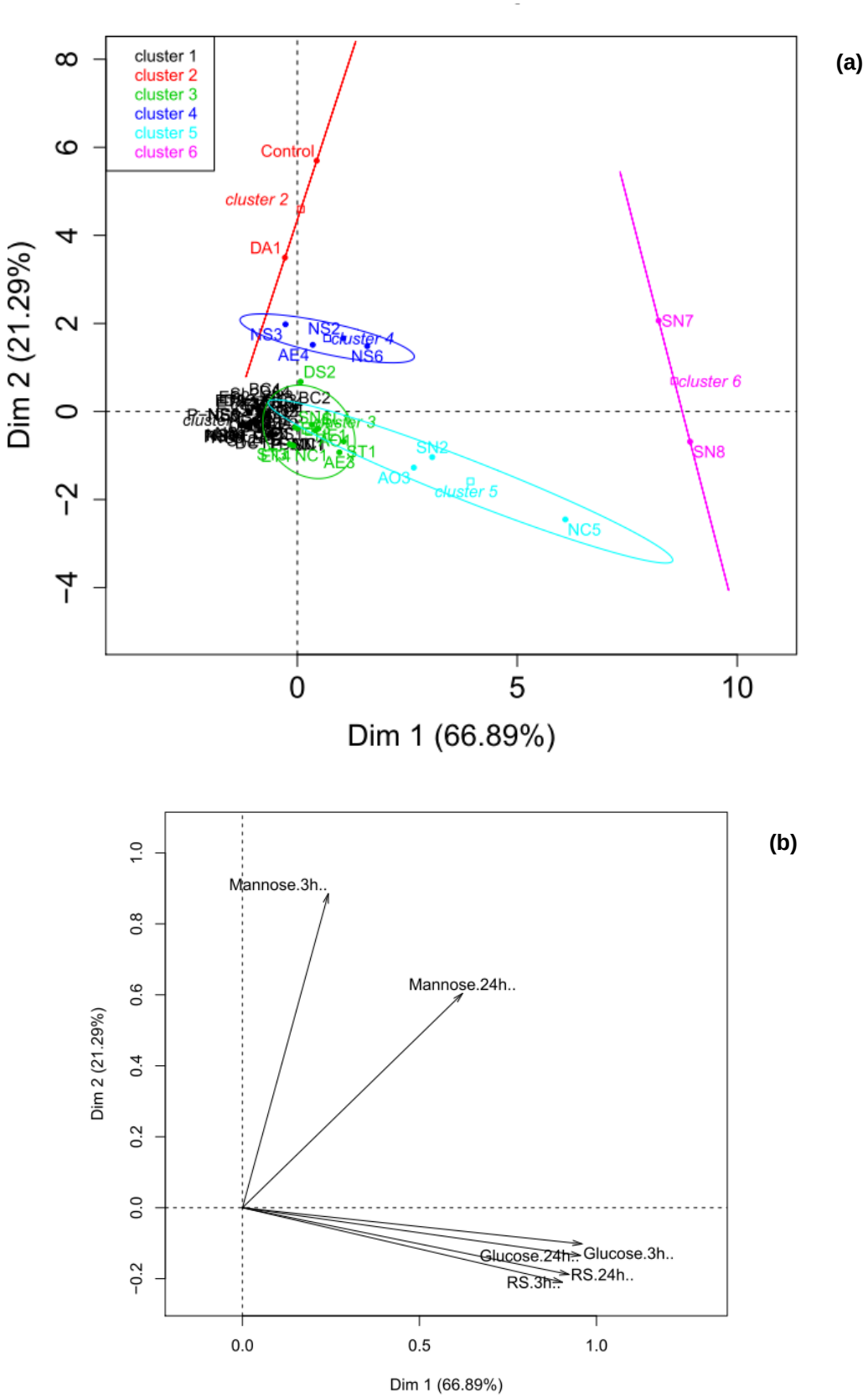


Figure 2.7 Autoscaling representation of global clusters of HYCW **(a)** induced by the first two principal components **(b)** in variables factor map (PCA).

The hierarchical clustering for HYCW starting material was done based on inertia gain (Appendix 8, Figure 8.1). The inertia gains shows that the tree can be cut in six clusters because even if we had made more clusters the main groups are already demarcated and the following groups will be similar. Obtained clusters will help us to characterize screened enzymes according with hydrolysates profile.

The partitioning in six clusters is represented on Figure 2.7 **(a)** and the two components related with the variables are represented on Figure 2.7 **(b)**. The barycenter of each cluster is represented by a square. The arrows indicate the correlation between one variable and the first two principal components [23].

In this case, the first principal component explains 66.89 % of the total variation and the second principal component an additional 21.29 %. So the first two principal components explain nearly ~ 88% of the total variance. The variables free glucose and RS correlate almost perfectly with the first component, while the variable free mannose correlates with the second component. Cluster 5 and 6 scores high on component 1. Cluster 2 and 4 seems be more connected with component 2.

In a simplistic overview, we could say that the following hydrolysate properties and enzymes are coloured according to their cluster:

Cluster 1 in **black** and cluster 3 in **green** are pretty similar: mid/low RS and mid/low free glucose and free mannose release (e.g. BC2 DS2, AE3 and NC1);

Cluster 2 in **red**: high free mannose release compared with the others groups, beside that it is important to remember that mannose concentration remains relatively low (e.g. Control and DA1);

Cluster 4 in **purple**: a bit more of free mannose release comparing with cluster 2, low RS and mid glucose release (e.g. NS3, AE4, NS2 and NS6);

Cluster 5 in **cyan**: no mannose release, high RS and free glucose release (e.g.SN2, AO3 and NC5);

Cluster 6 in **pink**: a bit of mannose release, very high RS and free glucose release comparing with the others groups (e.g. SN7 and SN8).

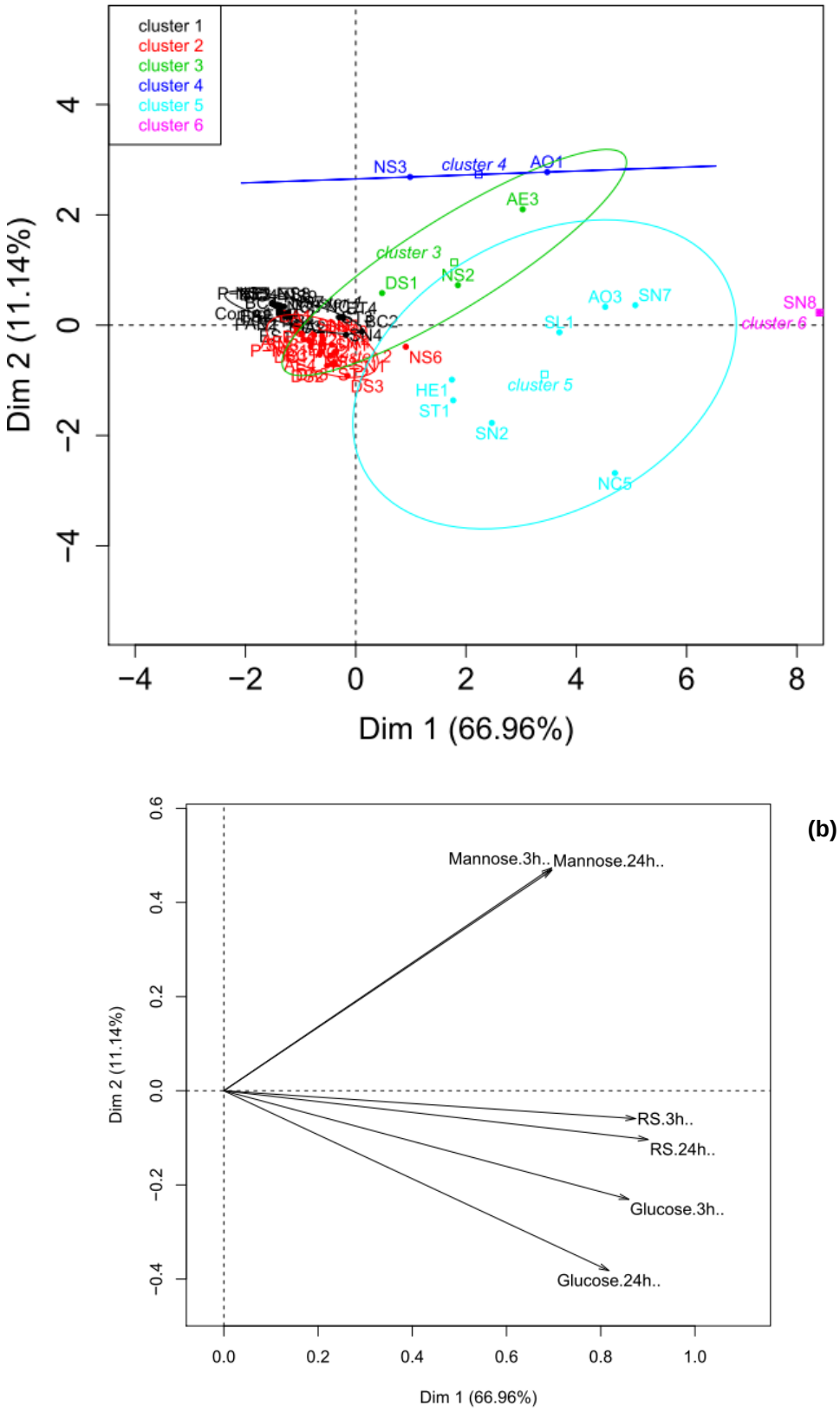


Figure 2.8 Autoscaling representation of global clusters of TYCW **(a)** induced by the first two principal components **(b)** in variables factor map (PCA).

The hierarchical clustering for TYCW was done, based on inertia gain (Appendix 9, Figure 9.1). The inertia gain show that the tree can be cut in six clusters as it was explained above for hierarchical clustering of HYCW.

The first principal component explains 66.96 % of the total variation and the second principal component an additional 11.14 %. So the first two principal components explain nearly ~ 78% of the total variance. RS and free glucose correlates almost perfectly with first component and the variable free mannose correlates with the second component.

Generally we could say that the following hydrolysate properties and enzymes are coloured according to their cluster (Figure 2.8):

Cluster 1 in **black** and cluster 2 in **red** are pretty similar: mid/low RS and mid/low free glucose and free mannose release (e.g. BC2, DS3 and NS6);

Cluster 3 in **green**: bit more of free mannose release comparing with cluster 4, low RS and mid glucose release (e.g. DS1, NS2 and AE3);

Cluster 4 in **purple**: high free mannose release compared with the others groups, beside that it is important to remember that mannose concentration remains relatively low (NS3 and AO1);

Cluster 5 in **cyan**: high RS and free glucose release (HE1, ST1, SN2, SL1 and NC5). Enzymes AO3 and SN7 release a bit of free mannose beside the same profile release;

Cluster 6 in **pink**: no free mannose release, very high RS and free glucose release (e.g. SN8).

2.4 Discussion and Conclusion

2.4.1 Yeast cell wall composition

During this thesis, YCW components for *S. cerevisiae* (HYCW) and *C. utilis* (TYCW) were analysed. The results have shown higher protein content and lower carbohydrate content (~60% and ~20% respectively) for TYCW and for HYCW around 30% of proteins and ~ 50% of carbohydrates. Lipids content was nearly the same for both YCW. As mentioned before, in Section 1.3, some studies have shown a resemblance of YCW model for both yeasts [12, 24]. Moreover, a report done by Miguel Otero *et al.* (2011) have shown an average composition as percentage per DM similar to the one that we have achieved, concluding the same. *C. utilis* YCW has a higher protein content and lower carbohydrate content than *S. cerevisiae* YCW (See Sections 1.2 and 1.3) [15, 21]. Moreover, the lipids amount can be explained by the similar lipids distribution on CW of those two different species (Table 2.12) [90].

Table 2.12 Lipids distribution on CW of *S. cerevisiae* and *C. utilis* expressed as percentage of the total lipids [90].

Yeast	Hydrocarbons (%)	Sterol Esters (%)	Triacylglycerols (%)	Fatty acids (%)	Sterols (%)	Phospholipids (%)
S. cerevisiae	-	16.0	25.0	6.0	3.0	49.0
C. utilis	-	52.8	11.2	-	5.6	30.4

2.4.2 Hydrolysates analytical characterization and release of sugars

Hydrolysates obtained from enzymatic reactions with HYCW and TYCW were analysed in order to have a fundamental understanding on the effect that various enzymes have on YCW conversion and which are the most interesting value-added products for possible applications in food and/or fermentation. Hydrolysates differed in physico-chemical properties, i.e., viscosity, turbidity, solubility, MW and sugars release. These properties have a significant influence on the biological activity of these hydrolysates which is important to develop applications in the future.

Viscosity, resistance to flow, is defined as the ratio of shear stress to shear rate. Usually polysaccharides solutions are associated to non-Newtonian flow, relating an increased shear rate to an increase or decrease of viscosity [91, 92]. Viscosity is highly influenced by the MW and solubility of β-glucan, a lower MW and/or solubility of β-glucan are expected to reduce its resultant viscosity [12, 17, 22, 93]. Viscosity is also associated to the porous matrix structure formed by polysaccharide chains, which can hold large amounts of water through hydrogen bonds [94]. Moreover, the decrease of viscosity of yeast β-glucan by heating and an increase by cooling was reported previously [91, 95, 96]. Byun *et al*. (2008) showed when there is an increase of gamma irradiation absorbed, a significant decrease in viscosity was observed [91, 97].

During this enzymatic assay, parameters as pH, temperature, shaking and enzyme dosage were the same for all enzymes screened. Temperature and pH and were defined according to the enzyme specifications data sheet, attached in Appendix 15. Some authors described that high pH may cause an unfavourable effect on viscous properties of extracted β-D-glucan due to the sensitivity of β-(1,3) bond to high pH, but in our case we did an enzymatic treatment which is a relatively less harsh treatment and it may responsible to kept the (1,3)-linkages intact, explaining the medium/higher viscosity in a general way [98]. Comparing with the respective control, it was verified that some hydrolysates had a higher viscosity ranging from 90 to 496mPa.s in the case of HYCW. Moreover, for TYCW the highest values range from 284 to 1200mPa.s. Regand *et al*. reported some average of physicochemical properties of β-glucans from starch, showing a high viscosity for values of ~429-796mPas at $30s^{-1}$, a medium viscosity for values between ~37 to 66mPas at $30s^{-1}$ and a lowest viscosity between ~4 to 13mPas at $30s^{-1}$, comparing with our results the range of values obtained are higher than these oat values which means that we have some hydrolysates with good potential use for applications [99]. Accordingly, TYCW seems to be more viscous than HYCW which can be explained by the starting DM used in reactions with both materials, ~21 % (w/w) and ~12% (w/w) respectively. However, most enzymes screened for both starting materials have shown a similar profile for viscosity during the assay.

From all enzymes screened, the results suggest a higher viscosity for DC1, DA3, NC3, NS5, NS6, NS8 and P-N1. Those enzymes have shown a medium/low rate of solubilisation and a low release of sugars comparing with others enzymes, except NS6 which shows some release of RS and free glucose. In a general way, we can conclude that these enzymes have a medium enzyme activity leading to a medium/low digestion of YCW and consequently the polysaccharides remained as non-degradable, explaining the high viscosity of the hydrolysates. A higher viscosity can be associated to a very strong intermolecular associations within the same molecule or with intermolecular association with other constituents of the hydrolysates [100]. Intermolecular interactions between β-(1,3)-D and β-(1,6)-D-glucan bindings are associated to a weak hydration of complex structure of helix of high MW β-glucans.

Enzymatic digestion can lead to the hydrolysis of long-chain of β-glucans, allowing a lower the degree of depolymerisation and their particle mass in relation to native form, which simultaneously increases their solubility and lowers viscosity in liquids. Thus, may explain our lowers results of viscosity as e.g. SN7, NC5, SN8, ST3, ET4 or NC1 [42]. The first 3 enzymes have shown the highest release of RS and free glucose and the last 3 had no free glucose and a high release of RS. All six enzymes had a high solubilisation rate associated to a high enzyme activity and YCW degradation [42, 44]. Lowering of DP of β-glucans with β-(1,3)/(1,6)-D-glucan bindings to DP below 20, results in the weakening of intermolecular interactions, leading to new bindings between β-glucan particles and solvent, causing its dissolution [42]. The presence of non-β-D-glucan components in the extracted β-D-glucan can be another important factor responsible for change in viscosity and perhaps associated to some lowest results in case of enzymes that are not pure and can release more than β-glucans [98].

Fractions with a MW between 100 to 200 kDa which exhibit a single and/or triple helix conformations have shown high biological activity, whereas a fraction from the same source with a low MW (5 to 10 kDa) have shown a limited activity [41, 44] (See Section 1.3.2). Some studies demonstrated that β-D-glucans having the same MW differ significantly in their biological potency depending on whether they are in single or triple-helical form. The single helical conformation of β-D-(1,3)-glucan was a contributor to the activation of limulus coagulation factor G. However, a study of correlation between antitumor activity, MW and conformation of lentinan demonstrated that samples with a lower MW had higher activity in vitro, while higher MW samples were more active in vivo [41]. As it was mentioned before a higher viscosity, turbidity and solubility are usually associated to a higher MW. However, the resolution in the high molar mass region was not sufficient to resolve the oligomer with a low molecular weight into single chains. It eluted like the higher molar masses do, in just one peak. In Figure 2.5 and Figure 2.6, section 2.3.4, it is possible to observe big areas between 200-5 kDa which does not correspond to reality. For a correct resolution we will need a different column with a resolution for molecules with low molar mass [101]. Regarding this information it was decided to not calculate the MW of the samples and just provide a profile overview. The MW profile, indicates three (ST3, ET4 and NC1) enzymes producing a molecule that corresponds to the same size as β-(1,3)-Laminaripentose.

Solubilization and phase separation displayed a profile of the water-soluble fraction (SN) which were the hydrolysates containing reducing and free sugars and the non-water-soluble fraction (pellet) which is mainly the remaining YCW. Accordingly, using the DM and SN volume was possible to quantify the solubilised content and sugar release over reaction time [102]. Samples with higher solubilization and sugars release were SN7, NC5, SN8, ST3, ET4 or NC1 having a good potential for possible applications. It was found out when COOH is substituted the solubility increases [91, 92]. Being a possible explanation for SN7, NC5 and SN8 RS results. ST3, ET4 or NC1 do not release any free glucose instead a glucose-pentaose. They result in a high RS release and are also able to ‘liquefy’ the pellet which probably means that all material was solubilized. This loose pellet also associated to a low viscosity, probably means that the non-hydrolysed material contain a less concentration of polysaccharides or small oligosaccharide chains. The oligosaccharides generated by those enzymes may also have decreased the aggregation tendency of the β-glucan molecules [102].

Limited solubility associated to a decrease of SN of some samples (e.g. NS1, ET2 or P-NE1) might be explained with the presence in the structure of long, side chains with bindings β-(1,6), which can cause high crystality and insolubility of this β-glucan [42]. Borchani *et al*. (2016) related insoluble polysaccharides with water binding capacity and swelling power in oat beta-glucan when the molecule was partially hydrolysed [103]. Therefore, this could be a hypothesis for the decrease of SN volume. For example, Byun *et al.* (2008) demonstrated that the water solubility of β-glucan from *Aureobasidium sp.* increased by the increase of absorbed dose of gamma irradiation [91, 92]. Gama irradiation is approved by FDA for uses on foods [104]. Thus, it will be an interesting procedure to complement our study in cases of low/medium solubility.

Turbidity gives a first visual impression of the quality of the product to the consumer [105]. Speers *et al.* (2003) found out that the increase in turbidity was proportional to the increase in MW, i.e., β-glucan at a size of 300 kDa increased turbidity in beer after filtration, which may be caused due to polymer aggregation and larger particle sizes [105, 106]. The effect of pH (4.0) on wort turbidity was related to the electrostatic properties of proteins with a high net positive charge that caused a high intermolecular electrostatic repulsion. As wort also contained high MW β-glucans, the increased haze was assumed to be derived from both proteins and β-glucans [106]. Our results showed a higher turbidity for NC3, DS3, P-SN1 for 3h and SN8, AO2, NS8 for 24h, in HYCW case. In TYCW case, the samples with a higher turbidity were BC4, ET1, NS4 for 3h and NS6, ST3 for 24h. Hydrolysates obtained with TYCW seem to be a bit more turbid at 24h than at 3h.The turbidity profile for both materials was similar for 24h of reaction time, having some major differences at 3h. Therefore, the samples which have an increased turbidity can be associated to the reaction pH 4.5. As in the CW the mannan-oligosaccharides are directly linked to the proteins, it is a hypothesis to assume that some protein were solubilized into SN and may be affected by the pH of the reactions leading to a turbid SN. A possible anwer to this problem will be an optimization of pH during the assay or degas/filter our samples to decrease haze [105].

Some discrepancies in the SN DM results were observed when comparing with the reducing and free sugars released. The results are may be associated to high turbidity of our samples. The volume of SN of the samples was reduced and in order to do all the measurements, the digital refractometer was the possible equipment to measure the SN DM. However, it is not an accurate instrument to measure the DM as, for instance, a moisture analyser.

^{1}H and ^{13}C NMR spectroscopy are especially powerful for structural characterization of natural (1→3)-β-D-glucans and can provide information on DP, DB and the structural features of the polysaccharides. Glucans with a DB between 0.20 and 0.33 appear to be the most active [41]. For a better understanding of enzymes characteristics and activity will be interesting to do an acid treatment that leads to the loss of fine structure of the branch-on-branch β-(1,3 or 1,6)-glucan in order to know the total sugars and the estimation of the number-average DP (defined as average number of monomeric units per molecule) which helps to find the best enzymes [37].

2.4.3 Enzymatic activity

For a better understanding of our results the main principle of the D-Glucose, D-Mannose and RS conversion will be described bellow.

D-glucose conversion

In the presence of the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP), D-glucose is phosphorylated to D-glucose-6-phosphate (G-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP) [80].

(5) D-glucose + ATP $\xrightarrow{HK}$ G-6-P + ADP

G-6-P is oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH), in the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH) [80].

(6) G-6-P + $NADP^+$ $\xrightarrow{G6P\text{-}DH}$ D-gluconate-6-phosphate + NADPH + H^+

The amount of NADPH formed in this reaction is stoichiometric to the amount of D-glucose [80].

D-glucose oxidation

The main principle of the D-glucose oxidation says that in presence of Glucose Oxidase (GOD) and oxygen from air, D-Glucose is oxidized to D-gluconate [82]:

(7) D-Glucose + O_2 + H_2O $\xrightarrow{(GOD)}$ D-gluconate + H_2O_2

The hydrogen peroxide (H_2O_2) is decomposed by a catalase:

(8) $2H_2O_2$ $\xrightarrow{(catalase)}$ $2H_2O + O_2$

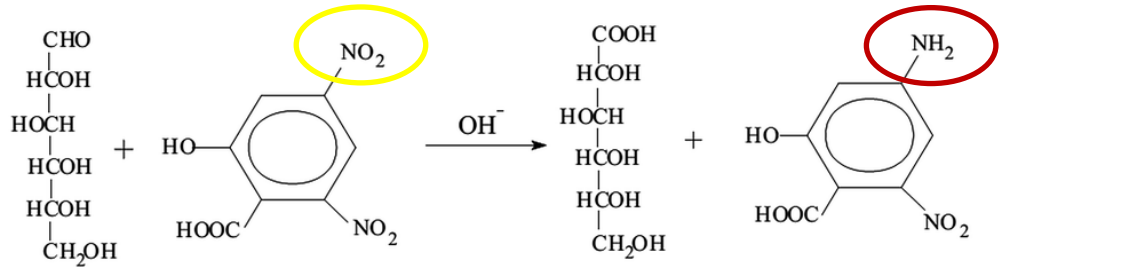
D-mannose conversion

The main principle of the D-mannose conversion starts with D-glucose phosphorylation and oxidation. On completion of reaction (6) of D-glucose conversion, M-6-P is converted to G-6-P by the PMI and PGI in the reaction mixture, leading to another further increase in absorbance that is the stoichiometric with the amount of D-mannose [84].

RS content

3,5-Dinitrosalicylic acid (DNS) reduced reagent is widely used in the measurement of RS, i.e., with free carbonyl groups (C=O), leading to a direct estimation of the number of glycosidic bond

cleavages and, consequently an estimation of enzyme activity. Monomeric sugars (e.g. glucose and mannose) are in aqueous solution in equilibrium between their open chain and ring structures nevertheless only the open structures are responsible for their reducing activity. The principle of the DNS reaction is the reduction of 3,5dinitrosalicylic acid (DNS) to 3-amino-5-nitro-salicylic acid and, simultaneously the aldehyde group of RS is oxidised to the respective carboxylic acid (Figure 2.9), under alkaline conditions. This substance builds an orange/red colourant with a high level of RS which can be detected at 530nm [107, 108].



D-glucose 3,5-dinitrosalicyclic acid Gluconic acid 3-amino-5-nitrosalicyclic acid

Figure 2.9 Conversion of 3,5-dinitrosalicyclic acid (yellow) into 3-amino-5-nitrosalicyclic acid (red-brown) by oxidation of glucose into gluconic acid as example of RS [107].

During the screening, the enzymes presented a similar profile of sugars release for both starting materials (HYCW and TYCW). Regarding the enzymatic hydrolysates, AO3, SN2, SN7, NC5 and SN8 suggest the highest release of RS and free glucose.

Trichoderma reesei is a producer of cellulose and hemicellulose degrading enzymes as SN2. AO3 is an endo-(1,4)-β-D-xylanase (EC 3.2.1.8), obtained from *Aspergillus niger*, which degrade the linear polysaccharide β-(1,4)-xylan into D-xylose into oligosaccharides. SN2 and AO3 presented a similar profile on sugars release, indicating that probably both contain side-activities which may explain the degradation of β-(1,3/1,6)-glucans. *T. reesei* can produce many enzymes at the same time. Even if the expression of some major enzymes (e.g. cellulase) has been knocked out, they still produce other enzymes. This enzyme mixtures present side-activities which are unknown or not reported and may explain our results [109]. Endoglycosidases, which can cleave between the two GlcNAc residues in the core of the N-linked sugar chains, are reported as an intrinsic side-activity of almost all commercial xylanases and cellulases [110, 111]. This trend may explain the side-activitie of SN2 and AO3 since it was observed a high release of free glucose and RS when those enzymes were used and as it is known they are specific to degrade plant CW and not YCW [112].

SN7 is a β-(1,3)-glucanase obtained from *Penicillium* sp., can be classified into exo-β-1,3-glucanases (EC 3.2.1.58) and endo-β-1,3-glucanases (EC 3.2.1.6 or 3.2.1.39), depending on the sugar cleavage manner. Endo-enzymes randomly hydrolyzes linear substrates, whereas exo-enzymes releases glucose residues from the non-reducing ends. This type of enzyme is associated to glycoside hydrolase (GH) families based on structural hydrophobic cluster analysis [113]. As it was mentioned before GHs are responsible for the hydrolysis and/or transglycosylation of a glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety

[76, 77]. Information about SN7 did not mention about presence of an endo or exo-glucanase, however this background may explain the good results achieved with SN7 because as its known β-(1,3)-glucan has several non-reducing ends allowing it to function as acceptor for β-(1,6)-glucan and chitin. [24, 37]. Liberating β-(1,3)-glucans makes chitin and β-(1,6)-glucan more accessible to enzymatic hydrolysis. SN8 is a β-(1,3)-glucanase wich can hydrolyze β-(1,3), (1,4) and (1,6) glycosidic bonds and it was obtained from *T. reesei*. Hydrolysate profile showed the highest sugars release which is supported by the activity of this enzyme that liberating glucose chains on β-(1,3)/(1,6)-glucans hydrolysed a higher part of glucans in YCW.

NC5 is a complex of enzymes (Appendix 15) with different hydrolysing capacities. According with information mentioned before about some of these activities is understandably the high solubilisation rate and release of sugars because β-glucans of YCW were hydrolysed into glucose and it was also probably released oligosaccharides from glucan/mannan. NC5 did not release a considerable amount of free mannose and in its characterization it is mentioned β-mannosidase activity which is associated to hydrolysis of β-mannans to mannose [35]. In the YCW, the mannan-oligosaccharides (MOS) are made of main chain of α-D-(1,6)-mannose with short α-(1,2)- and α-(1,3)-linked side chains [35]. Accordingly, a β-mannosidase may not display any degradative activity on MOS.

ET4, NC1 and ST3 also presented a high solubilization rate, releasing a considerable amount of RS and no free glucose. Those enzymes are considered purified β-glucanases and were obtained from *Streptomyces* sp. Laminaripentaose-producing β-1,3-glucanase (LPHase) obtained from *Streptomyces matensis* DIC-108, cleaves a long-chain polysaccharide β-1,3-glucan into specific pentasaccharide oligomers, including laminarin, into a specific pentasaccharide oligomer “laminaripentaose”. β-1,3-glucans such as laminarin have e interesting biological roles in immune-modulation. Exo-enzymes produce much smaller sugars (mono- or disaccharides), whereas endotype enzymes yield heterogeneous forms of oligosaccharides. This inverting glycosidase utilize a single-displacement mechanism where the general base polarizes a water molecule to develop a stronger nucleophile to attack the anomeric carbon (C-1), while the general acid protonates the glycosidic oxygen to accelerate the reaction. This mechanism explains the hydrolysis of long-chain oligosaccharide from the reducing end. Accordingly, ET4, NC1 and ST3 can be associated to LPHase group based on RS profile [114-116].

All enzymes screened did not release a high amount of free mannose, but some of them as e.g. AE3, NS2, SN8 or AO1 (in TYCW case) released a bit after 24h of reaction time. SN7 was the only hydrolysate able to release a bit of free mannose after 24h of reaction time for both starting materials. On specification sheet of enzymes was not reported α-mannanase activity which may explain the lower or non-release of free mannose. Moreover, HYCW control released also a bit of free mannose suggesting that probably pH (4.5), temperature of 50°C or shaking can be associated to free mannose release.

Most of the enzymes had no or very low activity towards the YCWs, meaning that enzyme mixtures did not have the adequate enzyme activities to break down the YCW. Those results may be associated to the enzyme activities specific for some substrates as e.g. plant CW or starch [117].

For example, chitinase (NC4) and phospholipases (P-MC1, P-NE1 and P-N1) showed a lower profile of release of sugars and also a low solubilization rate which can be associated with YCW structure, i.e. chitin chains are linked to the nonreducing end of β-1,3-glucan via glycosidic linkages which means that chitin remains inaccessible because is into the inner layern of β-glucan which is a moderately branched. This information can be also associated to lipid content (membrane) which is entrapped by the CW [27, 30, 37].

PCA analysis for HYCW

For a better understanding of the differences between enzymes from a single cluster and also the impact of the raw material used (HYCW and TYCW), an individual factor map of variables and component 2 and 3 was accomplished (Appendix 8, Figure 8.2 **(b)**), where only 27.94% of the variance is explained and a big part of the information contained in the first component is not taken in account in this situation. In this case, the second principal component explains 21.29 % of the total variation and the third principal component an additional 6.65 % which means that the third component will not be taken in account because variation value is too low. Free mannose release correlates with the second component and the third component divide mannose 3h from mannose 24h.

The difference between enzymes SN7, DS2, AE4, NS2, NS3, NS6 and their respective cluster in Appendix 8, Figure 8.2 **(a)** can be explained with second component correlation, which means that those enzymes are the ones that release more free mannose compared to others enzymes. We must take the graphics shown in Appendix 5 and 6 into account, because as its possible observe the range of values of free mannose release are low comparing with free glucose and RS values.

Analyzing the cleaving properties of the enzymes, it is interesting to notice that, except AE4 whose specifications indicate that it is able to hydrolyze galactomannans and other non-starch polysaccharides, all the other enzymes usually cleave 1,3-glucanase. This β-mannanase, produced by fungi, is part of a group called hemicellulases. The activities of this enzyme include catalysis random hydrolysis of β-1,4-D-mannosidic linkages in mannans, galactomannans and glucomannans of [EC 3.2.1.78] [24].

This information allows to conclude that those enzymes are a mix of enzymes and besides the known cleaving properties they can act in order to cut mannan. The higher release of RS is a differentiating feature characteristic of enzymes belonging to cluster 5 (NC5, AO3 and SN2).

PCA analysis for TYCW

The component 2 and 3 map of individuals and map of variables (Appendix 9, Figure 9.2) explained only 20.58 % of the variance and a big part of the information contained in the first component is not taken in account in this situation. The second principal component explains 11.14 % of the total variation and the third principal component an additional 9.44 %. Free mannose correlates with the second component, while free glucose and RS correlate with third component.

The individual factors map (PCA) highlights some enzymes that are distinguished from others. SN2, ST1 and HE1 present a profile with a high reducing sugars release, which is explained for their

cleaving properties (Appendix 15). NC5 is an endoglucanase, which explains the higher free glucose release comparing with the others enzymes (**cyan**).

NS3 and AO1 exhibit freer mannose release at 3h over reaction time than others. NS3 whose specifications indicate that it is able to hydrolyse 1,4 and 1,6-alpha-D-glucosidic linkages at the non-reducing ends of polysaccharides and AO1 can cleave peptides and proteins. In AO1 case we can presume that mannoproteins are being cleaved because AO1 is a protease but about NS3 profile we can only assume that enzyme is actually a mix of enzymes and probably NS3 is able to cleave chitin become the mannose release.

NS2 and AE3 are distinguished from others because they are able to release free mannose at 24h over reaction time. Both enzymes are known for cleaving glucans, therefore we can presume the same thing explained above. Maybe those enzymes are not purified and that's the reason way they present different cleaving properties whose specifications indicate.

Comparing PCA analysis with the sugars release profile

The differentiator component on PCA analysis was the release of free mannose allowing the different clusters and overview of hydrolysates, as it was mentioned before the release achieved was to low to be considered as differentiator factor on sugars profile. As it possible to observe ET4, NC1 and ST3 are not visible on this analysis, however PCA analysis support some of our results. SN8 was considered the hydrolysate with the higgest release and SN7 is in the middle of very high/high release. AO3, SN2 and NC5 are associated to a cluster of high RS and free glucose release.

Moreover, AE3, HE1, NS6, SL1 and ST1 were introduced has a good hydrolysates showing a high/middle solubilization rate and release of sugars. When comparing this PCA results with the sugars release profile its possible confirm the good enzyme activity. AE3 is a β-glucanase, which can cleave (1,3)/(1,6)-linkages, with high protease side-activities (a.k.a lytic enzymes), obtained from *Trichoderma* sp. Scott and Schekman (1980), reported that β-glucanase attacked β-(1,3)-glucans releasing oligosaccharides, while the protease hydrolyzed protein content of CW by liberating mannoproteins. As the β-glucan layer of the CW is covered by a layer of mannoprotein it was suggested that there it is a synergistic effect of both enzymes activities [118]. NS6 is an exo β-(1,3)-glucanase which hydrolyzes β-D–(1,3)-glucans from the non-reducing end, releasing α-glucose (obtained from *Trichoderma hazianum*). Exo-β-1,3-glucanase hydrolyzes β-glucans by sequencial cleavage of glucose residues from the non-reducing ends, releasing monomers of glucose [119]. Almeida *et al*. (2007) reported that β-1,3-glucanase activity has synergistic action with chitinases, and others CW dregrading enzymes [120]. Therefore it will be interesting for the future combine NS6+NC4. SL1 is a pectinase composed by a complex enzymatic system of polygalacturonase (PG; EC 3.2.1.15), pectinesterase (PE; EC 3.1.1.11), and pectin lyase (PL; EC 4.2.2.10), with side-activities because this enzyme is used to hydrolyze plant CW and not YCW [121]. HE1 and ST1 are mix of enzymes with galactomannanase and cellulose. The side-acitivities action was explained before for SN2 and it associated to an increase of CW solubilisation and sugars release. Galactomannanase did not influence free mannose release which it is in consonance with previously justification for mannose results.

In summary, all the properties analysed to characterize hydrolystes shown to be relevant for a better understanting of enzymes activity, hydrolysates with higher potential characteristics for future applications and some high lights for future studies about this enzymes and YCW as starting material. Besides the proposed ideas mentioned before, the process could be optimized (temperature, pH and enzyme dosage) to have a better enzymatic conversion of our best enzymes.

3 Yeast Extract from TYCW

3.1 Introduction

Yeast cells can be converted into variety of hydrolysates which are useful as ingredients in foods, feeds and fermentation media. Among the principal products are isolated fractions of yeast cell constituents, such as protein and CW [51]. Hydrolysates derivate from YEs or second-generation products are at moment known as standard type of flavour enhancers and extracts with exceedingly high flavour enhancing capability [65].

Trends in consumer preference for all-natural foods and a desire on the part of the food industry for a "clear label" has resulted in an increased demand for substitutes such as yeast extracts to serve the function of the commonly used flavour enhancers in processed foods [66]. As natural flavourings approved by the Food and Drug Administration, peptides, AA, trace minerals and vitamins of the B-complex group, YEs are nutritional additives in health-food formulations, baby foods, feed [51].

To obtain hydrolysates low production costs, reaction parameters must be optimized in order to have a high space-time yields and high stability of the biocatalyst. The most important improvements can be made by variation of some parameters: pH, temperature, solvents, buffer salts, cofactors, immobilization method, substrate and product concentrations, addition of antioxidants or stabilizers, reaction material or coating and physical treatment (stirring, pumping, etc.) [122].

TYCW is low sodium autolysed yeast /YCW obtained as a by-product of the Prime process. During the Prime process, the yeast cream is heated up and held for a specific amount of time, resulting in ˜25% yield of YE which is later converted to nucleotide-rich TYC and ~ 75% yield of YCW which is dried and sold as TYCW.

A consequence of the Prime process is that the YCW produced, still contain a significant amount of protein (~57% versus ~27% for baker’s YCWs). This is remarkable, as protein is the key parameter that determines the yeast extract yield. Currently, the YCW is mainly being sold for low-margin profit. However, based on the protein content of these YCW, the revenues could be much higher if yeast extract could be produced from it.

3.2 Material and Methods

3.2.1 Material

Table 3.1 List of equipment for analytical methods.

Equipment	**Producer**
Scale Quintix 5102-1Ceu (d=0.01g)	Sartorius, GmbH
Scale PS4500.R2 (d=0.01g)	RADWAG, Poland
Rapid dryer HB43-S Halogen	Mettler Toledo, GmbH
pH-meter 766 Calimatic	Knick, UK
Research® plus Pipettes (20-200µL, 100-1000µL and 0.25- 2.5mL)	Eppendorf, GmbH
Waterbath	GFL, GmbH
Heraeus Multifuge X3R centrifuge	Thermo Fisher Scientific, USA
Digital thermometer (EU 620-0912 NA 82021-160)	VWR, GmbH
Analog vortex mixer, 230V	VWR, GmbH
Magnetic stirrer, 220 V-50/60Hz	IKA®-Werke GmbH & Co. KG. Janke
Rotary Evaporator Bath RE300DB	Stuart, UK

Table 3.2 List of material for analytical methods.

Material	**Producer**
Disposable pipettes, 3mL	Nerbe plus, GmbH
250mL Erlenmeyer	Duran, GmbH
250mL and 1000mL beakers	Duran, GmbH
250mL and 1000mL beakers	Simax, Czech Republic
4cL Brandy glass (PET)	Sanitex, Poland
Price Labelling Starter Kit	Avery, China
1.7L Water Kettle (2400W)	WMF Stelio, GmbH
2L Thermos pot	Caterado, GmbH
GHP Acrosidisc membrane, 0.2µm	PALL, GmbH
1L Rotating flask	Schott Duran, GmbH

Table 3.3 List of reagents for analytical methods.

Reagent	Producer
Provesta®024	Ohly an ABF Ingredients Company
Maltodextrin(MaltoSweet180), B015L912010837	Taste & Lyle
Basic Vegetable Broth Without Pieces, 2016/HH 005/00	Ohly an ABF Ingredients Company
Mineral water	Bismarck, GmbH
Ohly® SYEAA, Lot-no. ES16-000096	Ohly GmbH
Provesta®512, Lot-no. 610301 EW	Ohly an ABF Ingredients Company
OHLY® YEHS	Ohly an ABF Ingredients Company
Solution with 45% of NaOH	Carl ROTH, GmbH
Solution with 50% of $C_6H_8O_7$	Merck KGaA, GmbH
$C_7H_6O_65*2H_2O$	Merck KGaA, GmbH
Buffer PF-SDB	VWR, GmbH
Lithium Amino Acid Calibration Standard Native Samples	Pickering Laboratories, USA

3.2.2 Methods

3.2.2.1 Enzymatic Reactions

Proteolytic enzymes (9) Carbohydrases (2) and Phospholipases (4) (Appendix 15) and TYC were tested on TYCW. The yeast cell were mixed with ultrapure water to reach a DM content of around 21%, 17%, 15%, 13% and 11% (w/w), and they were stirred for around 60 min at room temperature. The pH of the yeast mixture with a volume of 200mL was adjusted using a solution with 45% of NaOH or a solution with 50% of citric acid. No enzyme was added to the sample termed control. Hydrolysis was performed with a shaking of 120rpm and the phase separation of 20min at 4700rpm for the reactions in Appendix 10. The remaining trials were done with a shaking standardized at ~138 rpm and a phase separation performed for 20min at 4700rpm. The samples were inactivated at 80°C for 20min.

The hydrolysates characterization was done by analytical and chemical analysis, such as measurement of dry matter, protein analysis, peptide analysis, free AA, nucleotides and sensory analysis. The dry weight of the biomass was determined by using a Rapid dryer HB43-S Halogen.

With a view to achieve a higher yield (calculated as the dry weight of extracted supernatant/ dry weight of yeast biomass) and using the same starting material (TYCW), different DM contents of around 17% (w/w), 15% (w/w), 13% (w/w) and 11% (w/w) were tested. Enzyme NS11 was used at a standard dose rate of 0.5% (w/w) plus EN1 at a standard dose rate of 0.69% (w/w) with a pH of 7.0, at 60 °C. A pH of 5.1 and a temperature of 68°C were adopted and the enzyme AO5 was added at a

standard dose rate of 0.4% (w/w), 17h before the addition of EN1 and NS11. The reaction was allowed to proceed for more 8h.

According to results obtained and to attain a good YE derived from TYCW, it was chosen to use a DM content of ~11%.

Table 3.4 Reactions performed for TYCW with ~11% dry matter.

Reactions performed for TYCW with ~11% dry matter					
Enzymes dosages	**pH**	**T [°C]**	**Enzyme dosages**	**pH**	**T [°C]**
0.5% NS11 + 0.69% EN1	7.0	60	0.2% AO5	5.1	68
			0.15% AO5		
			0.1% AO5		
			0.05% AO5		
			0.01% AO5		
Reaction time	16/17h	-	-	8h	-

TYC contains high levels of 5'-IMP plus 5'-GMP and free glutamic acid. According with this information, was done a trial with enzyme NS11 at a standard dose rate of 0.5% (w/w) in conjunction with EN1 at a standard dose rate of 0.69% (w/w) with a pH of 7, at 60 °C, over 17h of reaction time. Afterwards different dosages: around 24% (w/w), 12% (w/w), 9% (w/w), 7% (w/w), 5% (w/w) and 2% (w/w) of TYC were added and mixed with 50ml of the previous solution.

3.2.2.2 Proteins analysis

The protein content in total solids of the yeast biomass was kindly carried out by QC people from Ohly GmbH, using Kjeldahl's Method in order to convert nitrogen content in total protein content. Indeed the total protein content in the biomass of the yeast cell was determined and the nitrogen content was converted into total protein content using the coefficient factor 6.25 [11].

When a strong base (NaOH) is added in excess, the ammonia in the digested substance is displaced. During the distillation, it is carried away by the vapor and can be titrated in the presence of a coloured reagent either in return or directly by recovery in boric acid [11].

The first result is presented 12min after the introduction of the sample, and the following results are obtained every 3min. The results of Kjelfoss determinations are in such good agreement with those of the Kjeldahl's Method that is indicated as the official AOAC (Official Methods of Analysis) method, regardless of the product analysed. The sample weighs for analysis were 0.5g and usually vary according to the nitrogen content [123].

3.2.2.3 Peptide analysis

The MW profiles were carried out with HPLC with size exclusion chromatography (SEC) and a wavelength detection (UV/VIS) used to detect proteins, according to an Ohly GmbH standard procedure, Table 3.6. The curve of the log of the solutions MW was plot according to the retention time. The MW of TYCW samples peaks were determined according with column calibration curve.

Table 3.5 Protein standards (Sigma Aldrich, USA).

Protein	**MW [kDa]**
Alcohol Dehydrogenase	150
Albumin	66
Carbonic Anhydrase	29
Cytrchrome C from equine heart	12.4
Aprotinin from bovine lung	6.511
Met-Ala-Ser	0.307
Gly-Tyr	0.238

Table 3.6 Conditions for MW analysis.

Eluent	Solution A+B
Flow rate	1 mL/min
Injection volume	20µL
Column	PROTEEMA SEC-column
Temperature	~ 23°C
Pressure	58 bar
Running time	~20min
Wavelength Detection	Dionex Ultimate 3000 Variable Wavelength Detector Thermo Scientific, λ= 210nm
Data System:	Chromeleon 6.8 version (Dionex, GmbH)

Eluent preparation

Solution A) 30 mmol Sodium dihydrogen phosphate monohydrate/0.5mol Sodium chloride

4.14 g/L $NaH_2PO_4*H_2O$

29.22 g/L NaCl

Fill up to 1000mL with ultrapure water

Solution B) 30mmol di-Sodium hydrogen phosphate/0.5 mol Sodium chloride

4.26 g/l NaH_2PO_4

29.22 g/l NaCl

Fill up to 1000mLwith ultrapure water

Around 600mL of solution A was added to 1000mL of solution B until setting pH-level to 6.6.

Samples preparation

Depending on the DM of the each examined sample, the amount used was adjusted. For a liquid sample with a DM ~ 100%, are usually added 0.05 g of sample into 10mL volumetric flask and fill up to 10mL with ultrapure water. The DM of TYCW samples was around ~ 10%, which means that it was necessary achieve a weight of 0.5g. Afterwards the solutions were filtered through a 1.5mL samples vials, using 0.45µm PES filters and 5mL syringe.

3.2.2.4 Amino Acid analysis

The measurement of free AA, performed by the Specialist Petra Priebe, was carried out with an Amino Acid Analyser L-8900 (Hitachi). The main principle of AA analyser suggest an ion-exchange resin applying post-column derivatization for the separation of AA with ninhydrin, as oxidizing agent [124]. All α-AA react with ninhydrin that builds a purple colorant with primary amines and ammonia which can be detected at 570nm. The AA proline and hydroxyproline also react with ninhydrin, but they build a yellow coloured complex which can be detected at 440nm [124]. For the calibration it was used Lithium AA Calibration Standard Native Samples and each run takes ~140min.

Preparation of samples

Depending on the DM of the each examined sample, the amount used was adjusted. For a liquid sample with a DM ~ 20%, are usually added 2.5g of sample into 50mL volumetric flask and fill up to 50ml with ultrapure water. Then the samples were filled in a vial in a 1:1 dilution with Buffer PF-SDB (acid buffer) and $C_7H_6O_65*2H_2O$ (salt) and left to react overnight letting larger peptides to precipitate out of the solution.

Afterwards the solutions were filtered through a 1.5mL samples vials, using 0.2µm GHP filters and 5mL syringe. The evaluation was carried out with the Agilent OpenLAB CDS EZChrom Edition software.

3.2.2.5 Nucleotide analysis

The 5'-mononucleotide content of the samples (AMP, CMP, GMP, UMP and IMP) was carried out by HPLC with UV-detection. The eluent and standards are described on Table 3.7.

Table 3.7 Eluent and standards used in nucleotide analysis.

Name	Manufacturer	Other
Sodium sulfate (anhydrous)	Merck	Eluent A
Potassium dihydrogen phosphate	Merck	Eluent A
Adenosine (5´monophosphate - Monohydrate)	Sigma Aldrich	AMP (Standard)
Cytidine (5´-monophosphate)	Sigma Aldrich	CMP (Standard)
Guanosine (5´-monophasphate disodium salt hydrate)	Sigma Aldrich	GMP (Standard)
Inosine (5´-monophosphate disodium salt)	Sigma Aldrich	IMP (Standard)
Uridine (5´-monophosphate disodium salt)	Sigma Aldrich	UMP (Standard)

Production of the buffer:

- Eluent A:

  0,35% KH_2PO_4 / 6% Na_2SO_4

Samples preparation

Depending on the DM of the each examined sample, the amount used was adjusted. For a liquid sample with a DM ~ 10%, are usually added 0.5g of sample into 10mL volumetric flask and fill up to 10mL with ultrapure water. After preparation the samples were filled into vials (without membrane filtration) and immediately measured. The evaluation was carried out using the Chromeleon 6.8 software.

3.2.2.6 Sensory analysis

The sensory evaluations to find out a good and cheap YE (starting material TYCW) with possible food applications was carried out by a panel of 4-5 expert assessors (age: 22-65) well training and able to detect and describe the perceived sensory attributes of the samples [71]. Tasting sessions were carried out in a dedicated, air conditioned sensory room where each participant was separated from each other by means of visual separators.

The different possible YEs were tested and compared in application (vegetable broth without pieces) and afterwards the final YE was compared with Ohly's YE products (SYEAA, TYC, and YEHP).

The aim of the tasting was to identify the difference between YEs and also between TYCW samples with enzymatic treatment in a food application which in this case was the vegetable broth. Additionally TYCW was tested and compared to the reference (vegetable broth). An existing recipe for vegetable broth without pieces at Ohly GmbH was used as application.

As referred to a characteristic application it is used SYEAA as a "base", 1.7g of vegetable broth mixed with 0.3g of SYEAA with 96% of dry base in powder for 100mL of water. The DM of each sample was measured and the final amount used in application was calculated having in account the previous information. E.g. for a DM= X% the final amount to mix with vegetable broth (1.7g) will be (1).

$$(1)\ C\ (g) = \frac{100 * 0.3}{X}$$

Then the mixture was diluted under stirring in boiling water. The optimum temperature for the gustation of samples is between 60-70 °C. The base was cooled down to 65 °C (+/- 2 °C) and filled into thermos bottles about half an hour before serving. All samples were labelled with a randomly-assigned 3-digit code [125]. The panellists were asked to rate the intensity compared to the reference, which was the application containing TYCW with enzymatic treatment. One reference sample was always served together with the sample to be investigated.

Panellists were asked to cleanse their palate between each sample with bottled still water and with non-salted crackers.

Quantitative Descriptive Analysis (QDA®) Method

QDA® method is based on statistical analysis to determine the appropriate terms, procedures, and panellists to be used for analysis of a specific product [71]. A typical scoresheet is shown in Appendix 11.

The results of a QDA test were analysed statistically and graphically presented as a "spider web" with a branch or spoke from a central point for each attribute [71, 126]. Quantitative Descriptive Analysis (QDA) using a 7-point scale which represent intensities of none, weak, middle, strong and very strong [127].

Ranking/ Rating Tests for Intensity

A raking test for intensity is usually done to categorize graded levels of intensity of a given attribute. Panellists were thoroughly familiarized with the attribute under test (umami taste). In those tests, candidates received a series of 5 samples in random order and they must rank the samples in ascending order, according to the level of the stated attribute [71]. A typical scoresheet is shown in Appendix 12.

Ranking tests have wide application, but with sample sets above three they do not discriminate as well as tests based on the use of scales. Statistical analyses were conducted with the Excel software. When data from a randomized block design are in the form of ranks, then a nonparametric analysis is accomplished using Friedman-type statistic for rank data, which takes the place of the F-statistic in the analysis of ratings [71]:

(1) $T = \left(\left[\frac{12}{bt(t+1)}\right]\sum_{j=1}^{t} x_{.j}^{2}\right) - 3b(t+1)$

- B = the number of panellists;
- t = the number of samples;
- x.j = the rank sum of sample j.

The test procedure is to reject the null hypothesis of no sample differences at the α-level of significance if the value of T (Equation 1) exceeds $X_{\alpha,t-1}^{2}$ and to accept H_0: otherwise, where $X_{\alpha,t-1}^{2}$ is the upper-α percentile of the X^2distribution with t-1 degrees of freedom. If X^2-statistic is significant, then a multiple comparison procedure is performed to determine which of the samples differ significantly. The nonparametric analog to Fisher's LSD for rank sums from a randomized block design is [71]:

(2) $LSD_{rank} = t_{\alpha/2,\infty}\sqrt{bt(t+1)/6}$

Two samples are declared to be significantly different at the α-level if their rank sums differ by more than the value of LSD_{rank} (Equation 2) [71].

3.2.2.7 Evaporation until reached a dry matter of ~50%

Higher solid contents prevent microbial contamination by reduction of water activity therefore the final product using TYCW with (0.5% (w/w) of NS11 + 0.5% (w/w) of EN1 +0.1% (w/w) of AO5) was evaporated until reached a DM of ~50% [128].

The rotary evaporator is used for the evaporation of a solvent under vacuum. The evaporator had heating bath with a temperature ~78°C and a rotating flask of 1L in which the liquid circulated under a thin film over the hot wall surfaces and was evaporated for a receiving flask. The evaporation rate can be regulated by the heating bath temperature, the size of flask, the pressure of distillation and the speed of rotation. The main principle of evaporation using this instrument consists in increasing the rate of evaporation of the solvent by reducing the pressure to lower the solvent boiling point, rotating the sample to increase the effective surface area and heating the solution [129].

3.3 Results

3.3.1 Hydrolysates analytical characterization

Carbohydrases and phospholipases were considered for the hydrolysis of the TYCW, however those enzymes did not result in a YE with superior analytical properties (Appendix 13). Those hydrolysates show in a general way a high DM, a low yield per DM and also low protein content per DM.

3.3.1.1 Free amino acids and peptide analysis

The content and profile of free AA and peptides is a significant indicator for the taste profile of a YE.

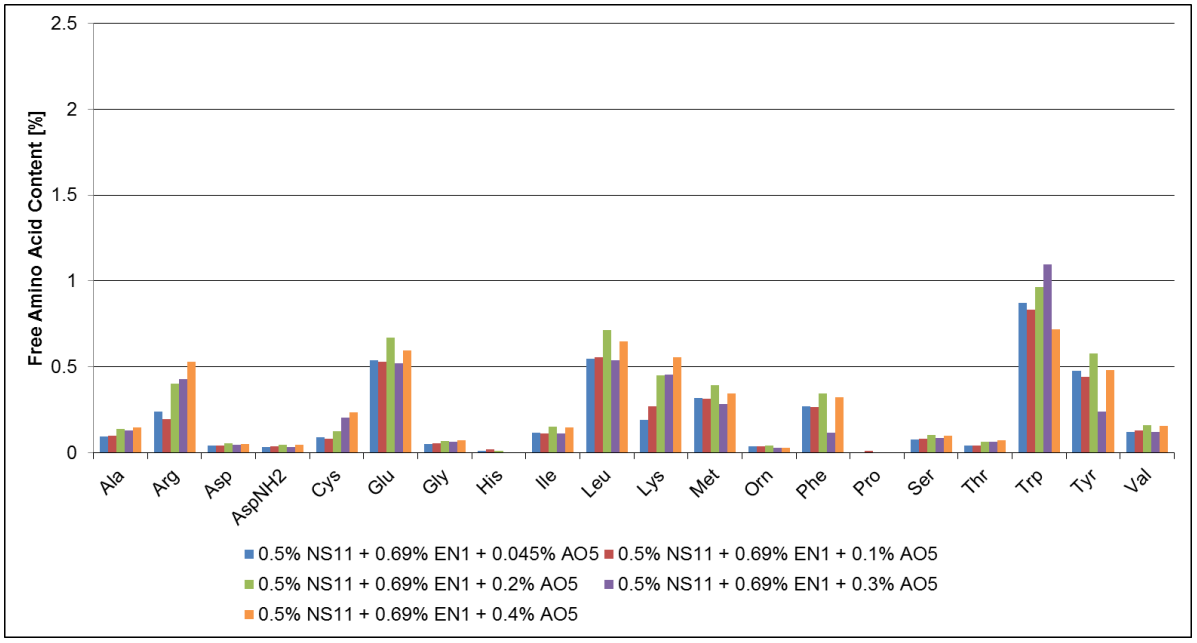


Figure 3.1 Free AA content for different concentrations of AO5 wit a starting DM= ~21%.

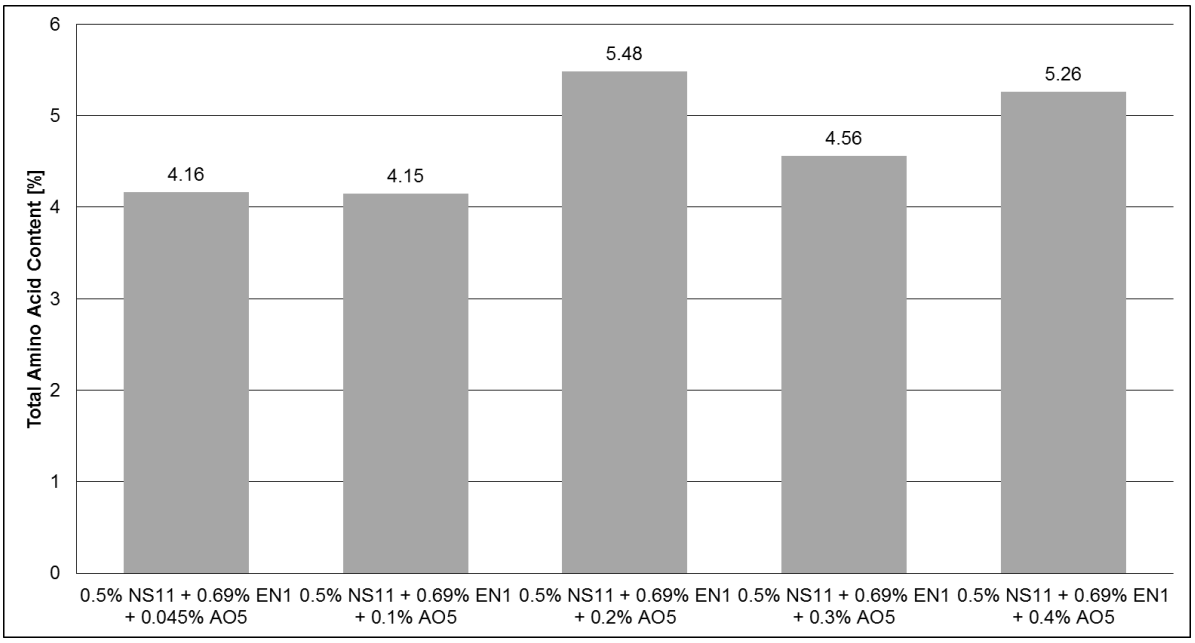


Figure 3.2 Total AA content for different concentrations of AO5 wit a starting DM= ~21%.

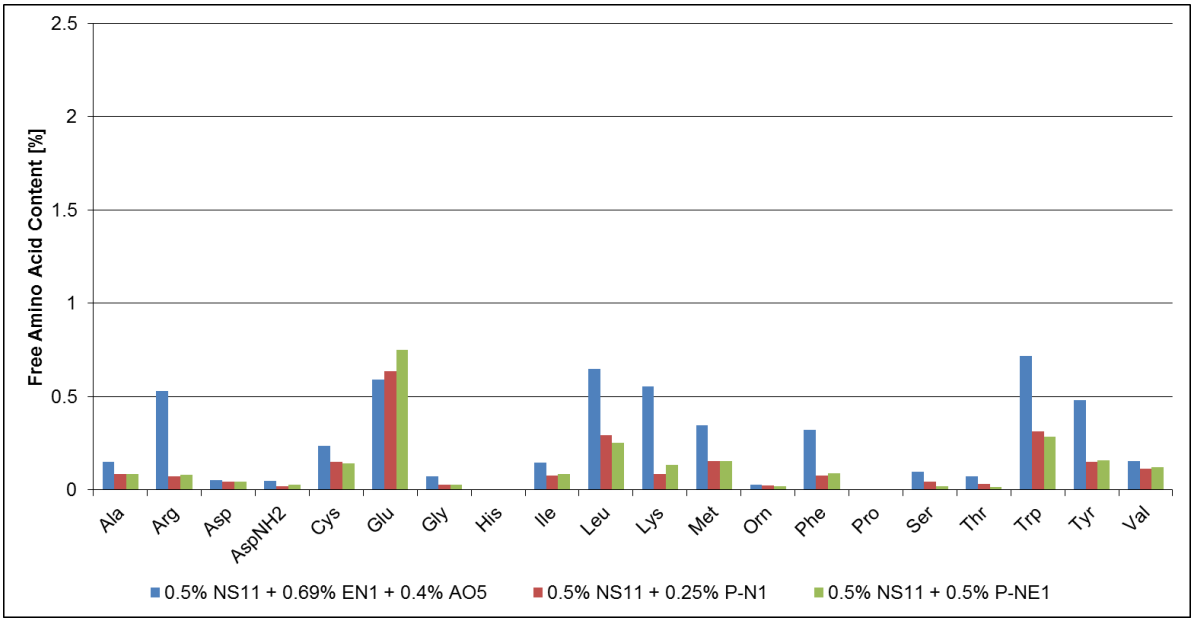


Figure 3.3 Free AA content for different concentrations of EN1 + AO5, P-N1 and P-NE1, with a starting DM= ~21%.

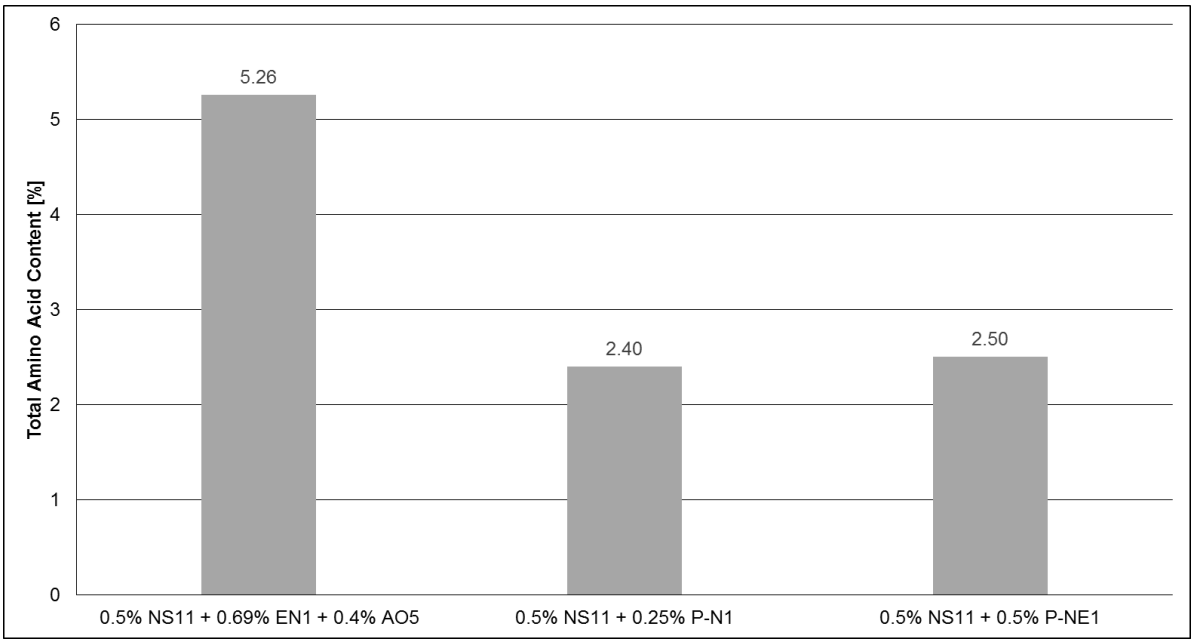


Figure 3.4 Total AA content for different concentrations of EN1 + AO5, P-N1 and P-NE1, with a starting DM= ~21%.

Endoenzymes as NS11 and EN1 are known for do not release a lot of free AA. Figure 3.1 and Figure 3.3 from reaction between enzymes and TYCW show low free AA content. P-NE1 is a phospholipase able to release fatty acids from membrane lipids and P-N1 is a phospholipase which cleave esterbonds (Appendix 15) [78]. It was suppose that hydrolysates obtained with phospholipases were able to release some free AA but as it is possible confirm in Figure 3.3 the release of AA profile is very low. The total content of AA from reactions with P-N1 and P-NE1 are slightly lower with ~2.50% (w/w) compared to ~4-5% (w/w) for the hydrolysates obtained from the reaction with EN1 and AO5 (Figure 3.2 and Figure 3.4). Glutamic acid (Glu), leucine (Leu) and tryptophan (Trp) are the AA represented at the highest concentration in the reactions with AO5, having ~0.5-0.6% (w/w), ~0.5-0.7% (w/w) and ~0.7%-1.0% (w/w) respectively.

Our hydrolysates had a clearly lower level of total AA when compared with total AA content from other Ohly YEs, e.g., SYEAA (data not shown).

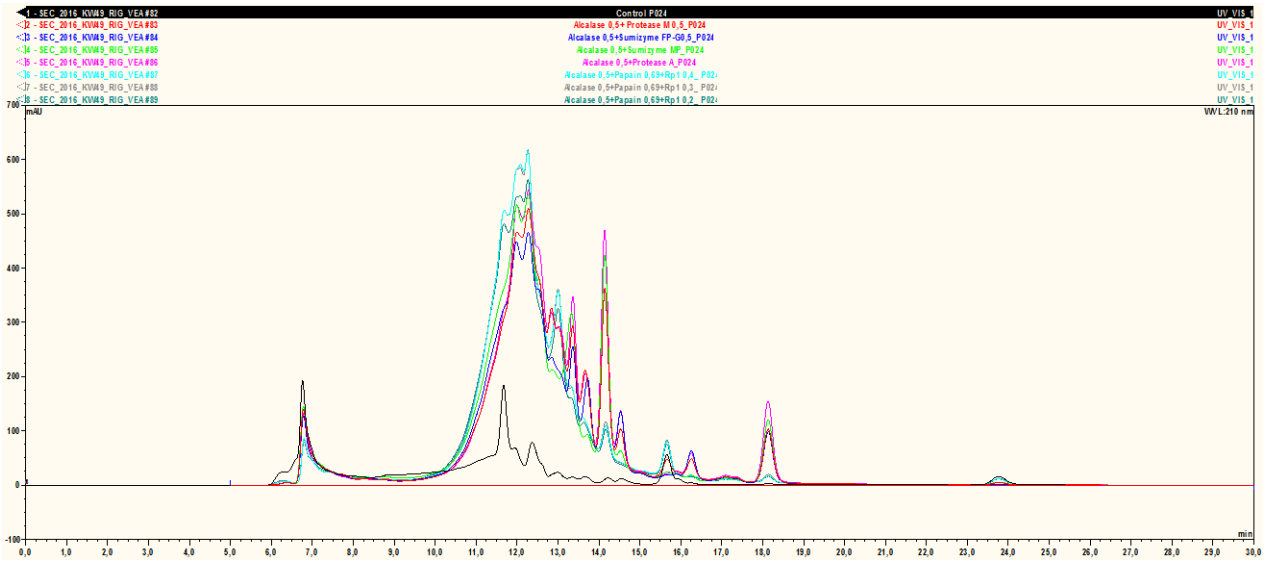


Figure 3.5 MW distribution of different hydrolysates using TYCW as starting material. The different substances in the standard solution have different retention times. The Profile is similar for all enzyme combinations. MW distribution of different hydrolysates suggests a higher content of soluble material. (black) which is the control (no enzyme added); (red) 0.5% NS11 + 0.5% AO1; (blue) 0.5% NS11 + 0.5% SN9; (green) 0.5% NS11 + 0.5% SN10; (pink) 0.5% NS11 + 0.5% AO4; (cyan) 0.5% NS11 + 0.69% EN1 + 0.4% AO5; (grey) 0.5% NS11 + 0.69% EN1 + 0.3% AO5 and (navy blue) 0.5% NS11 + 0.69% EN1 + 0.2% AO5.

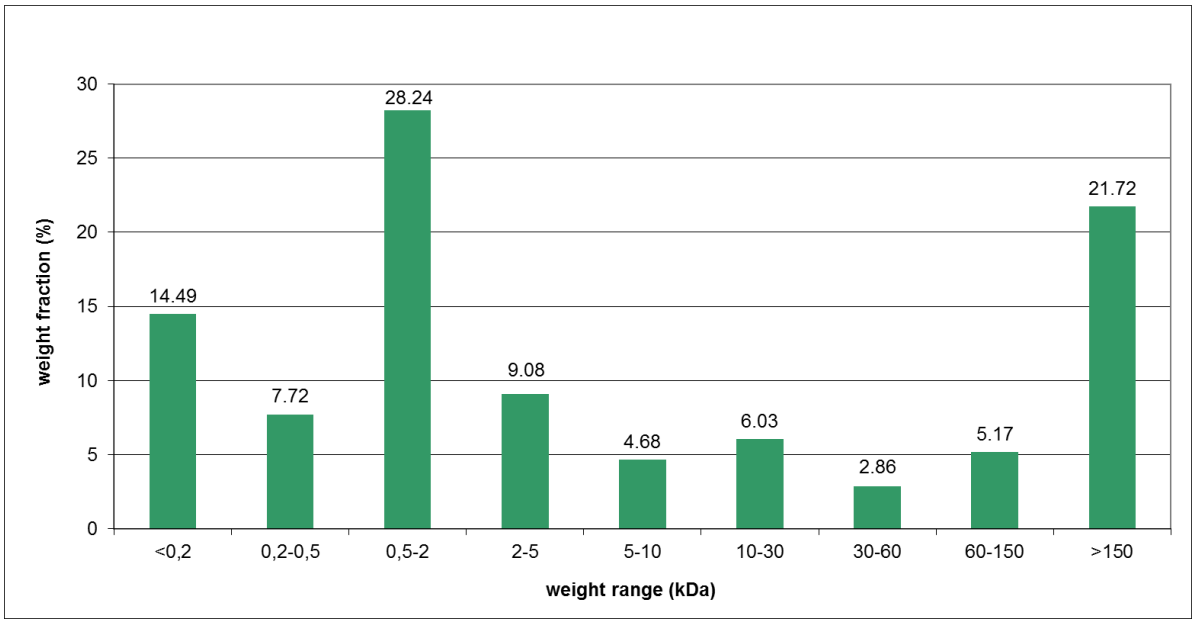


Figure 3.6 MW analysis of proteins for the sample named control, with a starting DM= ~17%.

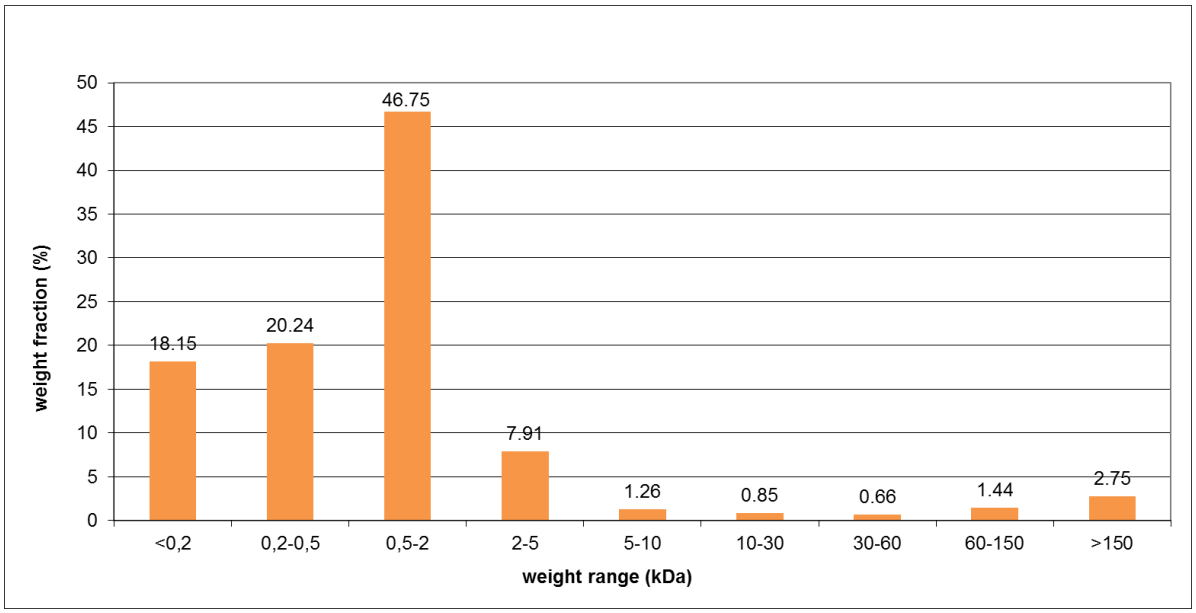


Figure 3.7 MW analysis of peptides for the sample 0.5% NS11 + 0.69 % EN1 + 0.2% AO5, with a starting DM=~17%.

Free AA should be present in the weight range lower than 0.2kDa because their MW is usually between 0.075kDa to 0.104kDa. MW analysis of peptides profile (Figure 3.5 and Figure 3.7) support the low values of free AA and comparing with control (Figure 3.6) is clear that the hydrolysates did not release high content of high size peptides.

The MW distribution is similar for all combinations of enzymes (Figure 3.5) and in case of hydrolysates obtained using AO5 suggests the higher content around 18% (w/w) of the protein in the hydrolysates is in the form of small size peptides less than 0.2 kDa, ~21% (w/w) of the protein is in form of peptides between 0.2-0.5kDa and ~47% (w/w) of the protein is in the weight range between 0.5-2kDa (Figure 3.7).

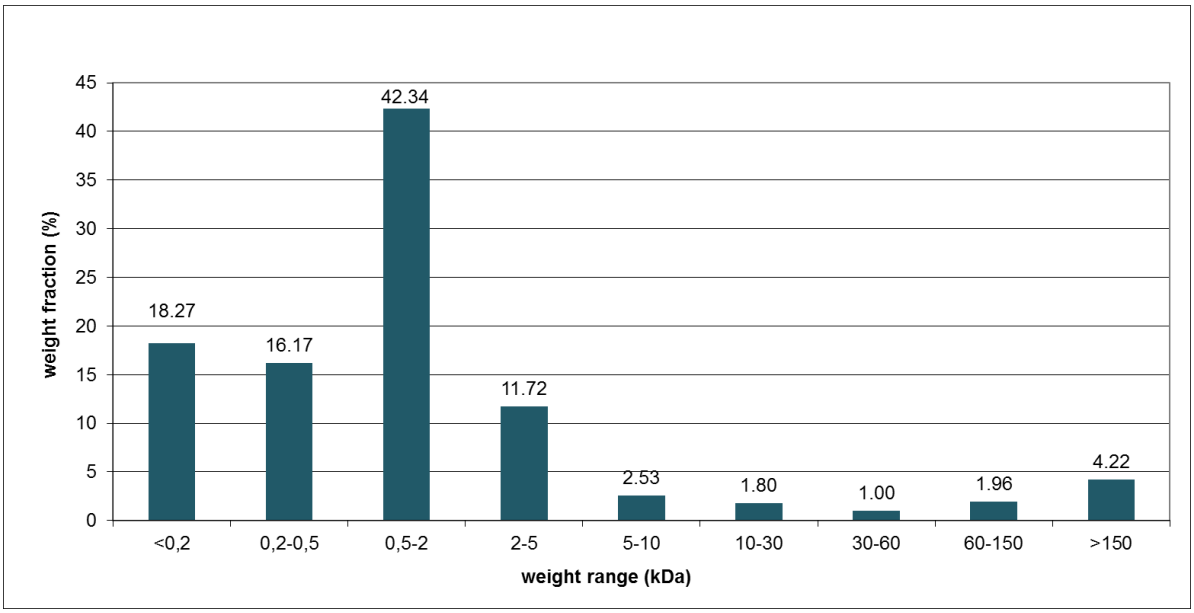


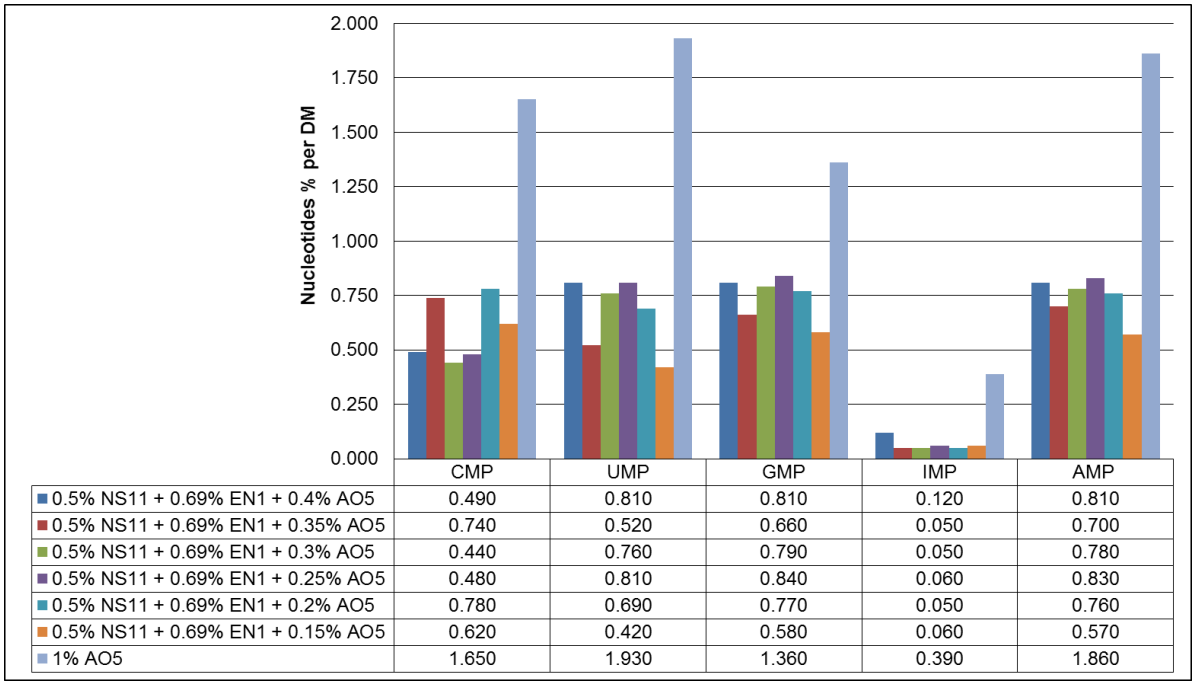
Figure 3.8 MW analysis of peptides for the sample 0.5% NS11 + 0.5% NS12 with a starting DM=~21%.

Exopeptidases as NS12 are known for release free AA by hydrolysis of the N-terminal (Appendix 15). However, as it is possible confirm in Figure 3.8 and comparing with the previous results mentioned, MW profile of hydrolysates with NS12 are really similar with the remaining hydrolysates which the profile shows the highest amount of proteins in the range of small size peptides.

MW analyses of peptides in a general way show that around 50% to 70% (w/w) of the protein in the hydrolysates is in the form of small size peptides between 0.2-2kD.

3.3.1.2 Nucleotide analysis

As stated in Section 1.7.2 the content of 5'nucleotides is an important component in provide certain flavour characteristics to a YE.



	CMP	UMP	GMP	IMP	AMP
0.5% NS11 + 0.69% EN1 + 0.4% AO5	0.490	0.810	0.810	0.120	0.810
0.5% NS11 + 0.69% EN1 + 0.35% AO5	0.740	0.520	0.660	0.050	0.700
0.5% NS11 + 0.69% EN1 + 0.3% AO5	0.440	0.760	0.790	0.050	0.780
0.5% NS11 + 0.69% EN1 + 0.25% AO5	0.480	0.810	0.840	0.060	0.830
0.5% NS11 + 0.69% EN1 + 0.2% AO5	0.780	0.690	0.770	0.050	0.760
0.5% NS11 + 0.69% EN1 + 0.15% AO5	0.620	0.420	0.580	0.060	0.570
1% AO5	1.650	1.930	1.360	0.390	1.860

Figure 3.9 5'mononucleotides content as % of total dry solids for different concentrations of AO5, with a starting DM= 17%.

The samples with different dosages of AO5 have a comparable nucleotide contents. The total level of nucleotides was not bigger than about 7% (w/w). Our results indicate that 5'-IMP was not taking part in combination of enzymes reaction, whereas 5'-GMP did.

Our hydrolysates with different concentrations of AO5 had a clearly lower level of nucleoties when compared with nucleotides content from other Ohly YE, e.g., TYC (data not shown).

3.3.2 Hydrolysates Sensory Characterization in application

Below, the average results of sensory evaluation are depicted in spider plots, and separated into taste attributes (sour, sweet, umami, bitter and salty). During the sensory analysis, hydrolysates obtained from TYCW were compared to a different controls: vegetable broth without pieces (without YE or enzymes), standard YE with high content in free AA (SYEAA), Torula yeast cell (TYC) with high nucleotides content and YE with high content of peptides (YEHP). Indeed, the results show vegetable broth without pieces as the only possible control because all the remaining possible controls had a distinct taste when compared with hydrolysates obtained from TYCW (data not shown).

Vegetable broth without pieces represented the control (without YE or enzymes), all samples were compared against. The aim of this experiment was to find out a new YE with good taste and

aroma characteristics. The standard deviation results of sensory analysis are attached in Appendix 14.

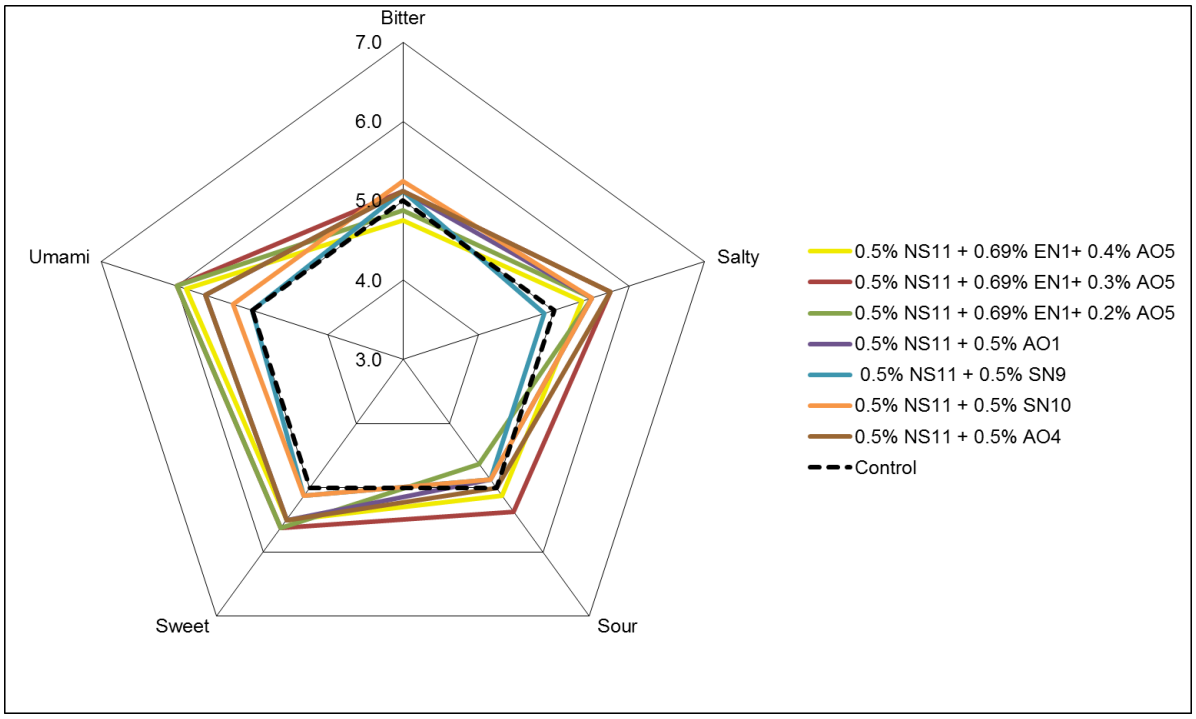


Figure 3.10 QDA analysis: sensory evaluations of TYCW possible YEs vs control (vegetable broth). Graphical display of sensory attributes based on QDA result. The scores of taste were 3-7, which represent intensities of none, weak, middle, strong and very strong.

The taste profiles in Figure 3.10 show that hydrolysates obtained with AO5 and AO4 in application led to a higher intensity of attributes umami, sweet and salty than the control and the others hydrolysates analysed. However, sample with AO4 is slightly less intense than others. Sample with 0.3% (w/w) AO5 show an intense sour taste, but besides that no major differences were noted between the samples with these various AO5 dosages. Sensory profile of sample with SN9 seems to be similar to the control. Moreover, the sensorial analysis found that the samples with AO1 and SN10 can enhance sweet and bitter/salty properties, respectively.

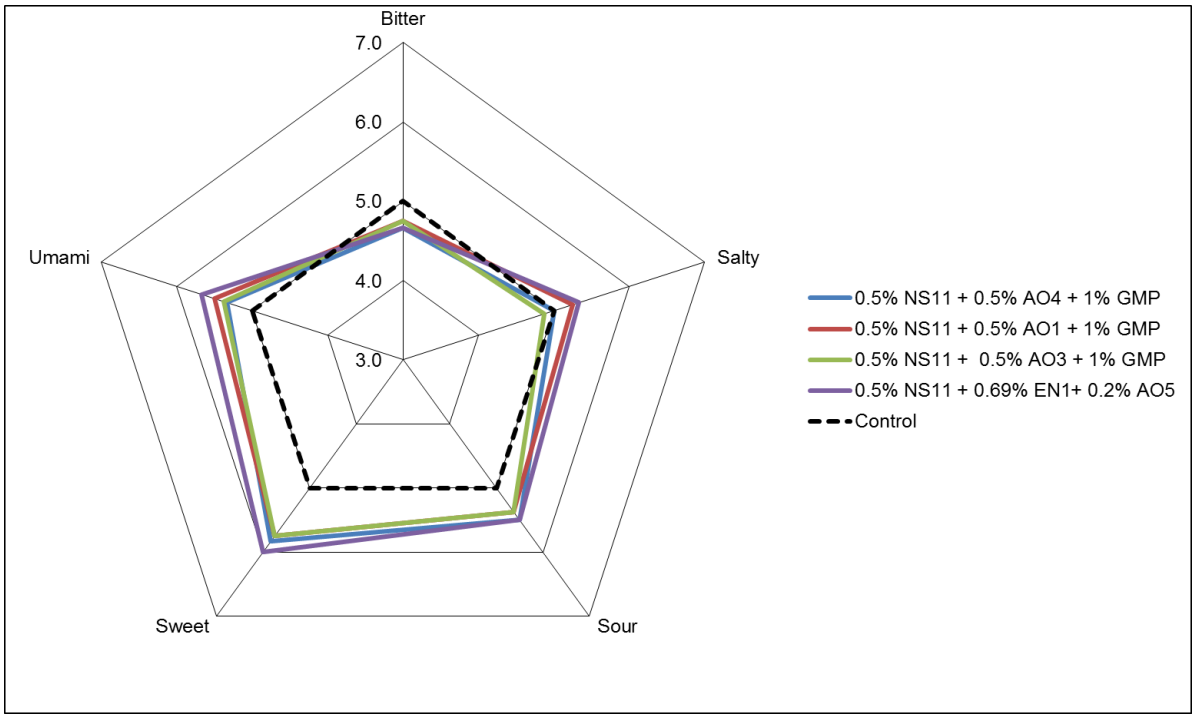


Figure 3.11 QDA analysis: sensory evaluations of TYCW with 0.2% AO5 vs other hydrolysates with 1% of GMP. Graphical display of sensory attributes based on QDA result. The scores of taste were 3-7, which represent intensities of none, weak, middle, strong and very strong.

The results related with hydrolysates with AO4, AO1 and AO3 had an admissible yield % (w/w), ~36.212% (w/w), ~35.154% (w/w) and ~24.326% (w/w), respectively (Appendix 13). According to this information was performed a sensorial analysis to understand the enhancer properties of these hydrolysates when added 1% (w/w) GMP. These percentage of GMP was the one associated to umami enhancer properties during this study as it is possible confirm in section 3.3.4.

The taste profiles in Figure 3.11 are similar among themselves, with some enhancer properties when compared with control. Hydrolysates obtained with AO5 and AO1 in application led to a higher intensity of attributes umami, sweet, salty and sour. AO4 and AO3 led to a higher intensity of umami, sweet and sour attributes. It is clear that AO5 presents the strongest umami, sweet and salty taste properties when compared with the others one.

Besides this potential results of sensory evaluations from all enzyme combination, NS11, EN1 and AO5 seem be the one with better yield % (w/w), good taste enhancer properties and protein yield per DM essential for a good and cheap YE.

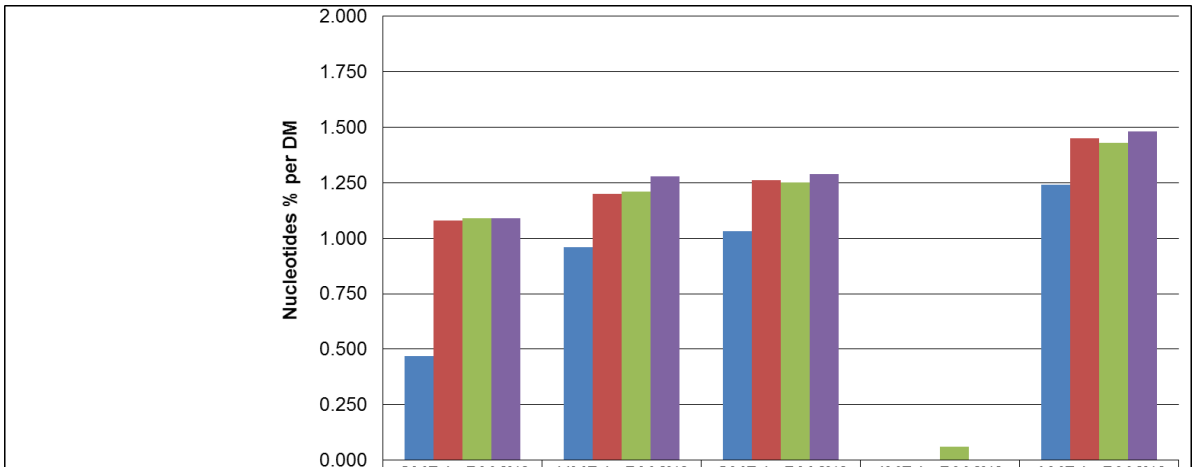
3.3.3 Dry matter of starting material

During this thesis the low yield % (w/w) was always an issue. Therefore, some changes (section 3.2.2) as shaking standardized at ~138rpm, a phase separation performed at maximum (for 20min at 4700rpm) and the use of different DM of TYCW, were made. As was shown in section 2.3.2, TYCW is viscous that was the reason way it was necessary a better homogenization in order to more efficient activity of the enzymes. The standard deviation results of hydrolysates with different starting DM are attached in Appendix 14.

Table 3.8 Hydrolysates characterization for different starting dry matter.

Enzymes	DM of starting material (%)	DM % in SN	Yield % per DM	Protein % per DM
0.5% NS11 + 0.69% EN1 + 0.4% AO5	17	8.873	22.009	64.417
	15	8.060	28.099	65.232
	13	6.633	30.467	67.032
	11	5.857	34.635	67.585

An enzymatic reaction (0.5% NS11 + 0.69% EN1 + 0.4% AO5) with the same conditions was done using different starting DM of TYCW (around 17%, 15%, 13% and 11%). Table 3.8 show that the starting DM had a major impact on the resulting yield after enzymatic hydrolysis, the higher the DM, the lower the yield. From all hypothesis, a starting DM of 11% seems to be the best option, presenting around 35% yield per DM also the highest value of protein content ~68% (w/w).



	CMP in DM [%]	UMP in DM [%]	GMP in DM [%]	IMP in DM [%]	AMP in DM [%]
0.5% NS11 + 0.69% EN1 + 0.4% AO5 (DM=17%)	0.470	0.960	1.030	0.000	1.240
0.5% NS11 + 0.69% EN1 + 0.4% AO5 (DM=15%)	1.08	1.2	1.26	0	1.45
0.5% NS11 + 0.69% EN1 + 0.4% AO5 (DM=13%)	1.09	1.21	1.25	0.06	1.43
0.5% NS11 + 0.69% EN1 + 0.4% AO5 (DM=11%)	1.09	1.28	1.29	0	1.48

Figure 3.12 5'mononucleotides content as % of total dry solids for different percentages of starting DM (around 17%, 15%, 13% and 11%).

The samples with different starting DM obtained from enzymatic hydrolysis with 0.5% NS11 + 0.69% EN1 + 0.4% AO5 have comparable nucleotide contents. Figure 3.12 show 5'-GMP levels from ~1% to 1.29% (w/w) and no 5'-IMP content. Starting DM of ~11% was found to contain the highest amount of 5'-GMP (1.29% (w/w)). Therefore our results indicate that 5'-IMP was not taking part in combination of enzymes reaction, whereas 5'-GMP did. The total level of nucleotides was not bigger than about 6% (w/w) for all samples.

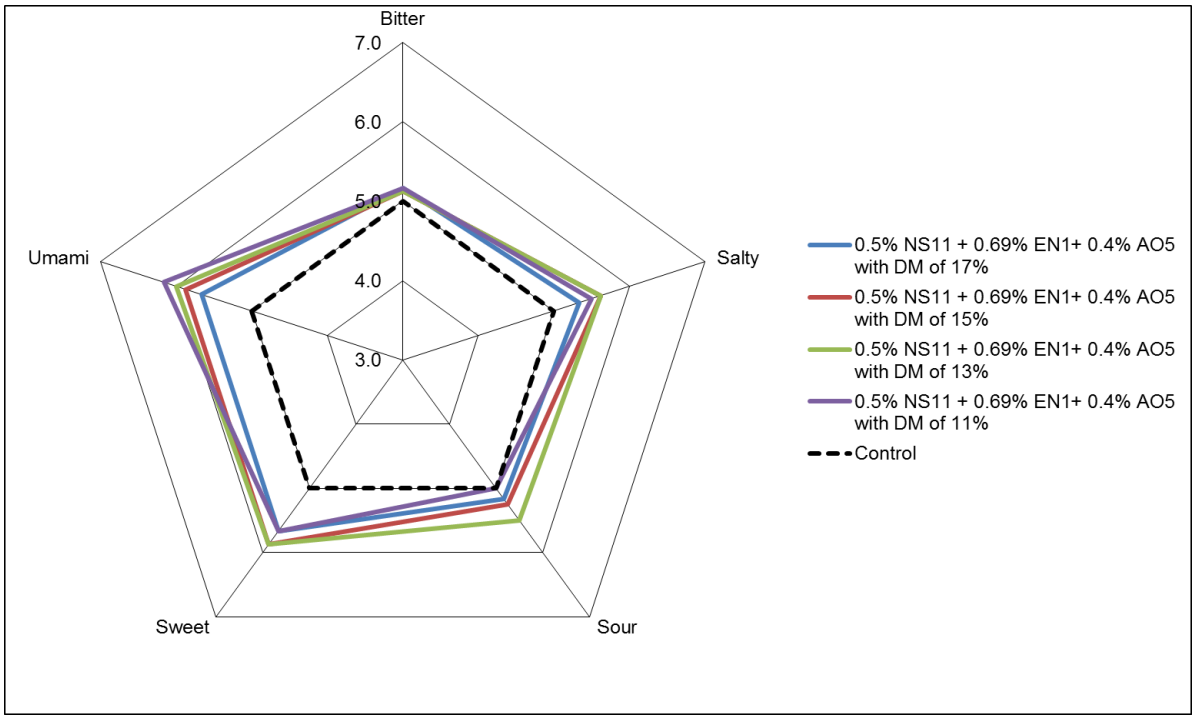


Figure 3.13 QDA analysis: sensory evaluations of TYCW with different starting DM (11%, 13%, 15% and 17%). Graphical display of sensory attributes based on QDA result. The scores of taste were 3-7, which represent intensities of none, weak, middle, strong and very strong.

Sensory evaluations of TYCW with different starting DM (around 11%, 13%, 15% and 17%), in application, have similar taste profiles as it is shown in Figure 3.13. Comparing with control (only vegetable broth) all samples show an increase of intensity of all attributes (umami, sweet, salty, sour and bitter). Sample with a starting DM of ~11% seems to be the one with higher umami taste profile which is something desirable for a good YE. The lower the starting DM, the higher the umami taste of the samples in application. Moreover, sample with starting DM of ~13% show a higher intensity of sweet and sour attributes.

Accordingly, after optimizing the conditions, a DM of ~11% (w/w) was chosen to continue the study to obtain a new YE.

3.3.4 AO5 dosage

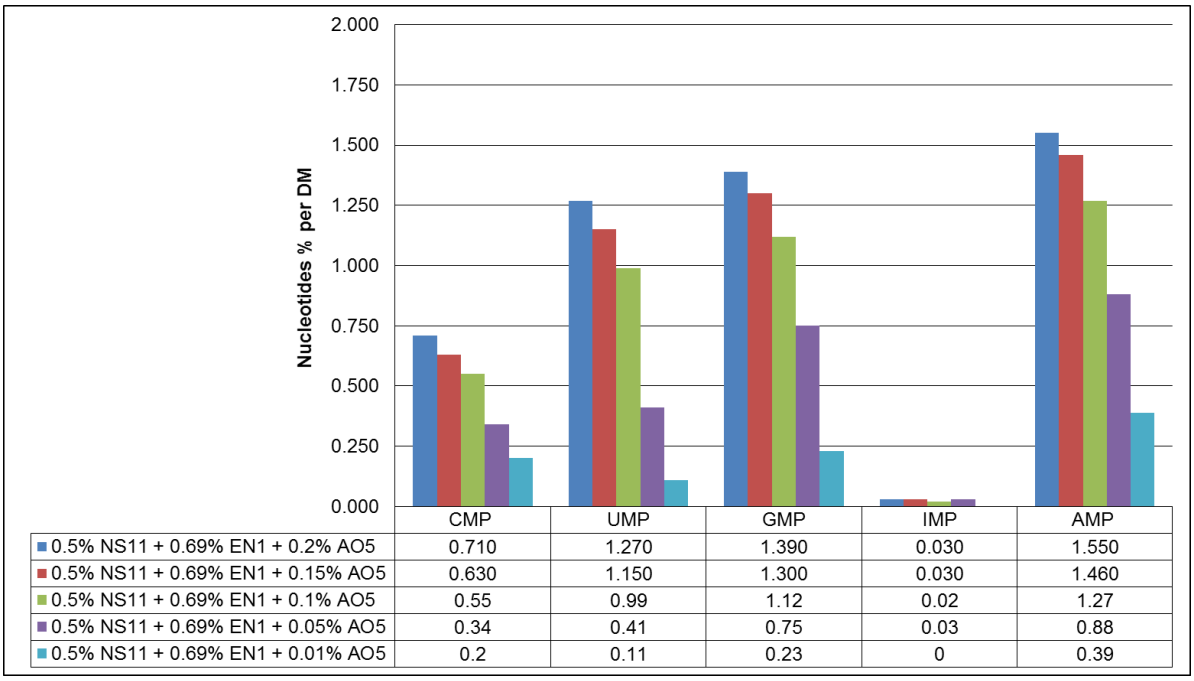
In section 3.3.2 was mentioned that NS11, EN1 and AO5 seem to be the best enzyme combination and in section 3.3.3 it was chosen to continue with a starting DM of ~11%. Therefore it was optimized the dosage of AO5 in order to obtain a better yield % (w/w), good taste enhancer properties and high protein yield per DM. The standard deviation results of hydrolysates with different AO5 dosage are attached in Appendix 14.

Table 3.9 Hydrolysates characterization for a starting material with ~11% DM.

Enzymes		DM % in SN	Yield % per DM	Protein % per DM
0.5% NS11 + 0.69% EN1 +	-	5.345	32.896	69.793
	0.2% AO5	5.880	36.743	69.910
	0.15% AO5	5.570	34.740	74.732
	0.1% AO5	6.280	38.869	62.998
	0.05% AO5	6.490	40.582	62.340
	0.01% AO5	6.510	40.733	61.231

Results presented in Table 3.9 show the influence of different dosages of AO5 on DM%, yield% (w/w) and protein yield during hydrolysis. It seems that NS11 and EN1 influenced the content of protein % per DM. Thus, in a general way all samples had a high content of protein % per DM. AO5 had a major impact on DM and yield values. The highest values were obtained as ~ 41% on dry solid basis for 0.01% (w/w) and 0.05% (w/w) dose rate of AO5. There was slightly difference on the remaining samples as ~39% at dose rate of 0.1% (w/w), ~35% at dose rate of 0.15% (w/w) and ~37% at dose rate of 0.2% (w/w).The amount of solid released from cells during hydrolysis considerably increased by use of lower dosages of AO5.

Protein yield, solid content and yield were affected by dosage, temperature, pH, shaking and centrifugation.



	CMP	UMP	GMP	IMP	AMP
0.5% NS11 + 0.69% EN1 + 0.2% AO5	0.710	1.270	1.390	0.030	1.550
0.5% NS11 + 0.69% EN1 + 0.15% AO5	0.630	1.150	1.300	0.030	1.460
0.5% NS11 + 0.69% EN1 + 0.1% AO5	0.55	0.99	1.12	0.02	1.27
0.5% NS11 + 0.69% EN1 + 0.05% AO5	0.34	0.41	0.75	0.03	0.88
0.5% NS11 + 0.69% EN1 + 0.01% AO5	0.2	0.11	0.23	0	0.39

Figure 3.14 5'mononucleotides content as % of total dry solids for different concentrations of AO5, with a starting DM= ~11%.

Reduction in the dose rate of AO5 (0.05% and 0.01% (w/w)) resulted in a lower rate of production and a lower final yield of 5'GMP (Figure 3.14) than the ones achieved with the dose rate of 0.2%, 0.15% and 0.1% (w/w). Therefore our results indicate that 5'-IMP was not taking part in combination of enzymes reaction, whereas 5'-GMP did. The total level of nucleotides was not bigger than about 5% (w/w) for all samples.

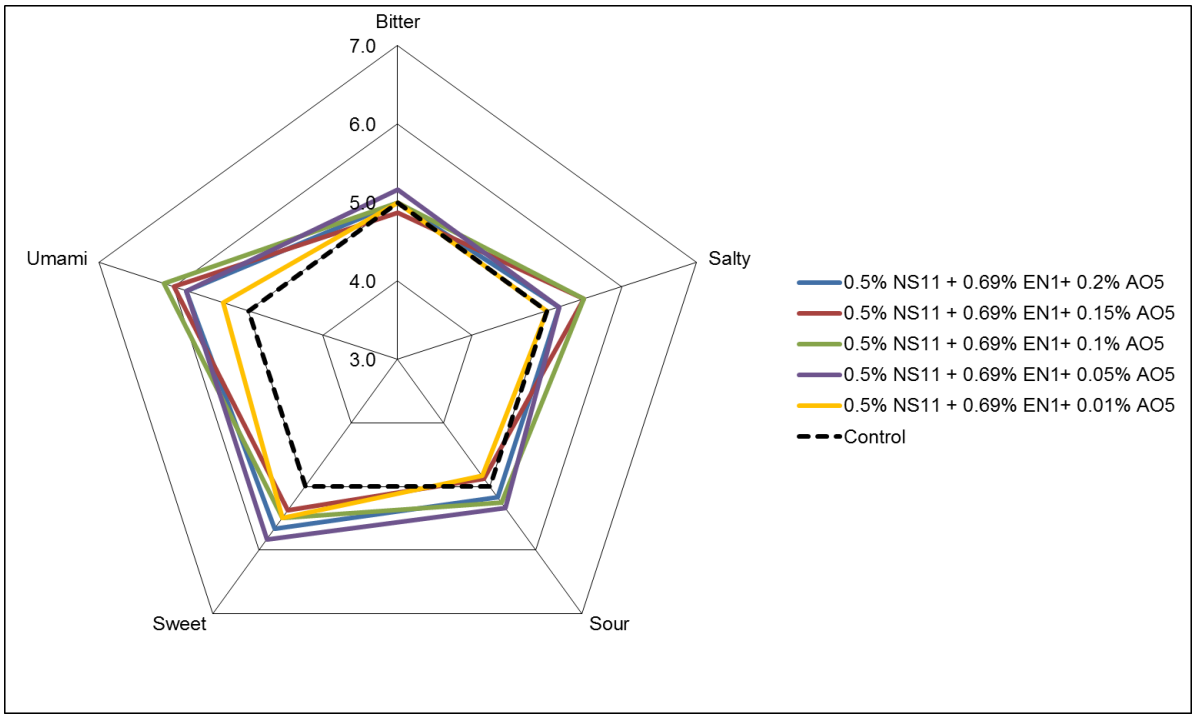


Figure 3.15 QDA analysis: sensory evaluations of hydrolysates with different concentrations of AO5. Graphical display of sensory attributes based on QDA result. The scores of taste were 3-7, which represent intensities of none, weak, middle, strong and very strong.

The taste profiles in Figure 2 show that 0.2% (w/w), 0.15% (w/w) and 0.1% (w/w) of AO5 in application led to a higher intensity of attributes “umami”, “sweet”, “salty” and “sour” than the control. The trend shows that the intensity of these attributes is nearly the same when using the dose rates of AO5 mentioned above. This indicates that is possible to achieve a savory YE using 0.1% (w/w) of AO5. Moreover, the sensorial analysis found that the samples with 0.05% and 0.01% of AO5 had weaker taste enhancing properties than the sample with 0.1% AO5.

Table 3.10 Hydrolysates (0.5% NS11 + 0.5% EN1 + 0.1% AO5) average characterization for TYCW with ~11% DM.

Enzymes	Starting DM %	DM %	Yield % per DM	Protein % per DM
0.5% NS11 + 0.5% EN1 + 0.1% AO5	11	5.755	35.63443	68.89122

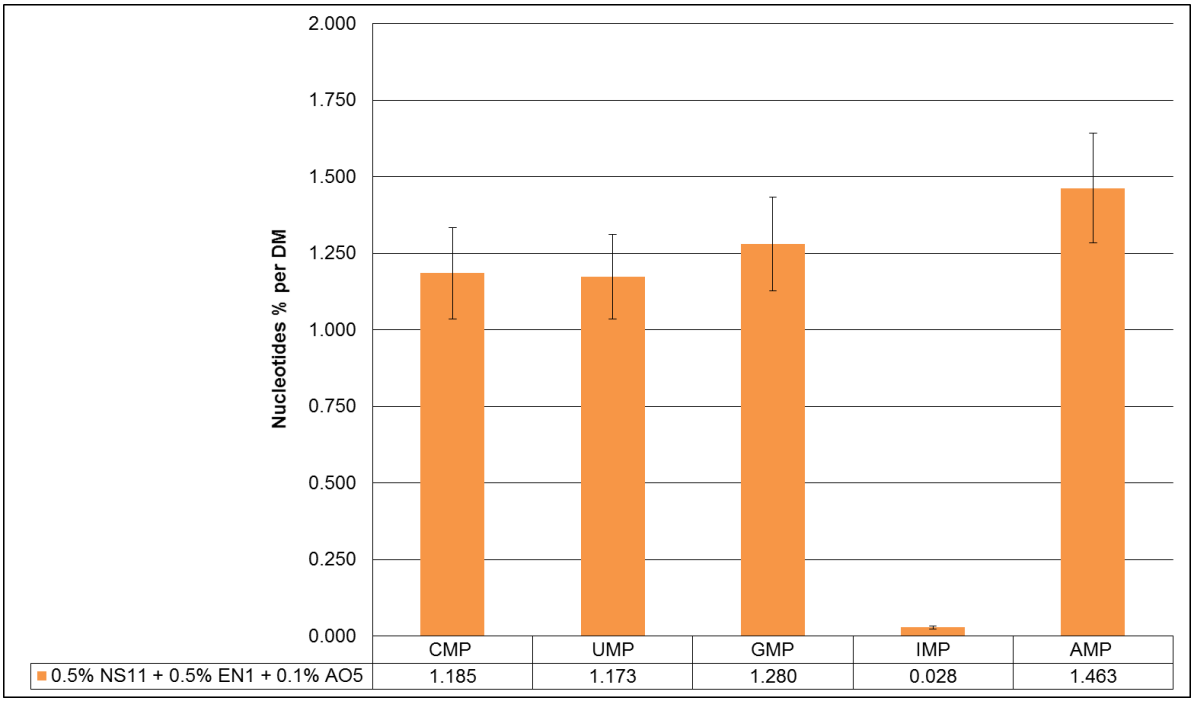
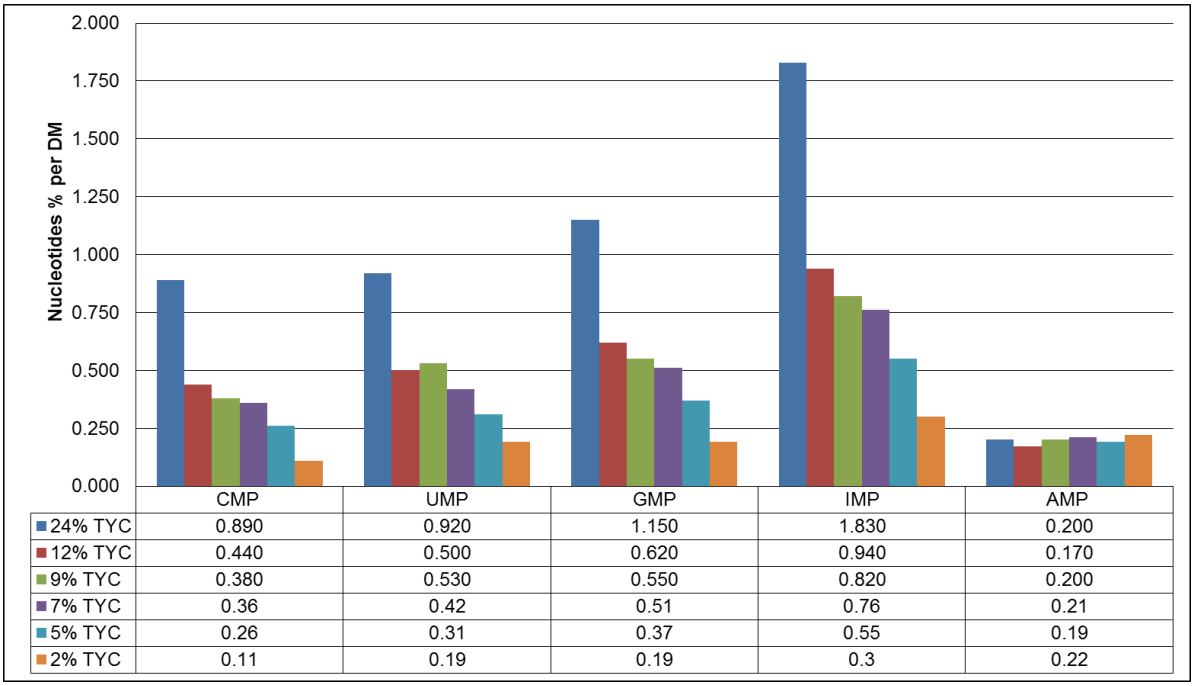


Figure 3.16 5'mononucleotides content as % of total dry solids for the possible final yeast extract (0.5% NS11 + 0.5% EN1 + 0.1% AO5), with a starting DM=~11%.

According with previous results it was found out that the best enzyme combination was 0.5% NS11 + 0.5% EN1 + 0.1% AO5 per DM. Thus, it was done an average of our results by a repetition of enzymatic reaction in order to support the possible final YE recipe. The dosage of 0.1% of AO5 was the lowest dosage achieved with a good taste, a DM ~5.8%, a yield around ~36% per DM, and a protein yield of ~69% per DM and content of 1.28% 5' GMP (of total dry solids) (Table 3.10 and Figure 3.16).

3.3.5 Supplementation with TYC

Torula yeast cell wall (TYC) contains high levels of 5'IMP and 5'GMP. In combination with TYCW with 0.5% (w/w) of NS11 and 0.69% (w/w) of EN1, TYC was used instead of AO5 to provide the 5'-nucleotides necessary for the good taste properties of the new YE.



	CMP	UMP	GMP	IMP	AMP
24% TYC	0.890	0.920	1.150	1.830	0.200
12% TYC	0.440	0.500	0.620	0.940	0.170
9% TYC	0.380	0.530	0.550	0.820	0.200
7% TYC	0.36	0.42	0.51	0.76	0.21
5% TYC	0.26	0.31	0.37	0.55	0.19
2% TYC	0.11	0.19	0.19	0.3	0.22

Figure 3.17 5'mononucleotides content as % of total dry solids for different dosages of TYC.

Figure 3.17 show that highest the dosage of TYC, the highest the 5'-GMP and 5'-IMP contents were taking part in combination of enzymes reaction. Reduction in the dose rate of TYC (2% and 5% (w/w)) resulted in a lower rate of production and a lower final yield of 5'-nucleotides than the ones achieved with the dose rate of around 7%, 9%, 12% and 24% (w/w). The total level of nucleotides was not bigger than about 5% (w/w) for all samples.

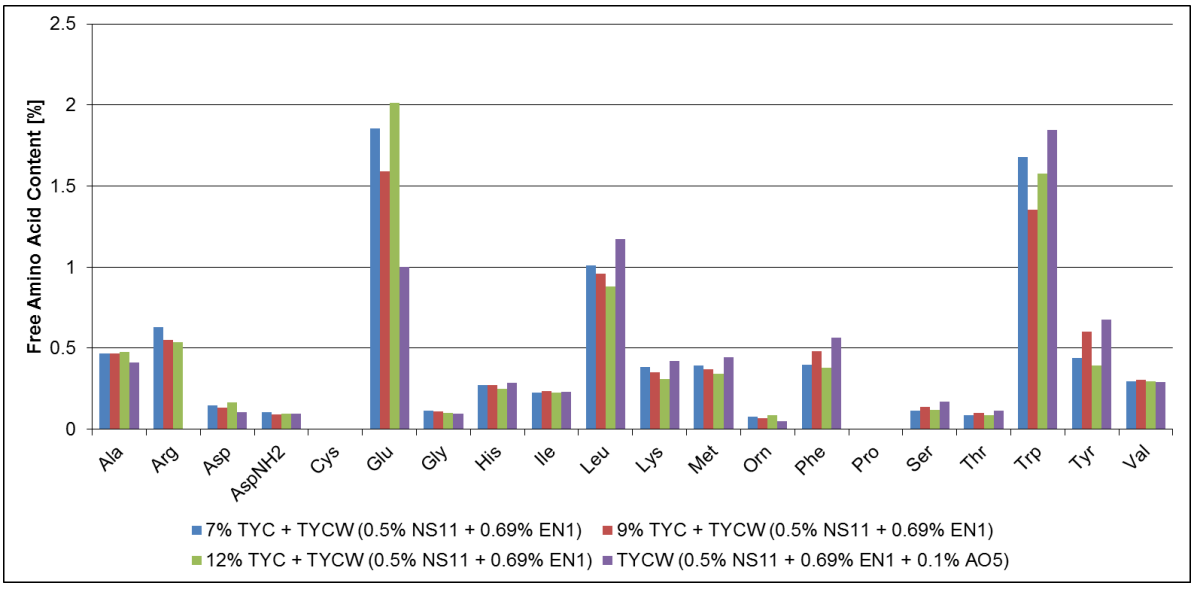


Figure 3.18 Free AA content for different concentrations of TYC and also AO5, with a starting DM= ~11%.

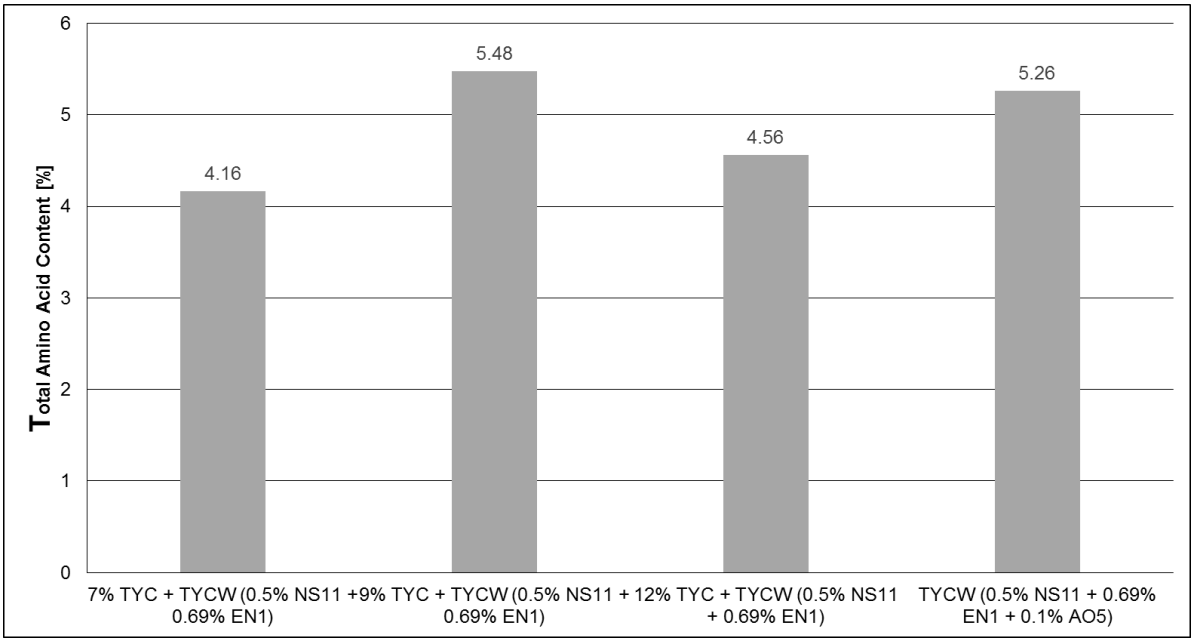


Figure 3.19 Total AA content for different concentrations of TYC and also AO5, with a starting DM= ~11%.

Figure 3.18 and Figure 3.19 show the profile of free AA and total AA, respectively. The content of free and total AA is low for all hydrolysates. In case of total content of AA the samples show a similar profile, ranging from ~4 to ~5.5% (w/w). The analysis for all 20 free AA analyzed show the highest amounts for glutamic acid (Glu) and tryptophan (Trp), however as it is possible confirm sample with AO5 has a profile a lit bit lower than the ones with TYC.

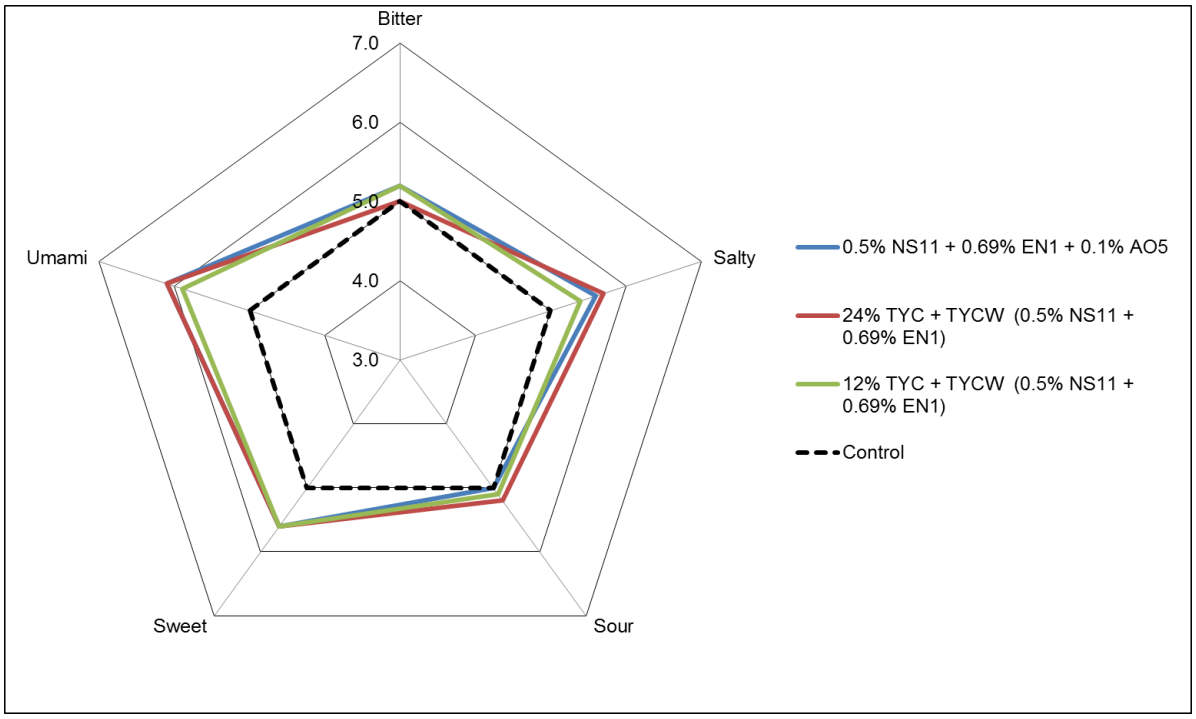


Figure 3.20 QDA analysis: sensory evaluations of hydrolysates with different concentrations of TYC vs the one with 0.1% (w/w) AO5. Graphical display of sensory attributes based on QDA result. The scores of taste were 3-7, which represent intensities of none, weak, middle, strong and very strong.

The taste profiles in Figure 3.20 show that hydrolysates obtained with AO5 and TYC, in application, led to a higher intensity of attributes umami, sweet and salty than the control (only vegetable broth). Samples with AO5 and ~12% (w/w) of TYC are a bit bitterer than control and the sample with TYC a bit sourer than control. There results allowed us to change the TYC dosage in order to found out a good YE but using the less dosage rate of components as possible.

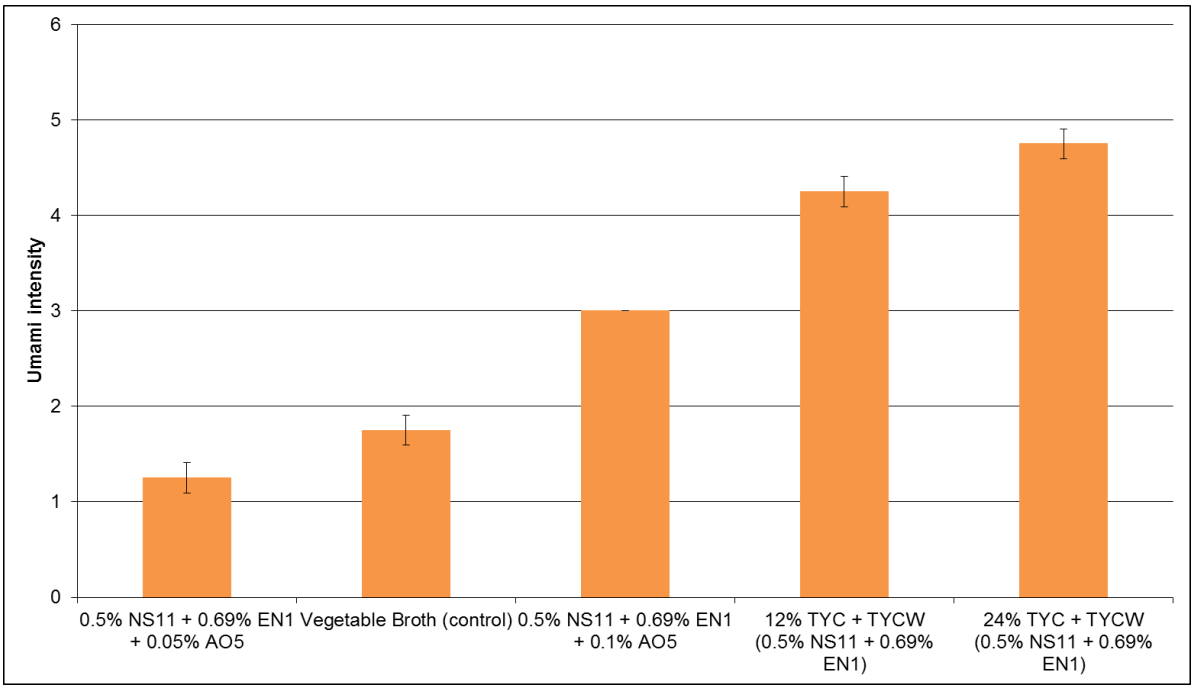


Figure 3.21 Ranking test for umami intensiy, comparing differents hydrolysates as % of total dry solids.

Table 3.11 Samples whose rank sums differ by more than LSD rank= 8.8 are significantly different at the α=0.05 (1: 0.5% NS11 + 0.69% EN1 + 0.05% AO5 per DM; 2: control; 3: 0.5% NS11 + 0.69% EN1 + 0.1% AO5 per DM; 4: 12% TYC + TYCW (0.5% NS11 + 0.69% EN1) per DM; 5: 24% TYC + TYCW (0.5% NS11 + 0.69% EN1) per DM.

Comparisons	Difference in sum of ranks	Significantly different
1vs2	2	No
1vs3	7	No
1vs4	7	No
1vs5	12	Yes
2vs3	5	No
2vs4	10	Yes
2vs5	12	Yes
3vs4	5	No
3vs5	7	No
4vs5	2	No

A ranking test for umami intensiy was done to found out the best combination for the new YE (Figure 3.21 and Table 3.11). Table Upper-α Probability Points of X^2-Distribution was used to find that α=0.05 (90% of confidence) critical value of a X^2 with 4 degrees-of-freedom is 9.49. As the value of T=14.8 was bigger than 9.49, it was concluded that the samples were significantly different. To determine which samples were significantly different, it was calculate the critical value of the multiple comparison Least Significant Difference (LSD) rank=8.8.

Any two samples whose rank sums differ by more than LSD rank= 8.8 are significantly different at α=0.05 [72]. Therefore, sample with 0.05% (w/w) AO5 shows a significantly difference from the one with ~24% (w/w) of TYC. Control (only vegetable broth) was significantly less intense from the samples with TYC. Samples with TYC and the one with 0.1% (w/w) of AO5 were not significantly different from each others.

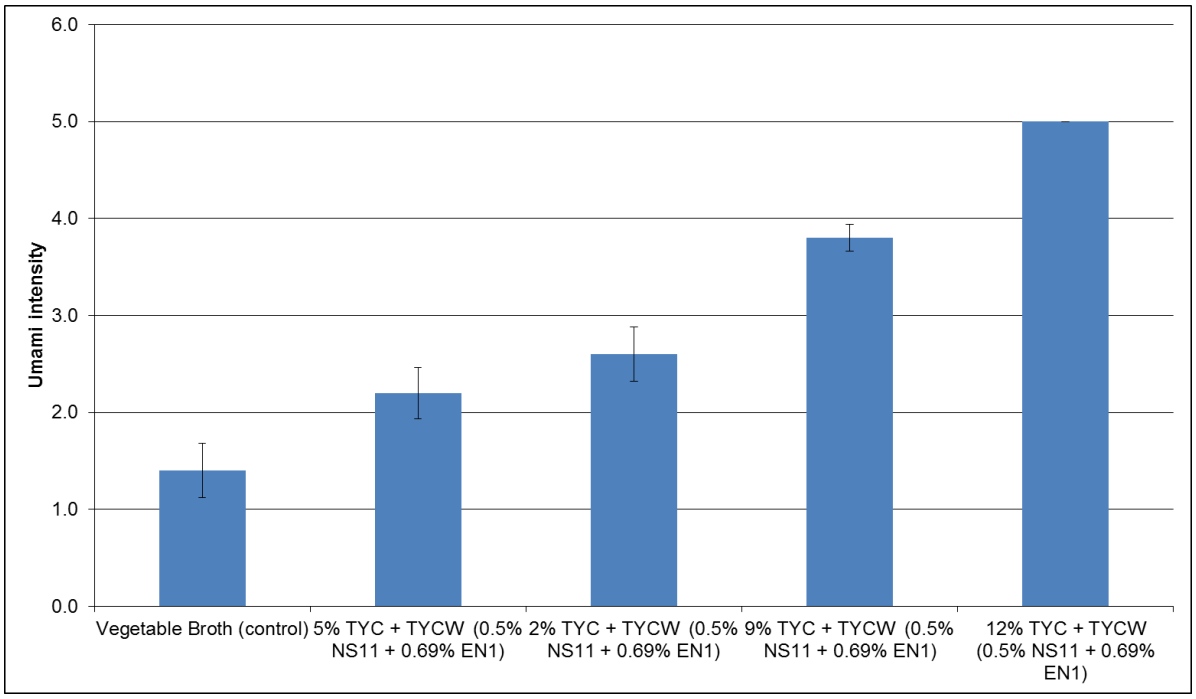


Figure 3.22 Ranking test for umami intensity, comparing hyrolysates with different concentrations of TYC as % of total dry solids.

Table 3.12 Samples whose rank sums differ by more than LSD rank= 9.8 are significantly different at the α=0.05 (1: control; 2: 5% TYC + TYCW (0.5% NS11 + 0.69% EN1) per DM; 3: 2% TYC + TYCW (0.5% NS11 + 0.69% EN1) per DM; 4: 9% TYC + TYCW (0.5% NS11 + 0.69% EN1) per DM; 5: 12% TYC + TYCW (0.5% NS11 + 0.69% EN1) per DM.

Comparisons	Difference in sum of ranks	Significantly different
1vs2	4	No
1vs3	6	No
1vs4	12	Yes
1vs5	18	Yes
2vs3	2	No
2vs4	8	No
2vs5	14	Yes
3vs4	6	No
3vs5	12	Yes
4vs5	2	No

According with previous results it was possible decrease the dosage of TYC for values lower than 12% (w/w) of TYC. As the value of T=16 was bigger than 9.49, it was concluded that the samples are significantly different. To determine which samples are significantly different, it was calculated the critical value of the multiple comparison LSD rank=9.8.

Any two samples whose rank sums differ by more than LSD rank= 9.8 are significantly different at α=0.05 [71]. Therefore, it was concluded that control shows significantly less persistence of umami taste than others four and it is significantly different from samples with ~9% (w/w) of TYC and ~12% (w/w) of TYC (Figure 3.22 and Table 3.12). Samples with ~5% (w/w) of TYC and ~2% (w/w) of TYC are significantly different from ~12% (w/w) of TYC. Sample with ~9% (w/w) of TYC is not significantly different from ~12% (w/w) of TYC. Therefore, the dosage of TYC was decreased to ~7% (w/w).

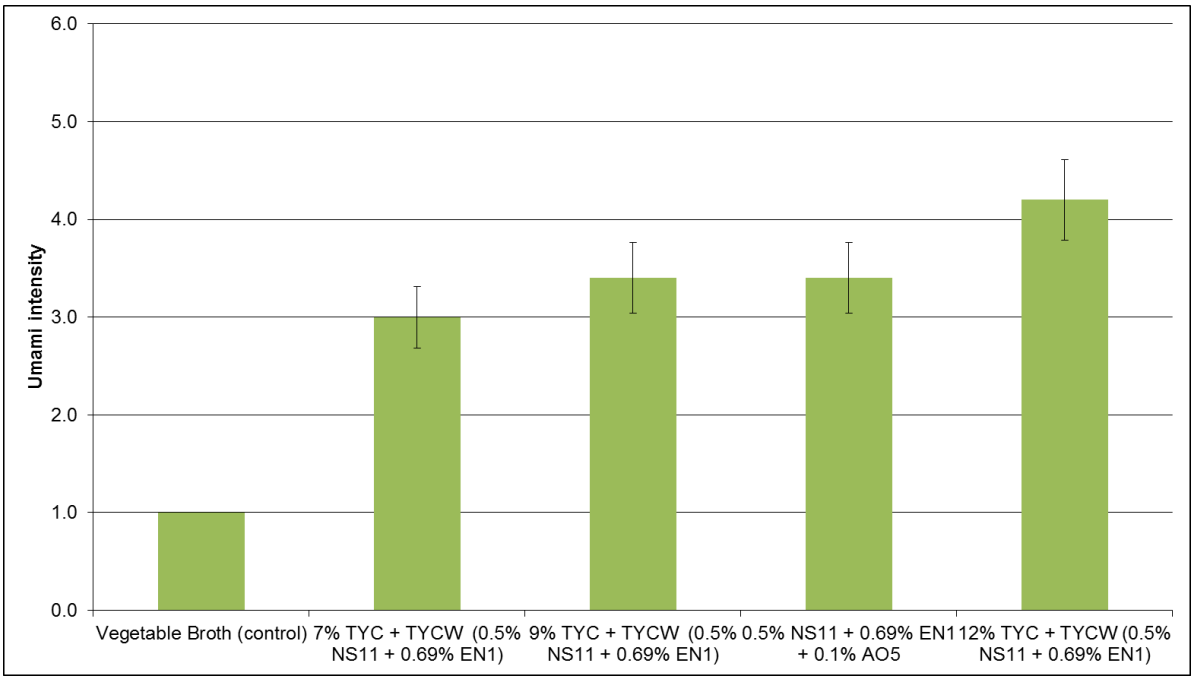


Figure 3.23 Ranking test for umami intensiy, comparing differentes hydrolysates as % of total dry solids.

Table 3.13 Samples whose rank sums differ by more than LSD rank= 9.8 are significantly different at the α=0.05 (1: control; 2: 7% TYC + TYCW (0.5% NS11 + 0.69% EN1) per DM; 3: 9% TYC + TYCW (0.5% NS11 + 0.69% EN1) per DM; 4: 0.5% NS11 + 0.69% EN1 + 0.1% AO5 per DM; 5: 12% TYC + TYCW (0.5% NS11 + 0.69% EN1) per DM.

Comparisons	Difference in sum of ranks	Significantly different
1vs2	10	Yes
1vs3	12	Yes
1vs4	12	Yes
1vs5	16	Yes
2vs3	2	No
2vs4	2	No
2vs5	6	No
3vs4	0	No
3vs5	4	No
4vs5	4	No

As the value of T=16 was greater than 9.49, it was concluded that samples are significantly different. To determine which samples were significantly different, it was calculate the critical value of the multiple comparison LSD rank=9.8 [71].

Any two samples whose rank sums differ by more than LSD rank= 9.8 are significantly different at α=0.05 [71]. Control shows significantly less persistence of umami taste than others four (Table 3.13). The remaining samples are not significantly different from each other which means that it's possible decrease the TYC dosage to ~7% (w/w).

Results presented in Figure 3.23 show a ranking test for umami taste intensity with different dosages of TYC comparing with best combination of enzymes used in TYCW (0.5% NS11+0.69% EN1+0.1% AO5) per DM. In this case the lowest dosage achieved was ~7% (w/w) of TYC. However due to the high cost price associated with TYC, this option was deemed non-feasible.

3.4 Discussion

3.4.1 Effects of different cell wall lysis enzymes and protease treatment on the recovery of solid and protein

As mentioned before as consequence of the Prime process, TYCW still contain a significant amount of protein which is ~57%. Based on this information and in a previous feasibility study it was decided to do a new YE.

Carbohydrases and phospholipases can cleave the YCW components and may increase protein yield by liberating more protein from the matrix source. A combination of CW-hydrolyzing enzymes has been used to cleave linkages within the polysaccharide matrix effectively and hence, liberate more intracellular protein from YCW. In the last few years, different proteases, alone or in combination, have been used to hydrolyze proteins to peptides, increasing their solubility and making them more easily extractable [130]. Moreover, proteases may hydrolyze selected peptide bonds to promote reduction of MW, possible conformation changes, and enhanced hydrophobicity due to newly expose amino and carboxyl groups. Proteolysis can increases solubility and decrease viscosity [7]. This means that protease treatment is very important in solubilization and recovery of cellular components [131].

The effect of different CW lysis enzymes (carbohydrases, phospholipases, proteases and nuclease) on the recovery of solid and protein of TYCW were investigated at first. The yield is the number of hydrolysed molecules per number of starting molecules which is an important parameter to understand how much of the YCW was solubilized in order to obtain the much as possible, to be sustainable produce the new yeast extract [122]. Carbohydrases and phospholipases were considered for the hydrolysis of the TYCW, however those enzymes did not result in a YE with superior analytical properties (Appendix 13). The recovery of solid and protein after disruption of YCW treated with carbohydrases and phospholipases were evidently lower than those treated by proteases and nuclease. SN8 (glucanase activity), AO3 (xylanase activity), P-NE1 (hydrolyzes phospholipids) and P-N1 (hydrolyzes esterbonds in glycerides) were chosen based on Chaper 2.3 results, however in this study the recovery of solids and proteins were not significant compared with action of others combinations [58]. For example, Kalum *et al*. (2007) did a study with 0.5% Acalase 2.4L + 0.25% of Lecitase ultra resulting in 67.4% and 66.8% of proteins and solids recovery, respectively [132]. In our case, we tried to combined a protesase+phospholipase but the results were lower than expected, e.g. using 0.5 % NS11+ 0.5% P-N1 have lead to ~48% and ~15% of protein and solids recovery, respectively. Phospholipases were study in Chapter 2 and the results have shown lower yields for the hydrolysates obtained which may explain our results in this second part. Moreover, we are doing a second extraction with enzymes treatment which in a general way can justify the lowers yields of solids and proteins in some cases.

Afterwards AO1, SN9, SN10, and AO4 which are enzymes with high protease activity were used, leading to a good recovery of solids and proteins. During sensorial analysis ~1% (w/w) of 5'-GMP was added to the hydrolysates containing AO4, AO1 and AO3 which resulted in a good taste profile. Therefore all enzymes combination with NS11 mentioned above did not result in a YE with

superior analytical or sensorial properties when compared with combination of NS11+EN1+AO5. Moreover, NS11 alone had a better recovery of protein than in combination with carbohydrases, phospholipases or other proteases. Treatment with CW lysing enzymes (e.g. glucanase like SN8) are not applicable on a large scale because of low yields and high costs associated [133].

NS13 is a serine endoprotease that hydrolyzes internal peptides bonds and shows a similar activity profile as NS11, leading to a good solid and protein recovery. Comparatively, NS12 is an exoprotease that liberates free AA by hydrolysis of the N-terminal and did not affected the recovery of solids and proteins as expected. Chain *et al*. (2001) reported high solid recovery ~48.3±53.1% and a good flavour profile when the yeast cells were treated using an optimal combination of endoprotease and exoprotease (0.6% Protamex and 0.6% Flavourzyme). In fact, they had the highest protein recovery with Flavourzyme instead Protamex, indicating that exoprotease rather than endoprotease was a key factor in yeast protein hydrolysis [131]. A possible explanation for NS12 results could be the disruption method first adopted and later standardized with a shaking at ~138rpm and a phase separation performed for 20min at 4700rpm, leading to a better mixture homogenization stimulating enzyme activity in case of enzymes combinations performed in this way. An additional information which may support this explanation is the results obtained during the feasibility study where the results have shown a good recovery of solids and proteins (data not shown). For instance, Verduyn *et al*. (1999) reported an increase of yield solids and proteins after high pressure homogenization pre-treatment in combination with enzyme action. Homogenization alone led to poor solids recoveries [133].

NS11 obtained from *Bacillus licheniformis* is a serine endopeptidase (EC 3.4.21.1) which is used to hydrolyze proteins with preferential cleavage at Tyr, Phe, Leu and Met resulting in smaller proteins and peptides [134]. This serine protease has a strong specificity for Glu residue and it has broad applications in the fields of protein analysis, peptide synthesis and recovery [135].

EN1 obtained from *Carica papaya* is a cysteine protease (EC 3.4.22.2) that hydrolyzes proteins into peptides and free AA. It is stabilized by three disulfide bridges in which the molecule is folded along these bridges creating a strong interaction among the side chains. Its three-dimensional structure consists of two distinct structural domains with a cleft between them. This cleft contains the active site, which contains a catalytic dyad that has been likened to the catalytic triad of chymotrypsin. EN1 occurs in all parts of the tree except the root [136].

In the study of Dolińska *et al.* (2012), vegetable protease EN1 was the most effective for the production of yeast hydrolysates. Similarily, EN1 and NS11 showed the highest hydrolysis efficiency in our assay. Therefore, it is a better choice for YE production [137]. The recovery using these enzymes was significantly improved, indicating that they degraded insoluble proteins and glycoproteins into soluble proteins, peptides and AA effectively [138]. However, NS11 seems to be more efficient on solids recovery than EN1. The increase of NS11 and EN1 dosage over 0.5% (w/w) provided no more improvement to the solid and protein recovery. Therefore, the optimal dosage of both enzymes was determined as 0.5% and the recovery of solid and protein under this condition was beteween 33 to 36% (w/w) and 62 to 69% (w/w), respectively. In this study, the dosage of EN1 was the used in the same range of values that some studies have already reported which ate

between 0.2 to 2.5% [55, 58, 138]. In the report by Guan *et al*. (2013) the optimal dosage for a protease as EN1 was 0.2% and the maximum solid and protein recovery was 67.02 and 61.62%, respectively [138]. The optimal dosage of protease reported by Milić *et al*. (2007) was 2.5% and under this dosage the highest recovery of solids was 59.84% [55]. In our case we used a dosage of 0.5% which we consider a good one because our hydrolysate resulted from a second extraction. In previous reports, the treatment time of protease for the production of YE was in the range of 16-24h, which was in the same range of values as ours, less in Guan *et al*. (2013) case which the treatment time was around 6h [58, 137, 138]. However, the protein recovery strongly depended on the enzyme dosage [131].

After CW disruption of TYCW, proteolytic enzymes were employed to hydrolyse intact proteins into small molecular peptides and free AA, followed by the utilization of the nuclease AO5 [143]. AO5, obtained from *Penicillium citrinum*, is an extracellular enzyme (EC3.1.30.1; Nuclease 5'-Nucleotidehydrolase, 3'-Phosphohydrolase). This enzyme cleaves 5'-nucleotides (e.g. 5'-GMP) successively from both 3'-5'-phosphodiester bonds in RNA, forming mononucleotides and oligonucleotides (which have 3'-OH and 5'-PO_4 termini). This enzyme can not cleave phosphodiester bonds with a basic 5'nucleotide, moreover the removal of the base of the 3'-nucleotide does not affect hydrolysis. However the aromaticity of the 5'-base affects the activity, suggesting that it may be involved in a stacking interaction with an aromatic protein side chain. Nuclease shows some dependence of its cleavage rates on the identity of the base 5' to the cleaved bond, reinforcing its importance. Taking into account economic constraints and facility to large-scale production, this enzyme is a good choice [139, 140].

From all enzymes combination, NS11, EN1 and AO5 seem be the one with the highest recovery of solids and proteins and also with the best sensorial properties as it will be discuss below.

3.4.2 Optimization of nuclease treatment

During this study the low yield % (w/w) was always an issue. Therefore, some changes as shaking standardized at ~138rpm, a phase separation performed at maximum (for 20min at 4700rpm) and the use of different dry matter of TYCW, were made. Four different starting DM (around 17%, 15%, 13% and 11% (w/w)) where analysed using the same conditions and enzymes combinations and dosage (0.5% NS11 + 0.69% EN1 + 0.4% AO5). From all hypothesis, a starting DM of ~11% seems to be the best option, resulting in a solid recovery of 35% (w/w), in a protein recovery of ~68% (w/w) and in 5'-GMP content of 1.29%. This can mean that a lower content of solids in the starting material leads to lower viscosity and better homogenization, resulting in higher activity of the enzymes combination.

Guo-Quing *et a*l. (2016) studied the effect of pH and temperature on enzyme activity and stability. The optimum pH of the purified nuclease p1 was determined as 5.4. The authors showed that a pH over 6.0 leads to a lost of more than 50% of the relative activity, conclunding that this nuclease needs acidic pH to perform its reaction. The optimum temperature reported for the nuclease was 69°C, using RNA as a substrate. The relative activity increased with the increasing temperature from 50° to 69°C and then drecreased [139]. Ying *et al*. (2007) reported that nuclease

p1 remained stable in a pH range of 4.0-6.0 and the optimum temperature was 68°C [141]. This studies and previous assays performed with AO5 at Ohly, supported the conditions defined for optimization of this nuclease activity.

Effects of enzyme combination, enzyme dosages and treatment sequence on the recovery of solid and protein, flavor and compositional characteristics were investigated [131]. To determine optimum nuclease (AO5) concentration for a good umami taste, the hydrolysate prepared with 0.5% of proteases (NS11+EN1) at pH 7.0 and 60°C for 16/17h was subsequently treated with AO5 at different concentrations (0.01-0.2% (w/w)) at pH 5.1 and 68°C for 8h. Proteases (0.5% NS11 + 0.69% EN1) treatment exhibited the highest solid recovery (~33%) and protein recovery (~70%). When trated with nuclease (AO5) the solid and protein recovery did not increase. This indicated that protease treatment is very important in solubilization and recovery of cellular components [139]. 5'-GMP content was strongly affected by the enzyme treatment sequence. Guo-Quing *et al*. (2016) reported a lower 5'-GMP content (0.03%) when the treatment was done only with protease. After the adition of the nuclease the 5'-GMP increased up to 0.11%. When TYCW is treated with proteases before nuclease, proteases break down the insoluble protein and protein-bound intracellular molecules to small molecules, facilitating action of nuclease. Then, the treatment of proteases (NS11 and EN1) followed by AO5 appeared to be desirable for hydrolysis of yeast components [139].

When the dosage of AO5 reached 0.1% (w/w), the 5'-GMP content peaked at ~1 % which was the value from which the umami taste started to be perceptible. The optimal dosage of nuclease reported by Guan *et al*. (2013) was 0.045%. At that point the content of 5'-AMP and 5'GMP were 2.45 and 1.70%, respectively which in total was 4.15%. In our study we did not use a deaminase to convert 5'-AMP into 5'-IMP after the treatment of cell disrupting, explaining the lowers values of AMP [138]. Chae *et al.* (2001) reported the solid and protein recovery and 5'-GMP content increased as the nuclease dosage increased, but reached a maximum value at 0.03% of the nuclease [131]. Reduction in the dose rate of AO5 (0.05% and 0.015% (w/w)) resulted in a lower rate of production and a lower final yield of 5'GMP than that achieved with the dose rate of 0.1% (w/w)). In our study, the lower dosage of AO5 achieved was 0.1% (w/w), leading to a recovery of solids and proteins ~36% and ~69%, respectively and to 1.28% of 5'-GMP.

3.4.3 Sensorial analysis: hydrolysates in application

YE usually require a high protein content in food applications where free AA and short peptides are important for the taste [142]. Dosage and combination of the enzymes used for the production of YE are important parameters in sensory properties of hydrolysates [131].

The sensory characteristics of the possible YE produced by the different enzymes combinations treatment were evaluated using a QDA analysis. The average results of sensory evaluation were depicted in spider plots, and separated into taste attributes (sour, sweet, umami, bitter and salty).

In the first screening, the hydrolysates obtained with NS11+EN1+AO5; NS11+AO4 and NS11+AO1 ranked highly in overall acceptance were selected for a second screening based on taste. In the second sensorial analysis it was added 1% of GMP to samples with AO4, AO1 and AO3,

resulting in high umami taste comparing with previous results however hydrolysates with AO5 resulted in the highest sensory profile of umami, salt, sweet and sour taste.

During the sensory analysis bitter taste was not evidenced, in some cases. Moreover, at the same time umami taste was more intense in those samples as e.g. hydrolysates with AO5. Studies about umami-bitter interactions with taste receptors, showed that umami peptides suppressed bitter taste by binding to bitter-taste receptor(s) [143, 144]. Kim *et al*. (2017) found out that that both free AA and Glu-enriched oligopeptides play an important role in enhancement of umami taste, and inhibit the binding of bitter material(s) to bitter taste receptor(s), e.g., hTAS2R43 and hTAS2R46 [145]. The decrease of unpleasant bitterness, derived from bitter free amino acids, hydrophobic bitter peptide fractions, $CaCl_2$ and $P(H_2PO_4)$ may beattributed to the diminishing or masking effect of saltiness, umami taste, sourness and sweetness [131].

Bitter-tasting AA contribute intense umami tastes to monosodium glutamate/NaCl mixtures being a possible explanation for the salty enhancement of our hydrolysates resulting in a low sodium YE [62, 146]. Our sensorial profiles for salty and sour tastes can be related with hydrogen ions (H+) for acidity and, mainly, sodium ions (Na+) for salt. Some studies related that many cells in the tongue might be able to pick up these signals, transmitting the information in a complex pattern of nerve signals to the brain. Although some studies reported that PKD1L3 and PKD2L1 heteromers may have function as sour taste receptors [72, 73]. The “receptor” for salt (NaCl) is apparently an epithelial-type Na+ channel on the apical membrane of some taste cells [74].

Sweet taste can be explained by stimulation by sugars such as glucose and by other sweet substances such as saccharin, aspartame, and Acesulfam K. The T1R2-T1R3 receptor can bind all sweet molecules, sugars, some D-amino acids and sweet proteins [60, 71]. Temussi (2012) reported aspartame as a peptide known for the sweet taste [60].

The MW analysis of peptides profile support the low values of free AA. The MW distribution was similar for all combinations of enzymes and in case of hydrolysates obtained using AO5 suggests the higher content around 18% (w/w) of the protein in the hydrolysates is in the form of small size peptides less than 0.2 kDa, ~21% (w/w) of the protein is in form of peptides between 0.2-0.5kDa and ~47% (w/w) of the protein is in the weight range between 0.5-2kDa. Free AA in the YE are known to exert a major influence on flavor however in our study we did not achieve a high content of free AA [131]. GMP alone and in presence with a low concentration of glutamate do not elicit the umami taste, thus our umami profile can only be explained by the low MW of peptides (< 2kDa) and in AO5 case, umami taste is more intense probably because it is a synergism between peptides and 5’GMP [69]. Quin and Ding (2007) reported that the content of MSG-like taste free AA and low MW peptide fractions in Chinese douchiba was increased by interaction among these taste components, enhancing the umami taste [147]. The MW distribution analysis (ranging 120±1355 Da), of Chae and Joo (2001) study indicated an umami extract contained mostly low MW peptides, ~25.9% of free AA and 3.67% of 5’-GMP+IMP content [136]. Kim *et al*. (2017) identified the key of umami-active fraction in Korean soy sauce (mJGN), showing that soy sauce contains material of MW smaller than 500 Da [145]. Doenjang water extract had a characteristic umami taste profile, and was composed by proteolytic peptides with low MW (1000 > MW ≥ 500 Da) and higher molar ratio of

bound-type Glu and Asp, considered important contributors to umami taste of this extract [148]. Thus, the high umami taste present during our sensorial analysis can be explained by the small size of peptides ranging (2 > MW ≥ 0.2 kDa). Temussi (2012) reported that isolated peptides, ranging from dipeptides (such as H-Asp-Glu-OH) to H-Glu-Pro-Ala-Asp-OH, had mainly a sour taste. Thus, supported the idea that the small peptides do not suggest that umami compounds represent an independent class [60].

Taste characteristics of peptides are complex, but an association exists between the standard of component AA and the tastes of di- or tri-peptides. Peptides containing N-terminal Glu residues and hydrophilic C-terminal L-Glu residues, showed umami taste. The dipeptides and their AA (Asp, Thr, Ser, and Glu) were probably involved in umami taste. Studies with fish protein hydrolysates as Glu-Glu, Glu-Asp, Thr-Gly, Glu-Ser, Glu-Gly-Ser, Ser-Glu-Glu, Glu-Gln-Glu, Glu-Asp-Glu, and Asp-Glu-Ser showed umami taste. It was reported the same for chicken protein hydrolysates as Glu-Glu, Glu-Val, Ala-Asp-Glu, Ala-Glu-Asp, Pro-Glu-Glu, and Ser-Pro-Glu [145, 148]. Rhyu and Kim (2011) associated the stronger umami taste characteristics of Doenjang water extract to taste-active fractions with the highest molar ratio of Glu, followed by Asp, Gly, Ser or Lys or Thr [148]. Zhang *et al*. (2017) reported that di- or tripeptides did not show umami taste on their own, but they showed umami taste when they were mixed with 0.02% IMP, suggesting interactions between IMP and peptides [61]. In order to support our study will be interesting to found out which peptides are associated to strong umami taste however the examples mentioned before are possible hypothesis to support theory of synergism between small size peptides and 5'-GMP.

T1R1-T1R3 was established to be umami receptor. T1R1 and T1R3 have three regions: the large extracellular region, the seven-spanning transmembrane region, and the cytoplasmic region. In addition, the extracellular region can be divided into the ligand-binding region, a.k.a Venus flytrap module (VFM), and the cysteine-rich domain. Biding site for glutamate and 5'-IMP or GMP are in Venus flytrap of T1R1 subunit. Here glutamate binds close to the VFM along the hinge-bending motion, which leads to stabilization of the active conformation. The 5'-nucleotide (in our case 5'-GMP) binds to an adjacent site close to the binding site for glutamate. This leads to further stabilization of the active conformation. The structure of T1R1 is in dynamic equilibrium, where the ratio between the closed (active) and the open (inactive) conformations is modulated by the presence/absence of ligand. Thus the synergism is produced by an allosteric regulation [69, 70]. Chapter 1.7.2 explains where and how umami reception is done to transmit their signals to gustatory nerve fibbers. [69].

TYC contains high levels of 5'IMP+GMP and free glutamic acid. This YE was used to replace AO5. In this case the lowest dosage achieved was ~7% of TYC however was not enough to compete with the price of the enzymatic combination used in TYCW (0.5% NS11+0.69% EN1+0.1% AO5). In the presence of glutamate, 5'-GMP and 5'-IMPcompounds are known to enhance umami taste [50]. Synergism between glutamate and 5' nucleotides can explain the good umami taste when using TYC instead AO5 to obtained a new YE [69]. Supplementation with TYC instead of addition of AO5 would be too cost prohibitive and therefore the option was not further analysed.

3.4.4 Final yeast extract

In the hydrolysis of YCW, 0.5% (w/w) of each endoprotease (NS11+EN1) at pH 7.0 and 60°C for 16/17h strongly affected the recovery of protein and solids. The combined treatment of endoproteases and 0.1% of nuclease (AO5) at pH 5.1 and 68°C for 8h, as shaking standardized at ~138rpm, a phase separation performed at maximum (for 20min at 4700rpm), resulted in high solid and protein recovery (~36 and ~68%, respectively) and the best flavor profile. New YE had good enhancement properties, specifically umami and salty. Among various treatment sequences using multiple enzymes, the treatment with proteases followed by nuclease contained mostly low MW peptides ranging (2 > MW ≥ 0.2 kDa) and low free AA content (8% free AA) and 1.28% of 5'GMP.

Finally and because higher solid contents prevent microbial contamination by reduction of water activity, the final product using TYCW with (0.5% (w/w) of NS11 + 0.5% (w/w) of EN1 +0.1% (w/w) of AO5) was evaporated until reached a dry matter of ~50% (w/w) [128].

For the future, it will be interesting discover which peptides are associated to our strong flavour profile, to do another sensorial analysis with a bigger sample (e.g. 40 people at least) in order to support our results, accomplished by another QDA analysis referring taste, aromas, chemical feeling factors, odour, look, and consistency using different food applications to understand the potential of our new YE [39, 71]. In long-term, a large scale production could be analyzed associated to a new study involving an application development, feedback from test customers, shelf-life test and product launch plan.

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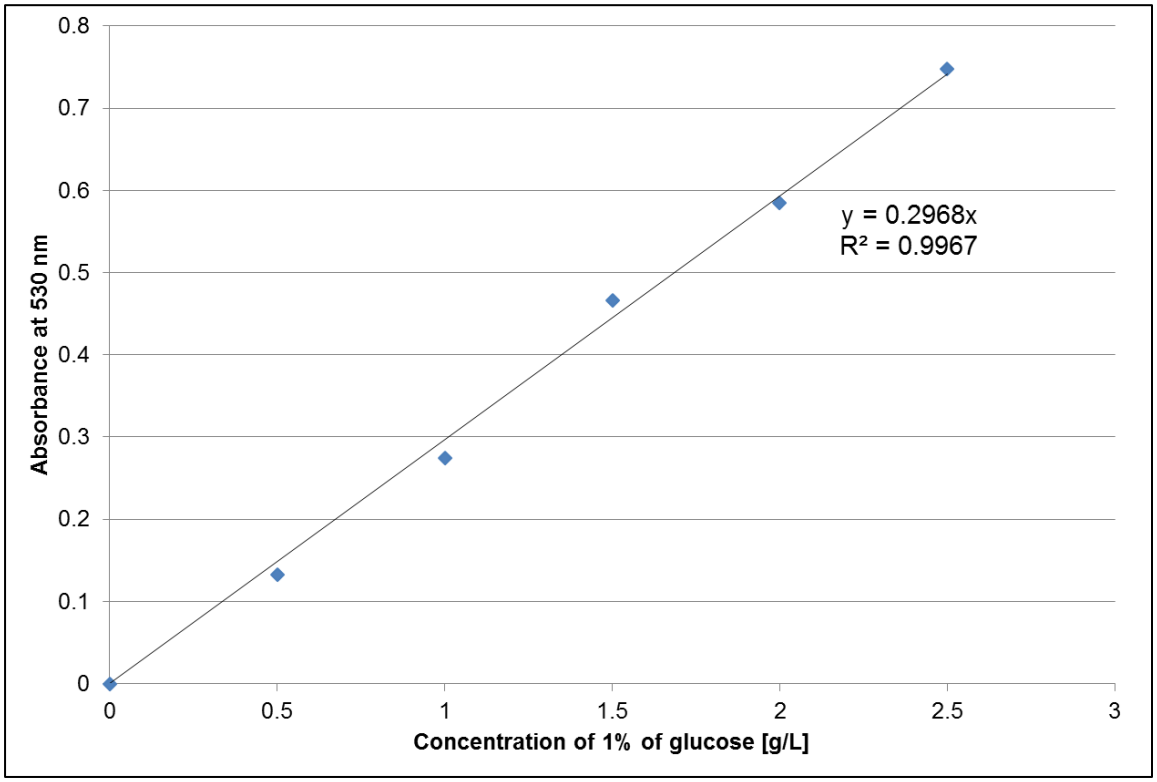
Appendix 1. Calibration curve for 1% of glucose stock solution, used to determine reducing sugars.

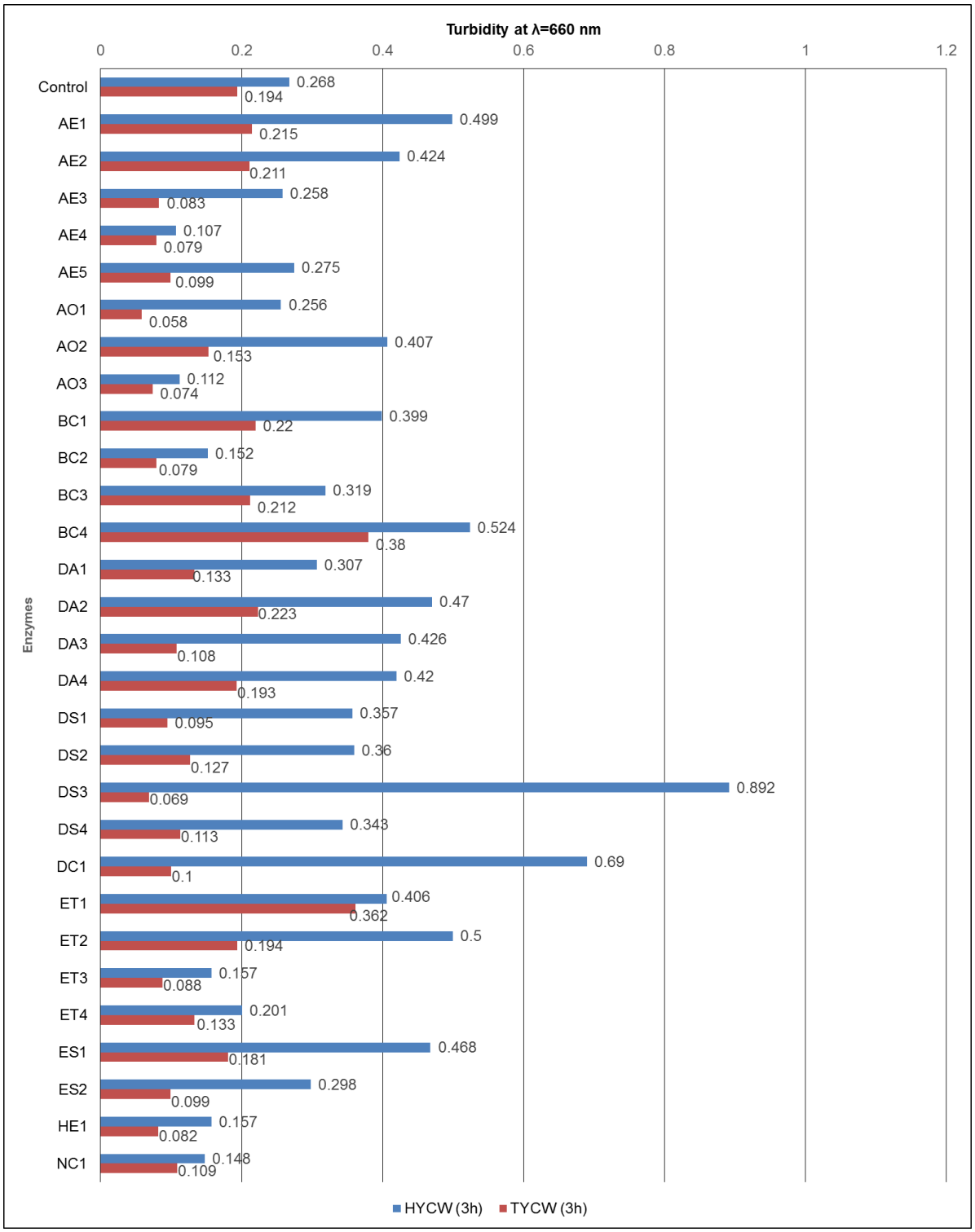


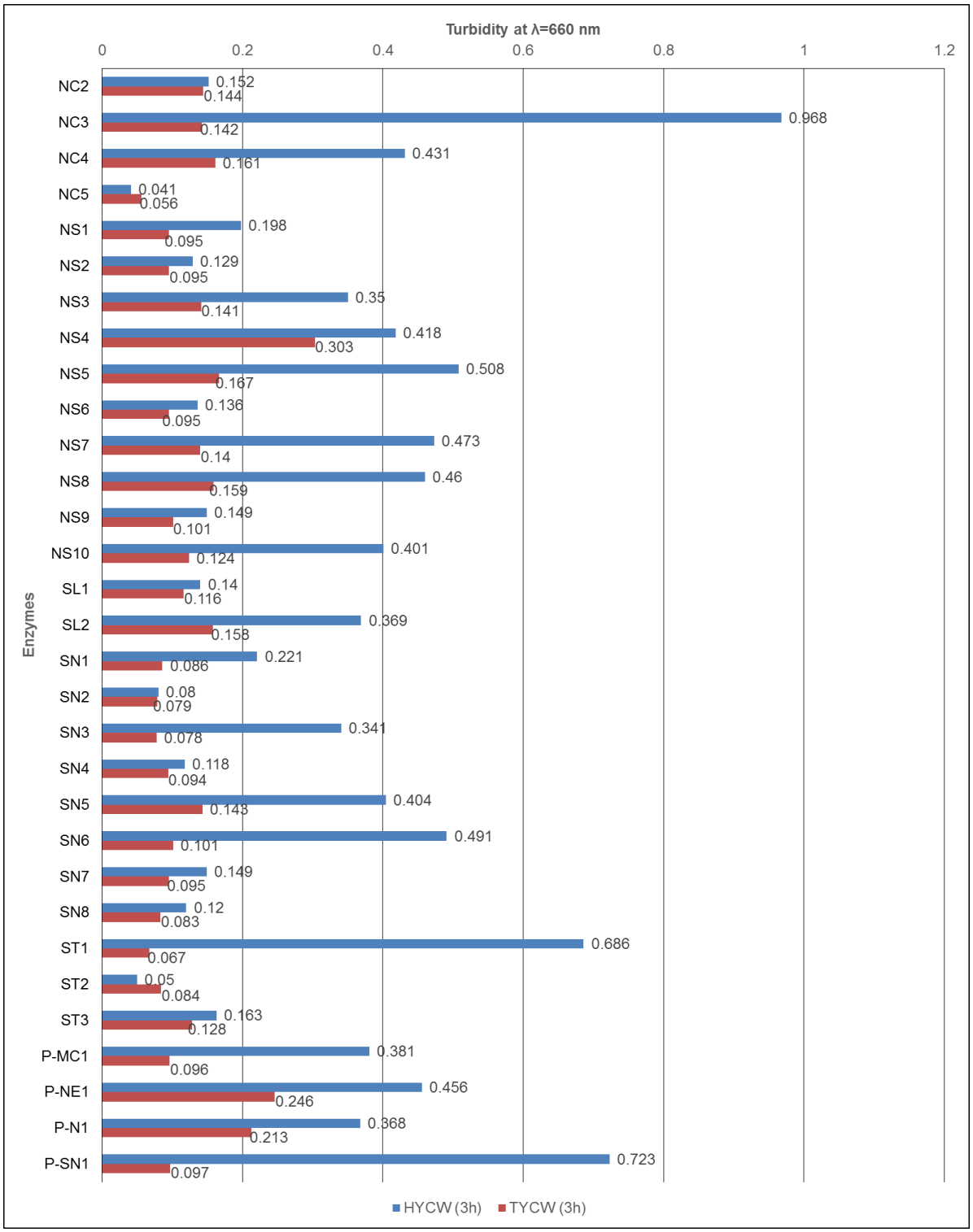
Appendix 2. Viscosity, μ [mPa.s] of hydrolysates of enzymatic screening at 25 °C for 24h of reaction time.

	24h reaction time at 25°C					
Starting Material	**HYCW**			**TYCW**		
Enzymes	**Viscosity (mPa.s)**	**Rotacional speed (rpm)**	**Spindle**	**Viscosity (mPa.s)**	**Rotacional speed (rpm)**	**Spindle**
Control	83	60	R2	274	60	R2
AE1	42	200		149		
AE2	98	60		151		
AE3	64			135		
AE4	47			314		
AE5	56			299		
AO1	69			69	100	
AO2	71			244	60	
AO3	54	200		188		
BC1	97	60		263		
BC2	163			840		R3
BC3	104			386		R2
BC4	103			382		
DA1	71			415		
DA2	90			243		
DA3	366			268		
DA4	208			162		
DS1	63	100		141		
DS2	73	60		151		
DS3	242			156	100	
DS4	73			497	60	
DC1	451			320		
ET1	21	100		284		
ET2	130	60		343		
ET3	46			263		
ET4	30			115	200	
ES1	154			451	60	
ES2	224			303		
HE1	225			395		
NC1	30			83	200	
NC2	63			341	60	
NC3	311			417		
NC4	108			650		R3
NC5	28			111		R2
NS1	42			173		
NS2	68	200		97	100	
NS3	140	60		1170	60	R3

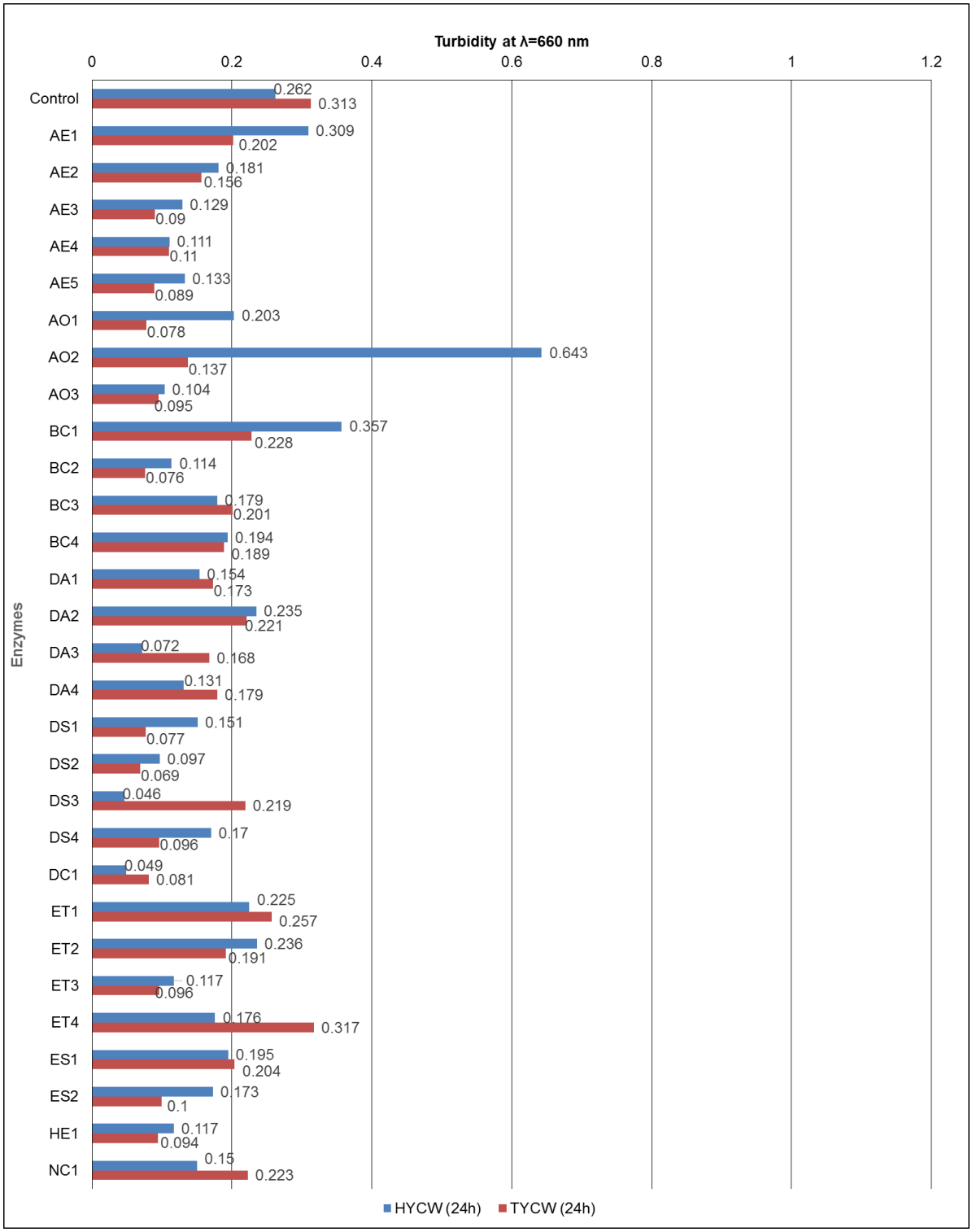
	24h reaction time at 25°C					
Starting Material	**HYCW**			**TYCW**		
NS4	78			394		
NS5	263			342		
NS6	347			860		R2
NS7	181			445		
NS8	331			413		
NS9	343			246		
NS10	428			500		R3
SL1	35			262		
SL2	124			483		
SN1	239			477		
SN2	69	200		140		
SN3	56			266		
SN4	75		R	346		
SN5	496			258		
SN6	295			1200		
SN7	22			140		R2
SN8	23			72	100	
ST1	83	60		427	60	
ST2	178			152		
ST3	28			79	100	
P-MC1	401			240		
P-NE1	378			333	60	
P-N1	366			517		
P-SN1	294			222		

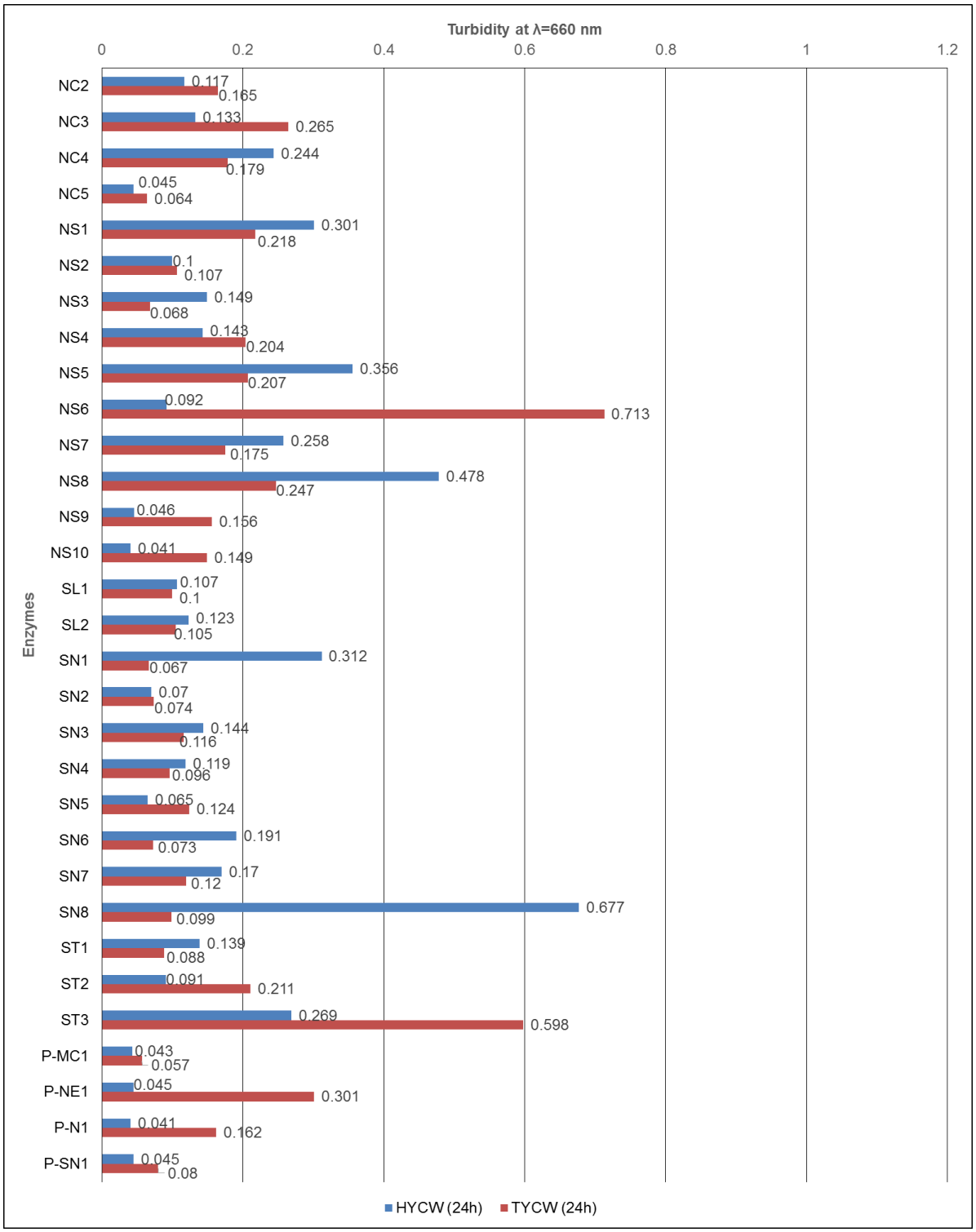
Appendix 3.Turbidity at 3h expressed as absorbance values at λ=660 nm, for 0.2% of dry matter of supernatant (P-N1 and P-SN1); for 0.8% of DM (DS3, DC1, NC3 and ST1) and for 1% of DM for the remaining enzymes.



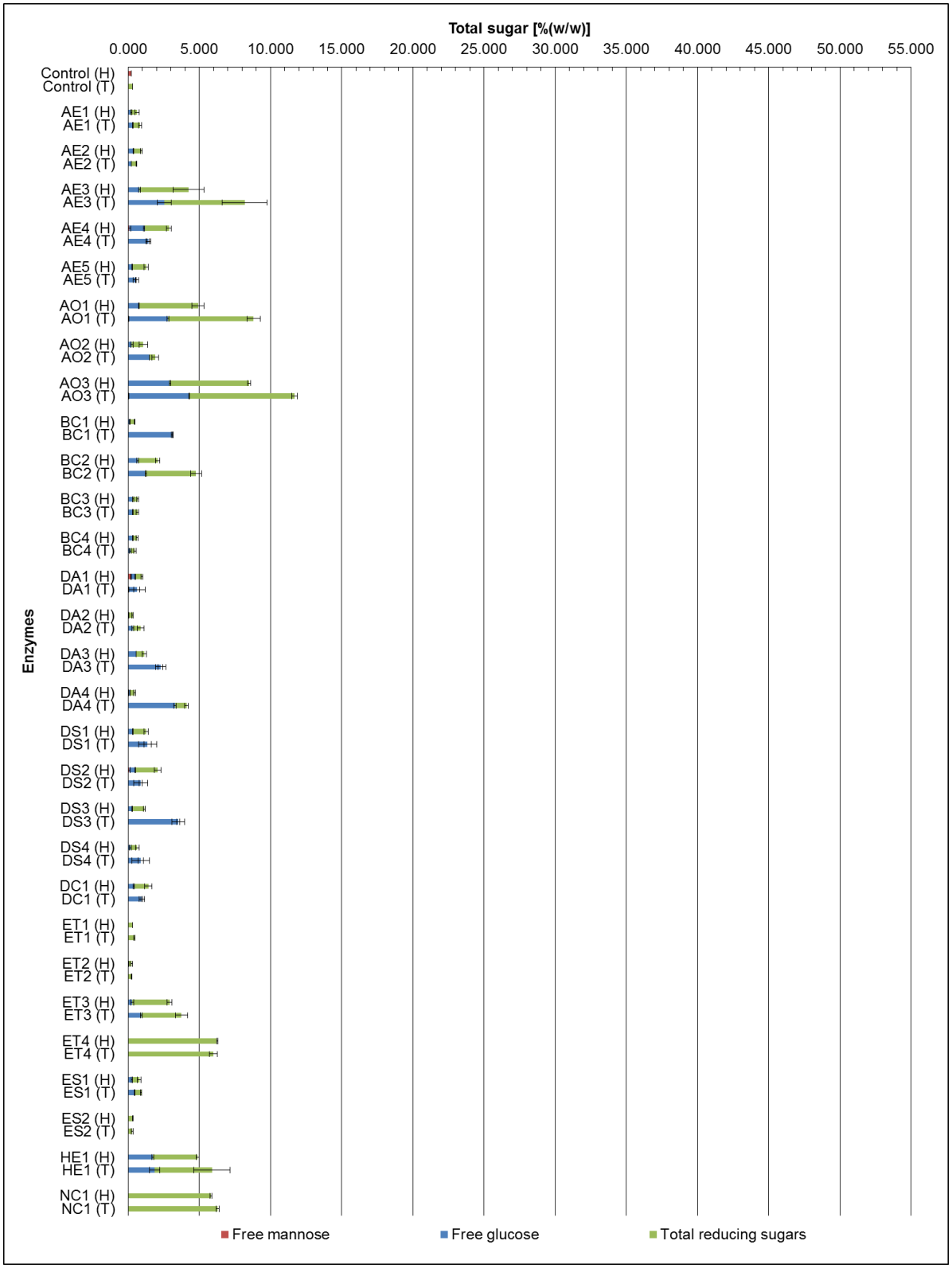

Turbidity at λ=660 nm
0
0.2
0.4
0.6
0.8
1
1.2
Enzymes
NC2 0.152 0.144
NC3 0.968 0.142
NC4 0.431 0.161
NC5 0.041 0.056
NS1 0.198 0.095
NS2 0.129 0.095
NS3 0.35 0.141
NS4 0.418 0.303
NS5 0.508 0.167
NS6 0.136 0.095
NS7 0.473 0.14
NS8 0.46 0.159
NS9 0.149 0.101
NS10 0.401 0.124
SL1 0.14 0.116
SL2 0.369 0.158
SN1 0.221 0.086
SN2 0.08 0.079
SN3 0.341 0.078
SN4 0.118 0.094
SN5 0.404 0.148
SN6 0.491 0.101
SN7 0.149 0.095
SN8 0.12 0.083
ST1 0.686 0.067
ST2 0.05 0.084
ST3 0.163 0.128
P-MC1 0.381 0.096
P-NE1 0.456 0.246
P-N1 0.368 0.213
P-SN1 0.723 0.097
HYCW (3h)
TYCW (3h)

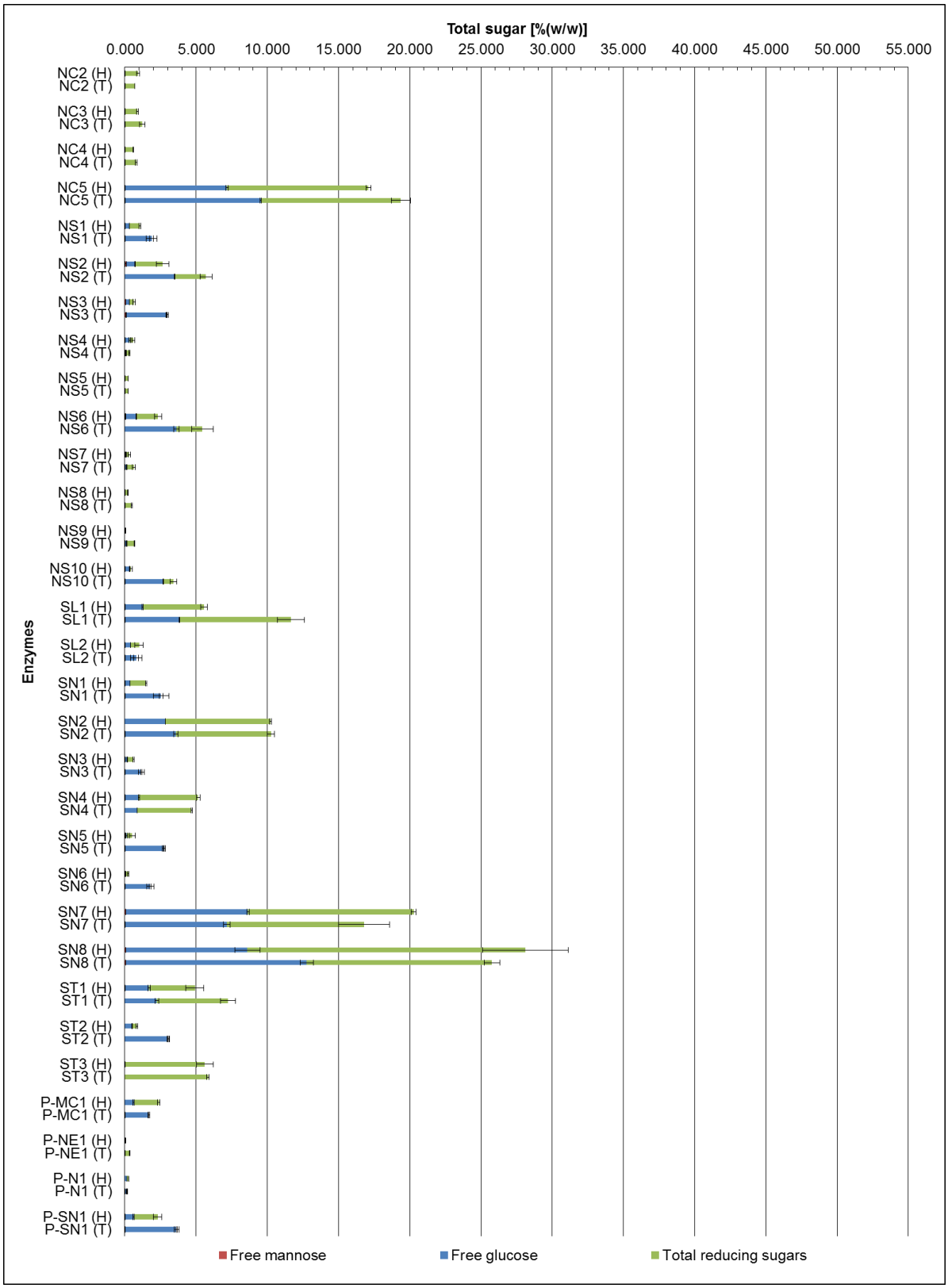
Appendix 4.Turbidity at 24h, expressed as absorbance values at λ=660 nm, for 1 % of dry matter of supernatant.



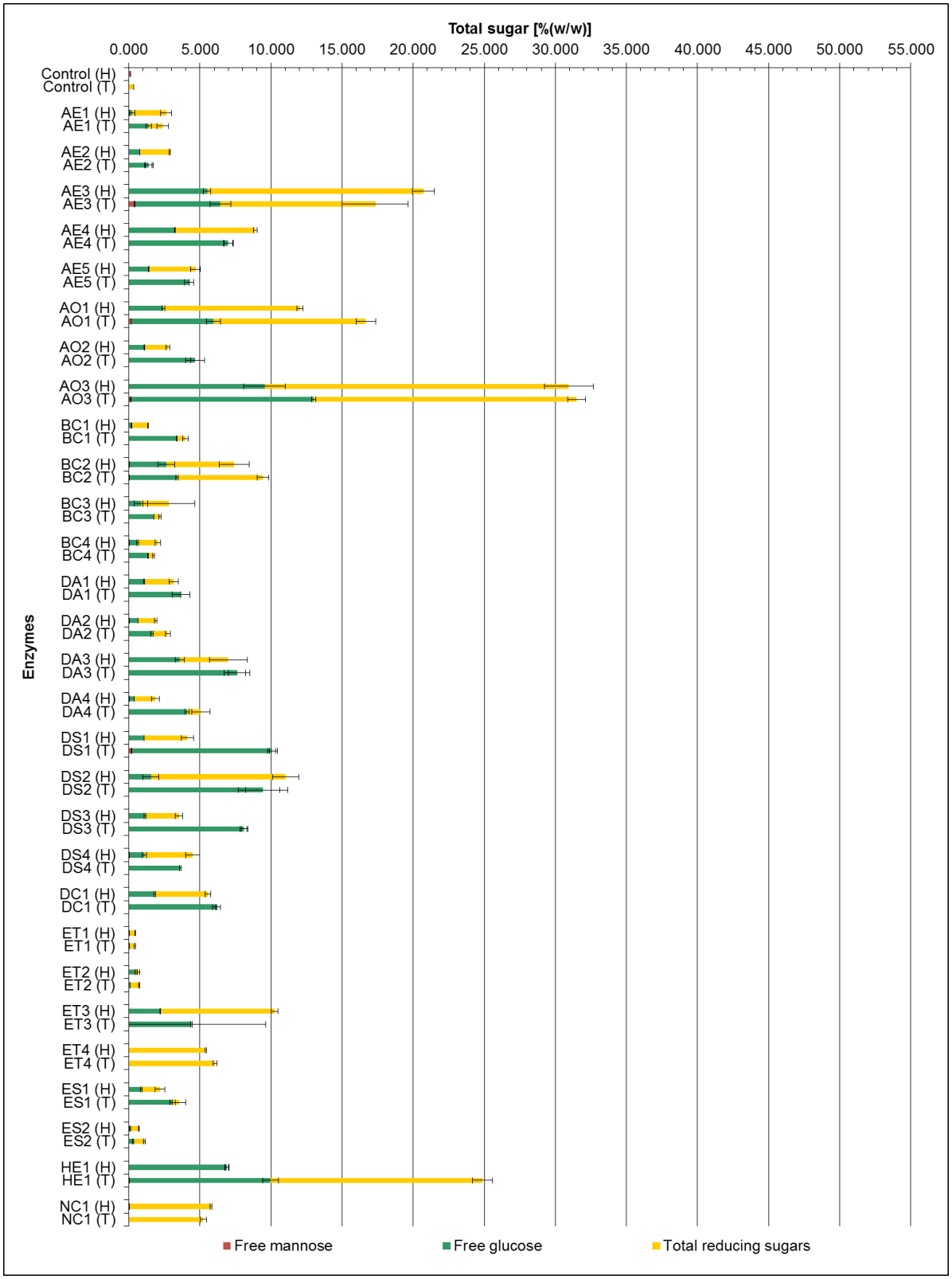

Turbidity at λ=660 nm
0
0.2
0.4
0.6
0.8
1
1.2
Enzymes
NC2 0.117 0.165
NC3 0.133 0.265
NC4 0.244 0.179
NC5 0.045 0.064
NS1 0.301 0.218
NS2 0.1 0.107
NS3 0.149 0.068
NS4 0.143 0.204
NS5 0.356 0.207
NS6 0.092 0.713
NS7 0.258 0.175
NS8 0.478 0.247
NS9 0.046 0.156
NS10 0.041 0.149
SL1 0.107 0.1
SL2 0.123 0.105
SN1 0.312 0.067
SN2 0.07 0.074
SN3 0.144 0.116
SN4 0.119 0.096
SN5 0.065 0.124
SN6 0.191 0.073
SN7 0.17 0.12
SN8 0.677 0.099
ST1 0.139 0.088
ST2 0.091 0.211
ST3 0.269 0.598
P-MC1 0.043 0.057
P-NE1 0.045 0.301
P-N1 0.041 0.162
P-SN1 0.045 0.08
HYCW (24h)
TYCW (24h)

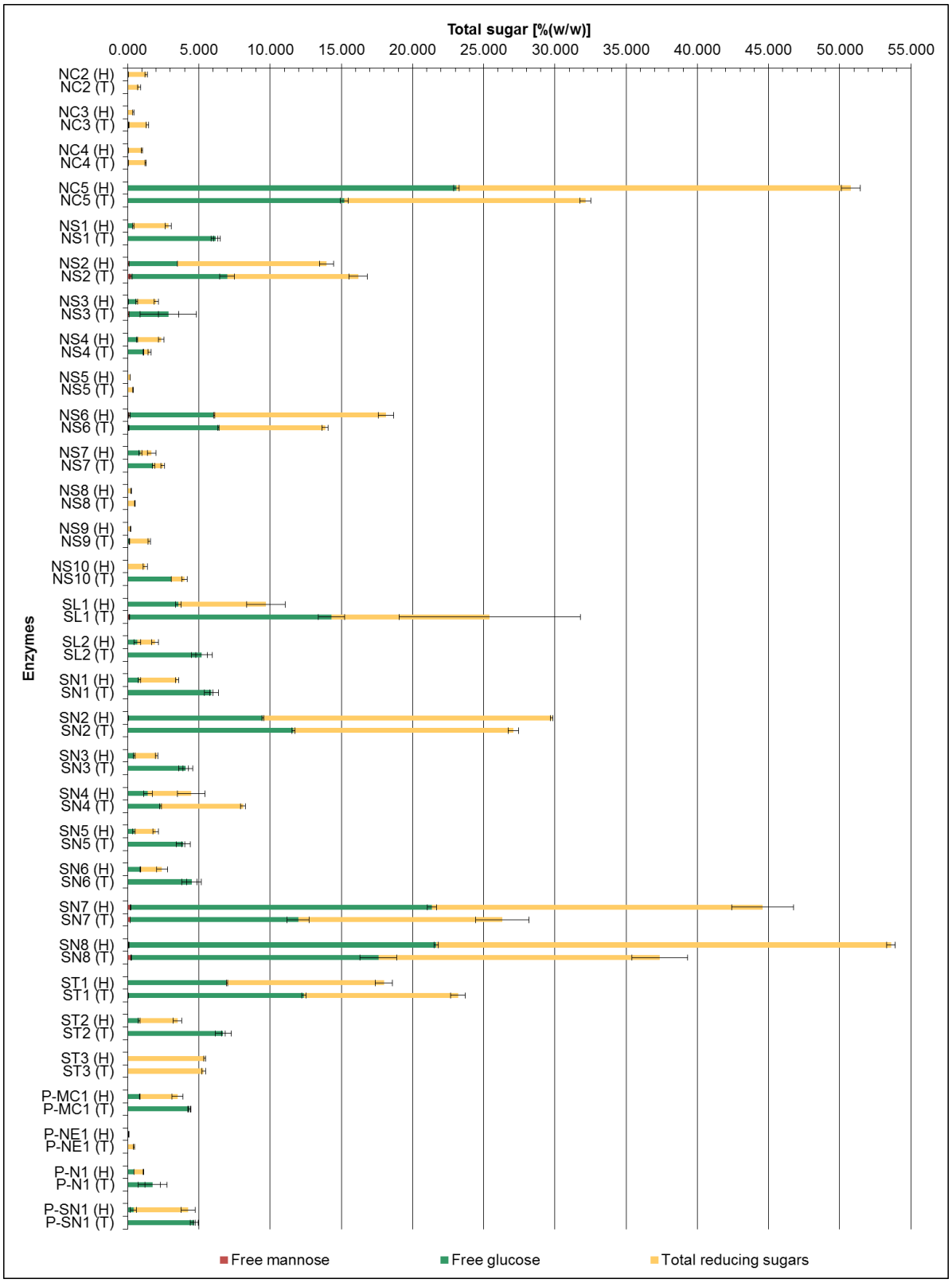
Appendix 5. Graphics of reducing sugars, free glucose and free mannose content over 3h of reaction time.




Total sugar [%(w/w)]
0.000
5.000
10.000
15.000
20.000
25.000
30.000
35.000
40.000
45.000
50.000
55.000
Enzymes
NC2 (H)
NC2 (T)
NC3 (H)
NC3 (T)
NC4 (H)
NC4 (T)
NC5 (H)
NC5 (T)
NS1 (H)
NS1 (T)
NS2 (H)
NS2 (T)
NS3 (H)
NS3 (T)
NS4 (H)
NS4 (T)
NS5 (H)
NS5 (T)
NS6 (H)
NS6 (T)
NS7 (H)
NS7 (T)
NS8 (H)
NS8 (T)
NS9 (H)
NS9 (T)
NS10 (H)
NS10 (T)
SL1 (H)
SL1 (T)
SL2 (H)
SL2 (T)
SN1 (H)
SN1 (T)
SN2 (H)
SN2 (T)
SN3 (H)
SN3 (T)
SN4 (H)
SN4 (T)
SN5 (H)
SN5 (T)
SN6 (H)
SN6 (T)
SN7 (H)
SN7 (T)
SN8 (H)
SN8 (T)
ST1 (H)
ST1 (T)
ST2 (H)
ST2 (T)
ST3 (H)
ST3 (T)
P-MC1 (H)
P-MC1 (T)
P-NE1 (H)
P-NE1 (T)
P-N1 (H)
P-N1 (T)
P-SN1 (H)
P-SN1 (T)
Free mannose
Free glucose
Total reducing sugars

Appendix 6. Graphics of reducing sugars, free glucose and free mannose content over 24h of reaction time.




Total sugar [%(w/w)]
0.000
5.000
10.000
15.000
20.000
25.000
30.000
35.000
40.000
45.000
50.000
55.000
Enzymes
NC2 (H)
NC2 (T)
NC3 (H)
NC3 (T)
NC4 (H)
NC4 (T)
NC5 (H)
NC5 (T)
NS1 (H)
NS1 (T)
NS2 (H)
NS2 (T)
NS3 (H)
NS3 (T)
NS4 (H)
NS4 (T)
NS5 (H)
NS5 (T)
NS6 (H)
NS6 (T)
NS7 (H)
NS7 (T)
NS8 (H)
NS8 (T)
NS9 (H)
NS9 (T)
NS10 (H)
NS10 (T)
SL1 (H)
SL1 (T)
SL2 (H)
SL2 (T)
SN1 (H)
SN1 (T)
SN2 (H)
SN2 (T)
SN3 (H)
SN3 (T)
SN4 (H)
SN4 (T)
SN5 (H)
SN5 (T)
SN6 (H)
SN6 (T)
SN7 (H)
SN7 (T)
SN8 (H)
SN8 (T)
ST1 (H)
ST1 (T)
ST2 (H)
ST2 (T)
ST3 (H)
ST3 (T)
P-MC1 (H)
P-MC1 (T)
P-NE1 (H)
P-NE1 (T)
P-N1 (H)
P-N1 (T)
P-SN1 (H)
P-SN1 (T)
Free mannose
Free glucose
Total reducing sugars

Appendix 7. Average of solubilisation profile for hydrolysates.

	3h reaction time								24h reaction time							
Starting Material	**HYCW**				**TYCW**				**HYCW**				**TYCW**			
Enzyme	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**
Control	S	50	50	1.2	S	50	50	2.4	S	50	50	1.2	S	52.5	47.5	2.3
AE1	S	50	50	1.35	S	62.5	37.5	3.95	S	45	55	2.7	S	52.5	47.5	6.95
AE2	S	47.5	52.5	1.75	S	62.5	37.5	4.4	S	45	55	3.2	S	55	45	7.3
AE3	S	43.75	56.25	4.25	S	53.75	46.25	8.9	S	31.25	68.75	5.4	L	56.25	43.75	11.75
AE4	S	42.5	57.5	3.3	S	62.5	37.5	6.3	S	37.5	62.5	5.2	S	55	45	9.7
AE5	S	50	50	2.7	S	62.5	37.5	5.4	S	40	60	4.45	S	55	45	9.45
AO1	S	48.75	51.25	5.35	S	40	60	11.65	S	36.25	63.75	6.05	L/S	36.25	63.75	15.4
AO2	S	50	50	1.55	S	62.5	37.5	3.75	S	43.75	56.25	2.1	S	57.5	42.5	5.65
AO3	S	40	60	4.1	S	51.25	48.75	9.05	S	32.5	67.5	6.5	S	42.5	57.5	13.25
BC1	S	50	50	1.35	S	62.5	37.5	3.8	S	40	60	2.5	S	61.25	38.75	5.4
BC2	S	50	50	3.1	S	62.5	37.5	7.25	S	36.25	63.75	5.25	S	61.25	38.75	10
BC3	S	50	50	1.5	S	62.5	37.5	3.6	S	60	40	3.55	S	60	40	6.7
BC4	S	48.75	51.25	1.65	S	62.5	37.5	3.3	S	45	55	2.55	S	60	40	5.85
DA1	S	48.75	51.25	1.7	S	62.5	37.5	3.55	S	55	45	3.55	S	58.75	41.25	6.35
DA2	S	50	50	1.65	S	62.5	37.5	4.15	S	45	55	2	S	60	40	6.65
DA3	S	68.75	31.25	1.8	S	62.5	37.5	3.75	S	57.5	42.5	4.55	S	52.5	47.5	7.8
DA4	S	50	50	1.5	S	62.5	37.5	4.4	S	37.5	62.5	2.7	S	52.5	47.5	6.3
DS1	S	46.25	53.75	1.75	S	52.5	47.5	6.65	S	62.5	37.5	4.25	S	50	50	9.75

	3h reaction time								24h reaction time							
Starting Material	**HYCW**				**TYCW**				**HYCW**				**TYCW**			
Enzyme	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**
DS2	S	47.5	52.5	2.5	S	60	40	5	S	33.75	66.25	5.95	S	50	50	8.65
DS3	S	61.25	38.75	2.2	S	62.5	37.5	5.55	S	56.25	43.75	4.9	S	56.25	43.75	9.85
DS4	S	50	50	2	S	62.5	37.5	4.4	S	42.5	57.5	3.8	S	57.5	42.5	8.15
DC1	S	62.5	37.5	2.8	S	62.53	37.47	5	S	56.25	43.75	5.65	S	57.5	42.5	9.55
ET1	S	50	50	1.5	S	63.75	36.25	3.3	S	50	50	1.65	S	61.25	38.75	3.85
ET2	S	48.75	51.25	1.6	S	62.5	37.5	3	S	47.5	52.5	1.95	S	63.75	36.25	4.35
ET3	S	48.75	51.25	3.2	S	61.25	38.75	6.75	S	37.5	62.5	5.1	S	50	50	8.65
ET4	L	23.25	76.75	5.85	L	53.75	46.25	8.1	L	23.75	76.25	6.5	L	50	50	8.9
ES1	S	50	50	5.1	S	63.75	36.25	3.15	S	50	50	3.05	S	62.5	37.5	6.4
ES2	S	48.75	51.25	2.15	S	63.75	36.25	5.05	S	50	50	2.9	S	62.5	37.5	9.95
HE1	S	57.5	42.5	3.4	S	61.25	38.75	8.3	S	50	50	6.55	L	50	50	12.7
NC1	L	26.25	73.75	6	L	38.75	61.25	7.15	L	23.75	76.25	6.95	L	42.5	57.5	7.35
NC2	S	51.25	48.75	2.65	S	62.5	37.5	4.2	S	47.5	52.5	3	S	61.25	38.75	4.1
NC3	S	62.5	37.5	1.25	S	63.75	36.25	3.05	S	63.75	36.25	1.55	S	68.75	31.25	3.5
NC4	S	53.75	46.25	1.4	S	62.5	37.5	3.25	S	48.75	51.25	1.7	S	62.5	37.5	4.25
NC5	S	37.5	62.5	5.6	S	37.5	62.5	7.2	S	22.5	77.5	7.6	S	37.5	62 5	9.25
NS1	S	46.25	53.75	2.3	S	58.75	41.25	6.1	S	63.75	36.25	1.65	S	48.75	51.25	9.2

	3h reaction time								24h reaction time							
Starting Material	**HYCW**				**TYCW**				**HYCW**				**TYCW**			
Enzyme	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**
NS2	S	47.5	52.5	3.1	S	60	40	7.4	S	35	65	5.6	L	50	50	9.15
NS3	S	58.75	41.25	1.5	S	62.5	37.5	3.5	S	50	50	2.7	S	62.5	37.5	5.75
NS4	S	58.75	41.25	1.35	S	62.5	37.5	3.15	S	50	50	2.5	S	62.5	37.5	4.65
NS5	S	50	50	1.3	S	62.5	37.5	3	S	62.5	37.5	1.45	S	61.25	38.75	3.55
NS6	S	50	50	3.1	S	61.25	38.75	7.2	S	37.5	62.5	5.7	L	66.25	33.75	9.5
NS7	S	60	40	1.4	S	62.5	37.5	3	S	52.5	47.5	2.35	S	60	40	4.9
NS8	S	56.25	43.75	1.45	S	62.5	37.5	3.05	S	56.25	43.75	1.5	S	67.5	32.5	3.5
NS9	S	65	35	1.1	S	65	35	4.1	S	75	25	1.5	S	62.5	37.5	5.4
NS10	S	68.75	31.25	0.85	S	62.5	37.5	3.45	S	63.75	36.25	2.6	S	58.75	41.25	5.2
SL1	S	40	60	4.1	S	51.25	48.75	8.5	S	70	30	6.4	S	45	55	12.65
SL2	S	50	50	1.55	S	62.5	37.5	3.6	S	61.25	38.75	4.25	L	62.5	37.5	7.05
SN1	S	61.25	38.75	2.15	S	62.5	37.5	4.85	S	50	50	3.7	S	60	40	9
SN2	S	37.5	62.5	5.5	S	38.75	61.25	7.9	S	27.5	72.5	6.5	S	37.5	62.5	10.4
SN3	S	50	50	1.85	S	62.5	37.5	5.3	S	60	40	3.8	S	60	40	10.1
SN4	S	37.5	62.5	4.8	S	60	40	9.8	S	63.75	36.25	6.4	L	61.25	38.75	12.05
SN5	S	60	40	1.55	S	63.75	36.25	4.1	S	50	50	3.15	S	57.5	42.5	7.45
SN6	S	60	40	1.4	S	62.5	37.5	4.6	S	57.5	42.5	2.75	S	66.25	33.75	8.45
SN7	S	36.25	63.75	5.65	S	50	50	9.05	S	18.75	81.25	7.55	L	52.5	47.5	13.45

	3h reaction time								24h reaction time							
Starting Material	**HYCW**				**TYCW**				**HYCW**				**TYCW**			
Enzyme	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**	**Pellet form**	**Vpellet (%)**	**Vsupernatant (%)**	**Supernatant DM (%)**
SN8	S	32.5	67.5	6.1	S	35	65	8.7	S	25	75	7.5	S	33.75	66.25	10.05
ST1	S	56.25	43.75	3.35	S	60	40	8.3	S	50	50	6.65	L	52.5	47.5	13.1
ST2	S	62.5	37.5	1.55	S	62.5	37.5	4.9	S	55	45	4.5	S	50	50	9
ST3	L	24.75	75.25	5.9	L	55	45	8.2	L	24.25	75.75	6.9	L	53.75	46.25	8.6
P-MC1	S	62.5	37.5	1.8	S	62.5	37.5	6	S	61.25	38.75	4.75	S	56.25	43.75	11.75
P-NE1	S	70	30	0.95	S	62.5	37.5	3	S	71.25	28.75	1.05	S	58.75	41.25	3.5
P-N1	S	67.5	32.5	1.3	S	61.25	38.75	3.65	S	62.5	37.5	3.6	S	55	45	6.55
P-SN1	S	71.25	28.75	1.9	S	70	30	5.65	S	60	40	5.55	S	56.25	43.75	12.25

L=loose; S=solid

Appendix 8. PCA analysis for HYCW

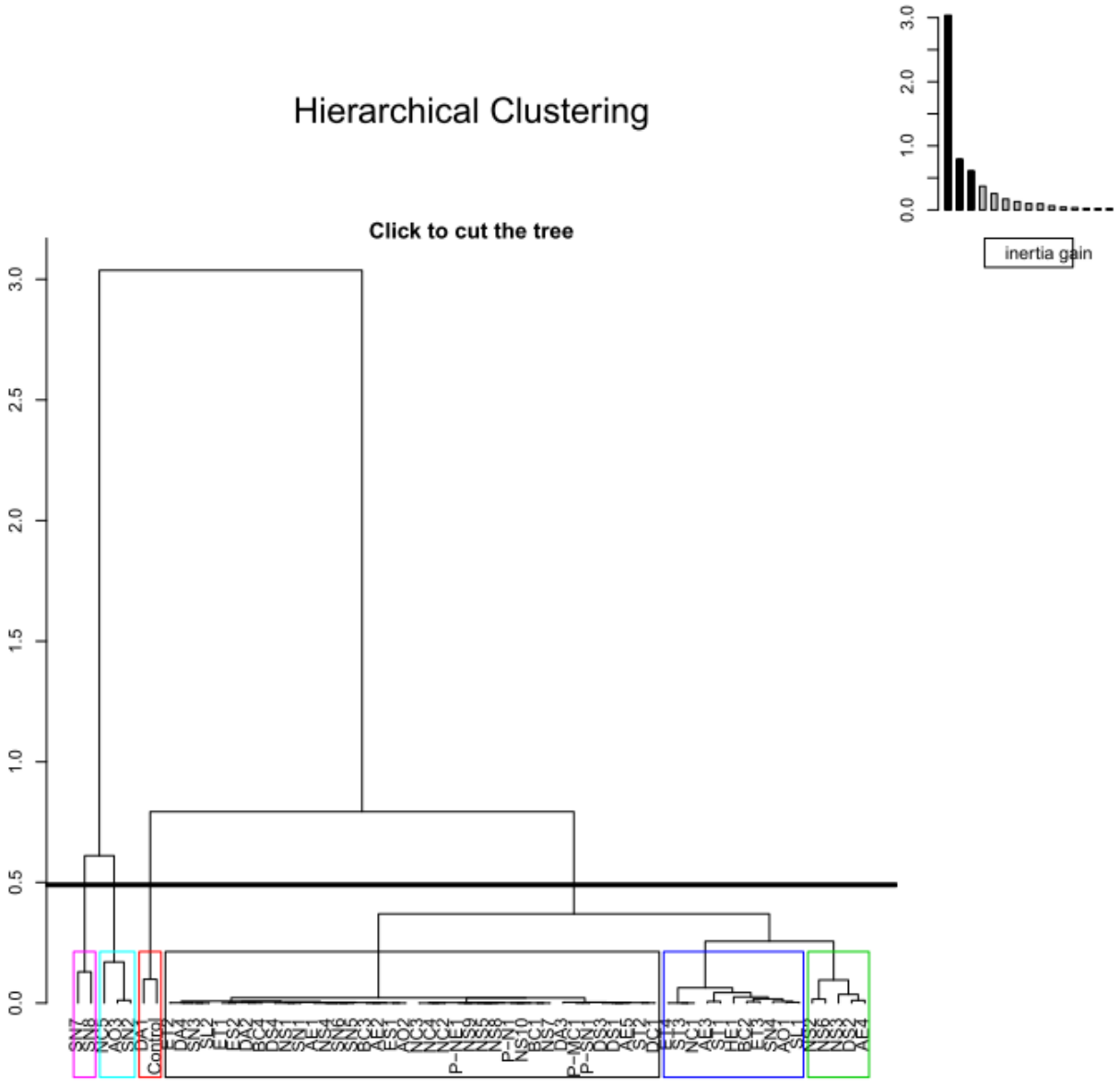
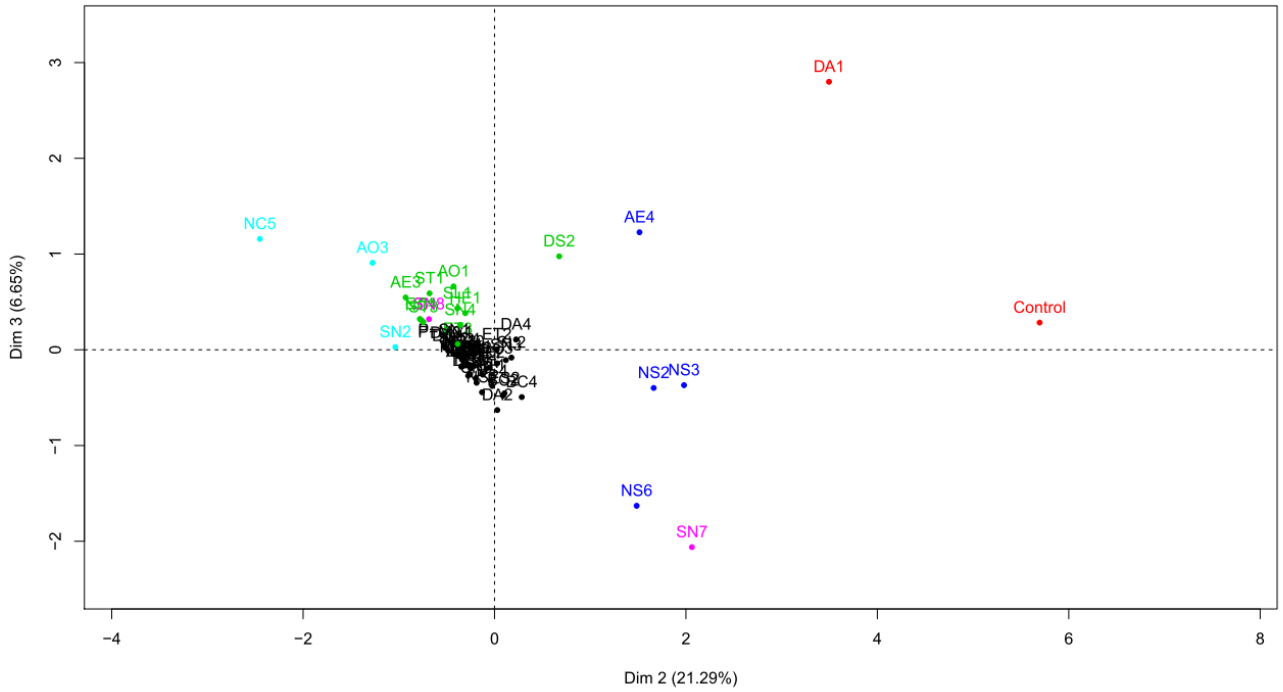


Figure 8.1 Representation of hierarchical clustering and inertia gain of HYCW (Autoscaling).



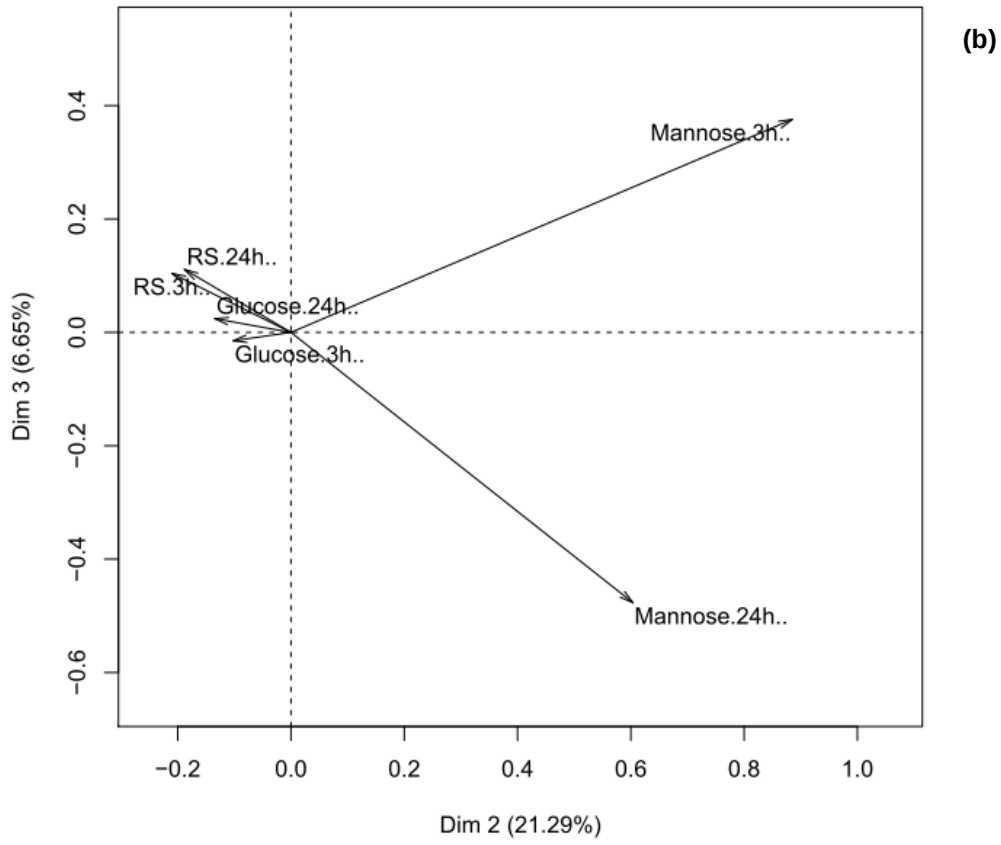


Figure 8.2 Autoscaling representation of individual factor map (PCA) (a) induced by the first two principal components (b) in variables factor map (PCA) for HYCW.

Appendix 9. PCA analysis for TYCW

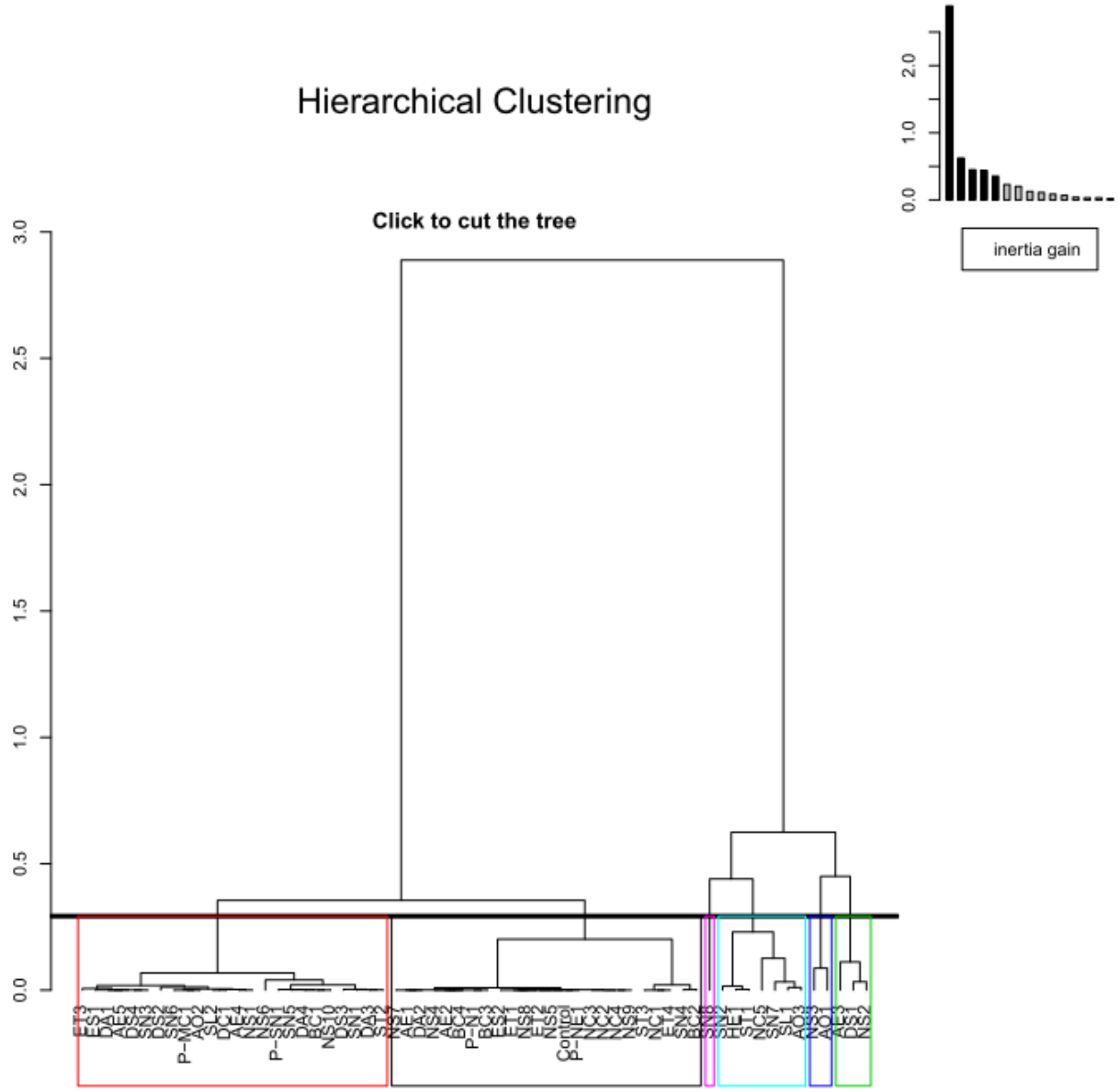


Figure 9.1 Hierarchical clustering and inertia gain representation of TYCW (Autoscaling).

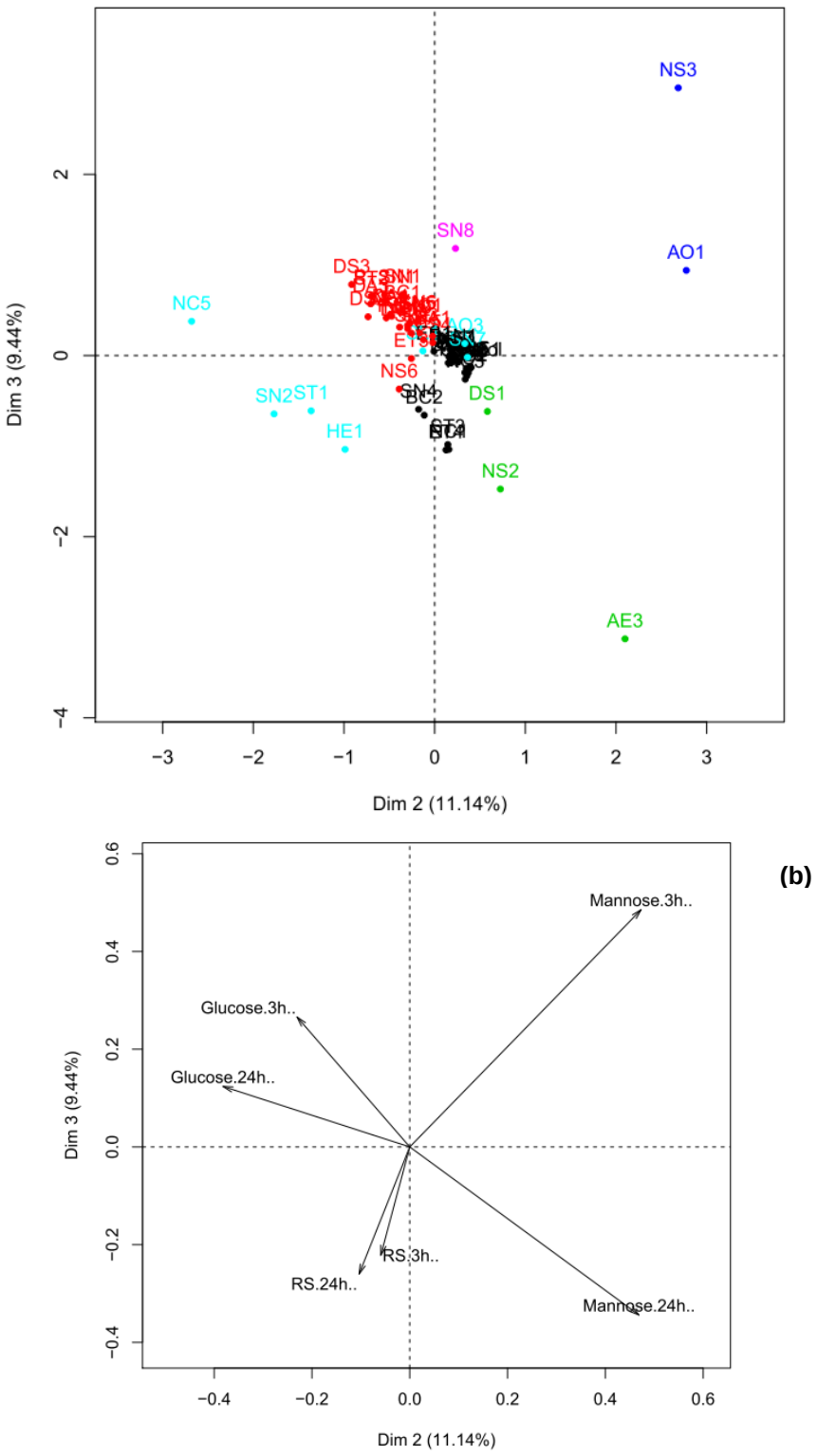


Figure 9.2 Autoscaling representation of individual factor map (PCA) (a) induced by the first two principal components (b) in variables factor map (PCA) for TYCW.

Appendix 10. Reactions performed to achieve the new yeast extract

Table 10.1 Reactions performed for TYCW with 21% (w/w) dry matter.

Reactions performed for TYCW with 21% (w/w) dry matter					
Enzymes dosages	**Start pH**	**T [°C]**	**Enzyme dosages**	**pH**	**T [°C]**
Control	7.0	60	-	5.1	68
0.5% NS11 + 0.69% EN1	7.0	60	0.4% AO5	5.1	68
	7.0	60	0.3% AO5	5.1	68
	7.0	60	0.2% AO5	5.1	68
	7.0	60	0.1% AO5	5.1	68
	7.0	60	0.045% AO5	5.1	68
0.5% NS11 + 0.5% P-N1	6.5	60	-	-	-
0.5% NS11 + 0.25% P-N1	6.5	60	-	-	-
0.5% NS11 + 0.1% P-N1	6.5	60	-	-	-
0.5% NS11 + 0.5% P-NE1	6.5	60	-	-	-
0.5% NS11 + 0.25% P-NE1	6.5	60	-	-	-
0.5% NS11 + 0.1% P-NE1	6.5	60	-	-	-
0.5% NS11 + 0.25% P-N1 + 0.25% P-NE1	6.5	60	-	-	-
1.0% NS11	7.0	60	-	-	-
0.5% NS11	7.0	60	-	-	-
0.5% NS11 + 0.5% NS12	6.5	60	-	-	-
Reaction time	16/17h	-	-	8h	-

Table 10.2 Reactions performed for TYCW with 17% (w/w) dry matter.

Reactions performed for TYCW with 17% (w/w) dry matter					
Enzymes dosages	**Start pH**	**T [°C]**	**Enzyme dosages**	**pH**	**T [°C]**
0.5% NS11 + 0.69% EN1	7.0	60	0.4% AO5	5.1	68
	7.0	60	0.35% AO5	5.1	68
	7.0	60	0.3% AO5	5.1	68
	7.0	60	0.25% AO5	5.1	68
	7.0	60	0.2% AO5	5.1	68
	7.0	60	0.15% AO5	5.1	68
-	**-**	**-**	1.0% AO5	5.1	68
0.5% NS11 + 0.5% EN1	7.0	60	-	-	-
0.5% NS11 + 0.5% NS12	7.0	60	-	-	-
0.5% NS11 + 0.5% AO1	6.5	50	-	-	-
0.5% NS11 + 0.5% SN9	6.5	50	-	-	-
0.5% NS11 + 0.5% SN10	6.5	50		-	-
0.5% NS11 + 0.5% AO4	6,5	50	-	-	-
0.4% NS11 + 0.5% EN1	7.0	60	-	-	-
0.5% NS11 + 0.5% EN1	7.0	60	-	-	-
0.6% NS11 + 0.5% EN1	7.0	60	--	-	-
0.7% NS11 + 0.5% EN1	7.0	60	-	-	-
0.8% NS11 + 0.5% EN1	7.0	60	-	-	-
0.9% NS11 + 0.5% EN1	7.0	60	-	-	-
1% NS11 + 0.5% EN1	7.0	60	-	-	-
0.5% NS11 + 1.0% EN1	7.0	60	-	-	-
0.5% NS11 + 1.5% EN1	7.0	60	-	-	-
0.5% NS11 + 2.0% EN1	7.0	60	-	-	-
0.5% NS11 + 2.5% EN1	7.0	60	-	-	-
0.5% NS11 + 3% EN1	7.0	60	-	-	-
0.6% NS11 + 0.6% EN1	7.0	60	-	-	-
0.5% NS11 + 0.5% AO3	7.0	60	-	-	-
0.5% NS11 + 0.5% SN8	7.0	60	-	-	-
0.5% NS11 + 0.5% NS12	7.0	60	-	-	-
0.6% NS13 + 0.6% EN1	6.5	50	-	-	-
0.5% NS11 + 0.5% AO3	6.5	50	-	-	-
0.5% NS11 + 0.5% SN8	6.5	50	-	-	-
0.5% NS11+ 0.5% NS12	6.5	50	-		-
Reaction time	16/17h	-	-	8h	-

Appendix 11. Questionnaires for sensory tests (QDA analysis)

a) Application “Vegetable broth without pieces”

Sample: **Name:** **Date:**

Please taste the sample (with a 3-digit-code) and rate the intensity of the attributes on the scale relative to the control. The "comments" section can be use if you notice an attribute not listed.

	weniger intensive less intense				**C**				**intensiver more intense**
Bitter	☐	☐	☐	☐	☐	☐	☐	☐	☐
Salzig/ salty	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sauer/ sour	☐	☐	☐	☐	☐	☐	☐	☐	☐
Süß/ sweet	☐	☐	☐	☐	☐	☐	☐	☐	☐
Umami/ umami	☐	☐	☐	☐	☐	☐	☐	☐	☐
Kommentar/ comments									

Appendix 12. Ranking test for umami intensity

Name: Date:

Please, rank the solutions in the coded cups in ascending order of umami taste.

	Code
Least umami	
Most umami	

Appendix 13. Hydrolysates DM, yield and protein per DM.

DM % of starting material	Enzymes		DM % SN	Yield % per DM	Protein % per DM
21	0.5% NS11 +	0.5% P-N1	11.300	14.931	48.310
		0.25% P-N1	12.400	12.228	47.516
		0.1% P-N1	12.500	13.251	49.200
		0.5% P-NE1	10.300	10.271	54.913
		0.25% P-NE1	9.900	10.256	49.717
		0.1% P-NE1	11.200	12.085	49.929
		0.25% P-N1 + 0.25% P-NE1	11.600	9.904	47.198
	1.0% NS11	-	13.700	13.330	53.818
	0.5% NS11	-	12.900	14.513	54.233
	0.5% NS11 +	0.5% NS12	13.200	15.267	52.902
	0.5% NS11 + 0.69% EN1 +	0.4% AO5	14.500	18.005	54.090
		0.3% AO5	13.800	15.990	52.051
		0.2% AO5	15.400	17.991	53.987
		0.1% AO5	15.800	18.311	52.981
		0.045% AO5	15.000	17.780	55.273
17	0.5% NS11 + 0.69% EN1 +	0.4% AO5	9.070	23.352	62.051
		0.35% AO5	8.560	19.117	61.530
		0.3% AO5	7.360	18.246	-
		0.25% AO5	8.920	21.438	78.038
		0.2% AO5	7.790	18.656	70.347
		0.15% AO5	8.400	18.379	69.702
	-	1% AO5	3.860	6.450	135.984
	0.5% NS11 +	0.5% AO1	10.810	35.154	62.581
		0.5% SN9	11.210	32.817	56.869
		0.5% SN10	10.030	27.742	66.431
		0.5% AO4	11.770	36.212	61.334
	0.5% EN1	0.4% NS11	7.340	17.813	60.899
		0.5% NS11	7.450	17.921	59.732
		0.6% NS11	7.250	17.785	66.345
		0.7% NS11	7.730	19.338	56.662
		0.8% NS11	6.200	14.972	82.581
		0.9% NS11	7.890	20.080	53.105
		1% NS11	6.680	15.446	66.168
	0.5% NS11	1% EN1	7.360	17.171	67.120
		1.5% EN1	7.050	17.039	72.057
		2% EN1	8.190	20.665	61.783
		2.5% EN1	8.410	20.339	67.301
		3% EN1	9.340	22.879	50.321
		0.5% AO3	8.170	24.326	52.109
		0.5% SN8	7.960	20.816	49.497

DM % of starting material	Enzymes		DM % SN	Yield % per DM	Protein % per DM
	0.6% EN1	0.6% NS13	8.360	19.774	67.937
	0.5% NS11	0.5% AO3	8.085	24.300	49.636
		0.5% SN8	8.980	26.423	39.532
		0.5% NS12	7.850	20.707	59.363

Appendix 14. Standard deviation values for hydrolysates DM %, yield % per DM, protein % per DM and sensory analysis.

Table 14.1 Standard deviation values for hydrolysates with different starting DM and different dosages of AO5.

DM % of starting material	Enzymes		DM in SN	Yield	Protein
17	0.5% NS11 + 0.69% EN1 +	0.4% AO5	0.087	2.207	1.612
15			0.332	2.261	0.094
13			0.313	2.657	2.047
11			0.236	2.237	0.754
		-	0.265	1.591	2.527
		0.2% AO5	0.040	0.233	1.819
		0.15% AO5	0.100	0.765	2.580
		0.1% AO5	0.020	0.292	1.129
		0.05% AO5	0.240	1.395	1.612
		0.01% AO5	0.370	2.156	2.059

Table 14.2 Standard deviation values for sensory analysis.

Related with Figure	Enzymes	Bitter	Salty	Sour	Sweet	Umami
3.10	0.5% NS11 + 0.69% EN1+ 0.4% AO5	0.387	0.487	0.371	0.316	0.371
	0.5% NS11 + 0.69% EN1+ 0.3% AO5	0.194	0.224	0.371	0.371	0.316
	0.5% NS11 + 0.69% EN1+ 0.2% AO5	0.194	0.316	0.371	0.371	0.316
	0.5% NS11 + 0.5% AO1	0.194	0.316	0.194	0.316	0.371
	0.5% NS11 + 0.5% SN9	0.194	0.194	0.194	0.194	0.316
	0.5% NS11 + 0.5% SN10	0.224	0.548	0.194	0.194	0.224
	0.5% NS11 + 0.5% AO4	0.194	0.224	0.000	0.316	0.371
3.11	0.5% NS11 + 0.5% AO4 + 1% GMP	0.204	0.612	0.354	0.540	0.540
	0.5% NS11 + 0.5% AO1 + 1% GMP	0.224	0.500	0.581	0.500	0.548
	0.5% NS11 + 0.5% AO3 + 1% GMP	0.224	0.371	0.581	0.224	0.487
	0.5% NS11 + 0.69% EN1+ 0.2% AO5	0.200	0.900	0.400	0.400	0.200
3.13	0.5% NS11 + 0.69% EN1+ 0.4% AO5 with DM of 17%	0.204	0.204	0.204	0.204	0.408
	0.5% NS11 + 0.69% EN1+ 0.4% AO5 with DM of 15%	0.194	0.194	0.387	0.194	0.371
	0.5% NS11 + 0.69% EN1+ 0.4% AO5 with DM of 13%	0.194	0.581	0.316	0.194	0.316
	0.5% NS11 + 0.69% EN1+ 0.4% AO5 with DM of 11%	0.204	0	0	0.204	0.204
3.15	0.5% NS11 + 0.69% EN1+ 0.2% AO5	0.000	0.204	0.408	0.204	0.204
	0.5% NS11 + 0.69% EN1+ 0.15% AO5	0.194	0.447	0.194	0.194	0.000
	0.5% NS11 + 0.69% EN1+ 0.1% AO5	0.000	0.316	0.224	0.316	0.371
	0.5% NS11 + 0.69% EN1+ 0.05% AO5	0.204	0.408	0.204	0.408	0.204
	0.5% NS11 + 0.69% EN1+ 0.01% AO5	0	0.354	0.204	0.354	0.540
3.20	0.5% NS11 + 0.69% EN1 + 0.1% AO5	0.400	0.800	0.316	0.374	0.490
	24% TYC + TYCW (0.5% NS11 + 0.69% EN1)	0.316	0.245	0.245	0.374	0.200
	12% TYC + TYCW (0.5% NS11 + 0.69% EN1)	0.400	0.583	0.200	0.374	0.374

Appendix 15. List with information about screened enzymes, including classes, enzyme code, cleaving properties, microorganism, pH and temperature range, activity and GMO.

Classes	Enzyme code	Cleaving Properties	Microorganism	pH range	T range (°C)	Activity	GMO
Carbohydrases	AE1	Pectinase with hemicellulolytic side activities. Can hydrolyze β-Mannanase, cellulose, pectin esterase and polygalacturonase	*Aspergillus* sp. *Trichoderma* sp.	4-7	50-65	9,600 PGU/mg	Self-cloned
	AE2	Cellulase and many side activities	*Trichoderma reesei*	3-6	50-65	15000 ECU/g	Yes
	AE3	β-glucanase with high protease side activities. Cleave 1,3- and 1,6-linkages.	*Trichoderma* sp.	4-7	50-60	minimum 2500 LAM/g	Yes
	AE4	Mannanase as main activity, β-glucanase, xylanase and cellulase side activity. Hydrolyses galactomannans and other non-starch polysaccharides.	*Trichoderma reesei*	2-7	40-75	minimum 1000000 MNU/g	-
	AE5	β-glucanase, breakdown 1,3-β-glycosidic linkages.	*Trichoderma citrinoviride*	3.5-7	50-70	50 LAMU/g	-
	AO1	Protease with peptidase activity.	*Aspergillus oryzae*	3-6	30-50	minimum 40000 U/g	-
	AO2	Protease, lipase, β-mannosidase and α-galactosidase as side activities.	*Aspergillus niger*	2-7.5	20-70	minimum 10000 U/g	-
	AO3	Endo-1,4-β-D-xylanase.	*Aspergillus niger*	4-9	30-55	minimum 90000 U/g	-
	BC1	A-amylase which cleaves α-1,4-glycosidic linkages.	*Aspergillus oryzae*	3-6	35-55	25000 U/g	No
	BC2	Blend of β-glucanases.	*Trichoderma* sp.	5-7	45-55	12000 U/g	No
	BC3	β-glucanases with side activity, cellulose and xylanase. Hydrolyzes β-1,4-	*Trichoderma*	3-6.5	50-65	12500 U/g	No

Classes	Enzyme code	Cleaving Properties	Microorganism	pH range	T range (°C)	Activity	GMO
Carbohydrases		linkages and complex polysaccharides.	*longibrachiatum*				
	BC4	Cellulase, cellobiase, β-glucosidase and β-glucanase with side activities.	*Trichoderma* sp.	3.5-6	50-70	1500 U/g	No
	DA1	Cellulase and β-glucanase which can cleave β-(1,3- and 1,6)-glucans and non-starch polysaccharides.	*Trichoderma reesei*	3.5-5.5	Opt. 60	minimum 6200 IU/g	No
	DA2	Cellulase and β(1,4)-glucanase.	*Trichoderma reesei*	4.9-5.3	-	mininum 2250 BGLU/mL	No
	DA3	β(1,4)-glucosidade.	-	5-6	-	minimum 3000 U/g	Yes
	DA4	Glucoamylase or amyloglucosidase which hydolyzes 1,4-α-glucans.	*Aspergillus niger*	3.5-5.5	Opt. 68	minimum 350 GAU/g	No
	DS1	Endo β-(1,3- and 1,4)-glucanase.	*Talaromyces emersonii* and *Trichoderma longibrachiatum*	4-5	-	minimum 100000 BGF/g	No
	DS2	Endo β-(1,3- and 1,4)-glucanase.	*Talaromyces emersonii*	4-4.5	-	minimum 40000 BGF/g	No
	DS3	Amyloglucosidase which cleaves α-1,4-glucans.	*Aspergillus niger*	-	-	80000 AGI	No
	DS4	Cellulase.	*Aspergillus niger*	-	-	7900 CXU/g	No
	DC1	Endo β-D-1,4-xylanase. Hydrolyzes β-1,3- xylanase and 1,4-glucanase and cellulose, pectinase, mannanase, xyloglucanase, laminarase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase as side activities.	*Trichoderma reesei*	4.2-6.5	40-57	Xylanase 110000 U/g / β-glucanase 35000 U/g / Cellulase	-

Classes	Enzyme code	Cleaving Properties	Microorganism	pH range	T range (°C)	Activity	GMO
Carbohydrases						140000 U/g	
	ET1	Purified pectinlyase.	*Aspergillus* sp.	-	up to 65	22500 PECTU/mL	Yes
	ET2	Pectinase and polygalacturonase.	*Aspergillus* sp.	-	10-50	46000 PGU/mL	Yes
	ET3	Pectinase and β-glucanase as side activity	-	-	-	2500 PGNU/g (Polygalacturonase) and 100 BGXU/g (exo-1,3-β-glucanase)	-
	ET4	β-glucanase	-	-	-	-	-
	ES1	Endo and exo 1,4-β-D-glucanase, and 1,4-β-D-glucosidase.	*Trichoderma reesei*	4-7	25-75	minimum 3000 U/g	Yes
	ES2	Exo and endo-peptidase, cellulase, hemicellulase and amylase side activities.	-	2.3-6	30-60	minimum 500 PAC/g	-
	HE1	Galactomannanase and cellulose.	*Aspergillus niger*	3-9	Opt 75	10000 GMA/g	-
	NC1	Glucanase	*Streptomyces* sp.	5-6.5	50-60	-	Self-cloned
	NC2	Endo-N-acetyl glucosaminidase. Cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins.	*Streptomyces violaceroruber*	-	-	-	-

Classes	Enzyme code	Cleaving Properties	Microorganism	pH range	T range (°C)	Activity	GMO
Carbohydrases	NC3	Pectatelyase.	*Bacillus* sp.	5-8	40-60	-	-
	NC4	Chitinase.	*Streptomyces* sp.	3.5-8	20-60	16 U/g	-
	NC5	Endoglucanase, α-galactosidase, α-arabinosidase, polygalacturonase, β-mannosidase, arabinase, galactanase, β-glucanase, β-glucosidase, β-xylosidase and α-amylase	-	-	-	-	-
	NS1	Polygalacturonase. Cleaves (1,4)-α-D-galactosiduronic linkages in pectate and other galacturonans.	*Aspergillus aculeatus*	4-5	45-60	3300 PGNU/g	No
	NS2	Endo (1,3- and 1,4)-β-glucanase, xylanase and hemicellulose side activities.	*Aspergillus aculeatus*	3.3-5.5	40-60	100 FBG/g	No
	NS3	Glucoamylase which cleaves α-D-(1,4- and 1,6)- glucosidic linkages at the non-reducing ends of polysaccharides.	*Aspergillus niger*	Opt 4.5	Opt. 60	300 AGU/mL	No
	NS4	Cellulase. Hydrolyzes β-D-(1,4)-glucosidic linkages in cellulose and other β-D-glucans.	*Trichoderma reesei*	4-6	55-65	700 EGU/mL	No
	NS5	β-mannanase and endo β-(1,4)-mannosidase.	-	5.5-7.5	-	4 MIUM/g	Yes
	NS6	Exo β-(1,3)-glucanase. Hydrolyzes β-D–(1,3)-glucans from the non-reducing end releasing α-glucose.	*Trichoderma hazianum*	3-5	40-60	46 BGXU/mL	No
	NS7	Endo β-(1,3- and 1,4-)-glucanase and cellulose side activity.	*Trichoderma reesei*	-	-	250 FBG/g	No
	NS8	Endo α-D-(1,4)-amylase and glucanohydrolase.	*Bacillus amyloliquefaciens*	6-10	45-80	480 KNU/g	-

Classes	Enzyme code	Cleaving Properties	Microorganism	pH range	T range (°C)	Activity	GMO
Carbohydrases	NS9	Endoamylase that hydrolyzes α-D-(1,4)-glucosidic linkages in starch polysaccharides endo-β-glucanase that hydrolyses (1,3)- or (1,4)-linkages in β-D-glucans metallo endoprotease that hydrolyzes internal peptide bonds xylanase that hydrolyses β-D-(1,4)-xylosidic linkages in xylans.	*Humicola insolens* and *Bacilus amyloliquefaciens*	-	-	0,7 AU-N7g; 270 FXU/g; 200 KNU-B7g;	No
	NS10	Glucoamylase. Hydrolyzes α-D-(1,4- and 1,6)-glucosidic linkages at the non-reducing ends of polysaccharides.	*Aspergillus niger*	4-5	Opt. 70	400AGU/g	No
	SL1	Pectin lyase, polygalacturonase, PE and PGL	*Aspergillus Niger*	2.5-4.5	25-65	-	No
	SL2	β-(1,2-; 1,3-; 1,4- and 1,6)-glucanase.	*Trichoderma reesei*	3.5-5.5	30-70	-	-
	SN1	α-galactosidase.	*Aspergillus niger*	4-8	Opt. 60	30000 U/g	-
	SN2	Cellulase and hemicellulose.	*Trichoderma reesei*	3-6	Opt. 50	4000 U/g	-
	SN3	β-mannanase, cellulose, hemicellulose and β-Galactomannase	*Aspergillus niger*	3-6	50-60	15000 U/g	-
	SN4	Protease, endo-β-(1,3-and 1,6)-glucanase and exo-glucanase.	*Rhizomucor miehei*	Opt. 6-7	Opt. 30-40	-	-
	SN5	Glucoamylase. Hydrolyzes α-(1,4- and 1,6)-glucosidase.	*Aspergillus niger*	3-9	20-60	1500 U/g	-
	SN6	Pectinase and endo and exo-arabanase	*Aspergillus niger*	3.5-5.5	40-65	5000 U/g	-
	SN7	β-1,3-glucanase	*Penicillium* sp.	3.5-5.0	Opt. 50	300 u/g	-
	SN8	β-1,3-glucanase and Botrytis glucanase. Can hydrolyze β-(1,3), (1,4) and (1,6) glycosidic bonds.	*Trichoderma reesei*	3.5-8	Opt. 50	100 u/g	-

Classes	Enzyme code	Cleaving Properties	Microorganism	pH range	T range (°C)	Activity	GMO
	ST1	Glucanase, galactomannanase, cellulase and β-glucosidase.	*Aspergillus niger*	3-9	50-75	-	No
	ST2	Glucoamylase.	*Aspergillus niger*	3.5-6	50-60	10000 U/g	No
	ST3	Purified glucanase.	*Streptomyces* sp.	Opt. 5-6.5	Opt. 50-60	-	Yes
Phospholipases	P-MC1	Phospholipase A1. Hydrolyzes the bond between the fatty acid and the glycerine residue at the 1-position of the phospholipid.	*Aspergillus oryzae*	4-10	30-60	10000-13000 U/g	No
	P-NE1	Purified phospholipase. Hydrolyzes phospholipids.	*Streptomyces* sp.	7-10	40-55	10000 - 13000 UN/mL	-
	P-N1	Lipase. Hydrolyzes ester bonds in glycerides.	-	-	-	10 KLU/g	No
	P-SN1	Phospholipase A1.	*Aspergillus oryzae*	3.5-6	Opt. 55	3000 U/g	-
Proteases	NS11	Protease, endopeptidase	*Bacillus licheniformis*	7.0-9.0	30-65	2,4 AU-A/g	No
	EN1	Vegetable protease, endopeptidase	*Carica papaya L.*	4.8-6.2	60	Minimum 100 TU/mg	No
	NS12	Exopeptidase that liberates amino acids by hydrolysis of the N-terminal peptide bond	*Aspergillus oryzae*	-	-	500 LAPU/g	No
	AO4	High protease and peptidase activity	*Aspergillus oryzae*	5.0-8.0	40-60	minimum 1000000 U/g	No
	SN9	High acid carboxypeptidase activity (GTGase) and aminopeptidase activity	*Aspergillus oryzae*	4.0-7.0	30-40	Minimum 300~700 u/g	-
	SN10	High semi-alkaline proteases activity	*Aspergillus melleus*	5.0-9.0	30-50	130000 u/g	-

Classes	Enzyme code	Cleaving Properties	Microorganism	pH range	T range (°C)	Activity	GMO
	NS13	Endoprotease that hydrolyzes internal peptide bonds	-	-	-	1.4 AU-N/g	No
Nuclease	AO5	Nuclease, convert RNA to 5'nucleotides	*Penicillium citrinum*	5	68	-	No