PRODUCTION OF THERMOPHYLIC α-AMYLASE FROM *ASPERGILLUS SP.* AND ITS UTILIZATION FOR VARIOUS APPLICATIONS

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ABSTRACT

PRODUCTION OF THERMOPHYLIC α-AMYLASE FROM ASPERGILLUS SP. AND ITS UTILIZATION FOR VARIOUS APPLICATIONS

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The objective of this study was to scale up the production of thermophilic α-amylase and its use to obtain fermentable sugar as the main carbon source for microorganisms such as baker's yeast and *Lactobacillus casei.*

Aspergillus niger N402 was the strain used for determining the fermentation parameters (carbon and nitrogen source, fermentation volume/total flask volume ratio (FV/TFV), pH & temperature of the fermentation and spore inoculum level.) Different strains (Am13, AmC18, and AmC28) were also tested to find out the strain producing the highest activity enzyme for scale up experiments that were conducted at 1.5L, 60L, and 80L volumes. The best strain was selected as AmC28 with the parameters of: starch and maltose syrup as the carbon source and yeast extract as the nitrogen source, temperature was set to 30 °C at pH 5.50 with 10^5 spores/ml and with 0.5 FV/TFV ratio at a 250 mililiter volume flask. One milliliter of these secreted amylases were capable of hydrolyzing almost 100 mg of soluble starch in one hour at 80 °C at pH of 5.0. Following the production of the enzyme at large scale, saccharification of raw starch (%10 solid load) was accomplished using these α-amylases. The produced fermentable sugar was used to grow Baker's yeast and to produce lactic acid from *L.casei.*

Keywords: *A.niger*, α-amylase production, scale up, hydrolysis

ÖZ

ASPERGİLLUS SUŞUNDAN TERMOFİLİK α-AMİLAZ ÜRETİMİ VE FARKLI UYGULAMALARDA KULLANIMI

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Bu çalışmanın amacı, termofilik α-amilaz enzimini büyük ölçekte üretip elde edilen enzim ile ekmek mayası ve *Lactabacillus casei* gibi mikoroganizmalar için fermente şeker üreterek karbon kaynağı sağlamaktır.

Aspergillus niger N402 suşu fermentasyonun karbon ve azot kaynağı, fermentasyon hacminin toplam flask hacmine oranı (FH/TFH), ekilen spor miktarı, pH ve sıcaklık gibi parametrelerini belirlemek için yüksek a-amilaz aktivitesi eldesinde kullanılmıştır. Am13, AmC18, AmC28 şuşları belirlenen parametrelerde büyük ölçekte, 1.5L, 60L, 80L, yüksek aktivede, enzim üretimine göre karşılaştırılmıştır. AmC28 suşu (nişasta ve maltoz şurubu karbon kaynağı olarak, maya özütü nitrojen kaynağı olarak, 30°C sıcaklıkta, 5.50 pHda, 10^5 spor miktarı ile 0.5 FH/TFH oranında 250 mililitre hacimdeki flask deneylerinde belirlenen parametrelerde en yüksek amilaz aktivitesini göstermiştir..

Üretilen α-amilazların bir mililitresi ile 80°C de ve 5.0 pHda saatte yak1aşık 100mg nişasta hidroliz edilebilmektedir. Üretilen bu amilazlar ile %10luk nişasta şekere hidroliz edilmiştir. Üretilen şeker ekmek mayası üretmede ve *L.casei*'den laktik asit üretiminde denenmiştir.

Anahtar Kelimeler: *A.niger,* α-amilaz üretimi, büyük ölçek, hidroliz

To My Family

&

To the Beautiful Hearts at the Backstage

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CHAPTER 1

INTRODUCTION

1.1 The Genus Aspergillus

One of the oldest named genera of fungi, Aspergillus received its name from Pier Antonio Micheli in 1729. He was inspired by asperges that was used by Roman Catholic clergy to sprinkle holy water during the liturgy (C.Ainsworth, 1977). Micheli showed a scanty identification and simple drawings, but a clear illustration of the characteristics of the genus. (Elmerich, 1989; Farias, Monteiro, Rodrigues, Fernandes, & Pinto, 2010)

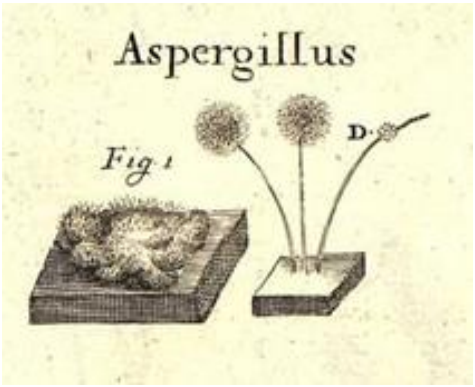

Figure 1.1.1 First illustration of Aspergillus genera by P.A Micheli (Houbraken, Spierenburg, & Frisvad, 2012)

After that Thorn and Church (1926) reported 69 species with their descriptions. Later in 1945, Thom and Raper introduced 77 more species (Thom & Raper, 1945).

In 1965 Raper and Fennell submitted 132 species to the genus of Aspergillus (Powell, Renwick, & Peberdy, 2014).

Now there are around 250 named species in the genus Aspergillus including industrially important species (*A.niger*, *A.oryzae*, *A.awamori*, *A.sojae*, *A.terreus*) and pathogenic species for animal/plant (e.g. *A.fumigatus*, *A.parasiticus*, *A.flavus*). (Geiser et al. 2007). Because of their remarkable metabolic adaptability, aspergilli are utilized as a part of biotechnology for the generation of an assortment of items, for example, organic acids, pharmaceuticals, proteins and enzymes (Meyer, 2008; Lubertozzi & Keasling, 2009; Raper & Fennel, 1965; Kis-Papo, 2003; Machida & Gomi, 2010).

Aspergillus sp. become prominent from other microbial cell factories of bacterial or yeast origin due to its high tolerance to the extreme cultivation conditions. To illustrate, Aspergillus can grow over a wide range of temperatures (10–50°C), pH (2–11), salinity (0–34%), and water activity (0.6–1) and under oligo- trophic or nutrient-rich conditions. Therefore, Aspergillus was handled for solid-state or submerged fermentations. Moreover fermentation protocols are established for large-scale industrial processes (Lubertozzi & Keasling, 2009).

The technologies and methodologies depicted are mainly centered on *Aspergillus niger* since being a model organism for filamentous fungi and being the leading genus for enzymes (Guimaraes et al., 2006). Moreover it has GRAS (generally recognized as safe) status (Lubertozzi & Keasling, 2009). On a basic level it could be connected to any of the other industrially relevant filamentous fungi (Meyer, Ram, & Punt, n.d.).

1.1.1 *Aspergillus Niger*

Aspergillus niger is the most widely recognized type of the genus Aspergillus. The taxonomical hierarchy is given at Figure 1.1.1

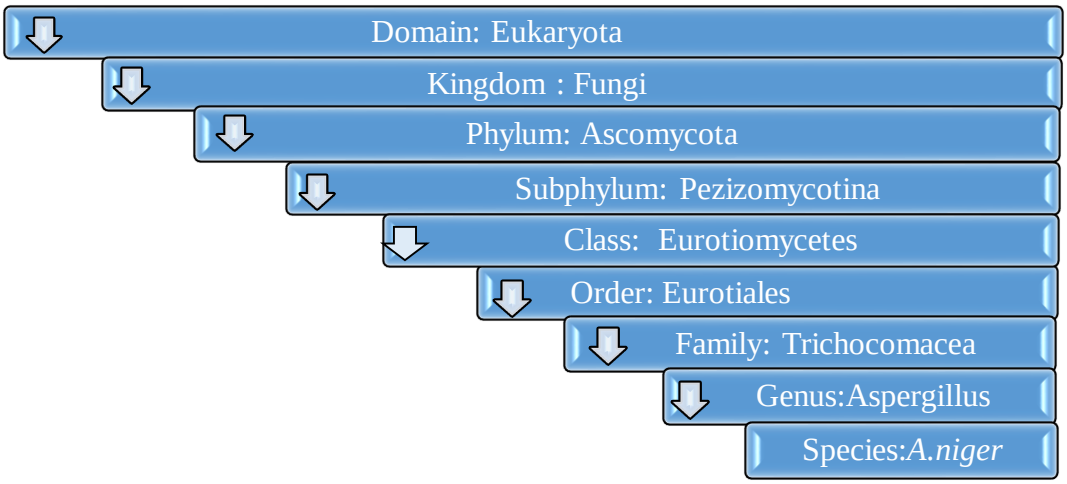

Figure 1.1.1.1 Lineage of *Aspergillus niger*. (Taxonomy Database of National Center for Biotechnology Information (NCBI) Taxonomy ID: 1608278)

A.niger is a multicellular fungi and can reproduce only asexually. (Casselton & Zolan, 2002; Pontecorvo, Roper, & Forbes, 1953) which makes it a perfect candidate for biotechnology since no or little variation occurs at the genome of the next generations. Vegetative reproduction happens when somatic mycelium differentiates and produces spores that contain nuclei from mitotic division. Figure 1.1.1.2 shows schematically the different phases of spore growth, germ tube outgrowth, germ tube elongation and branch development.

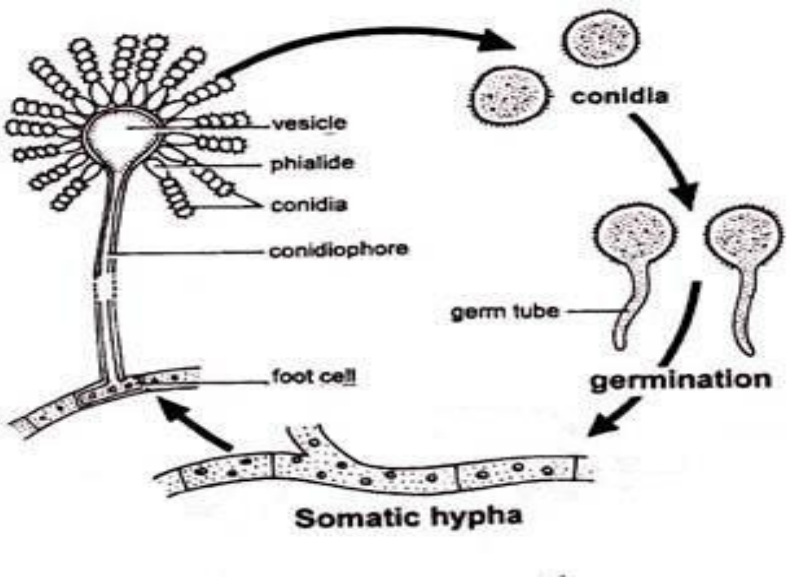

Figure 1.1.1.2. The life cycle of *Aspergillus niger*.(Khandelwal, 2016)

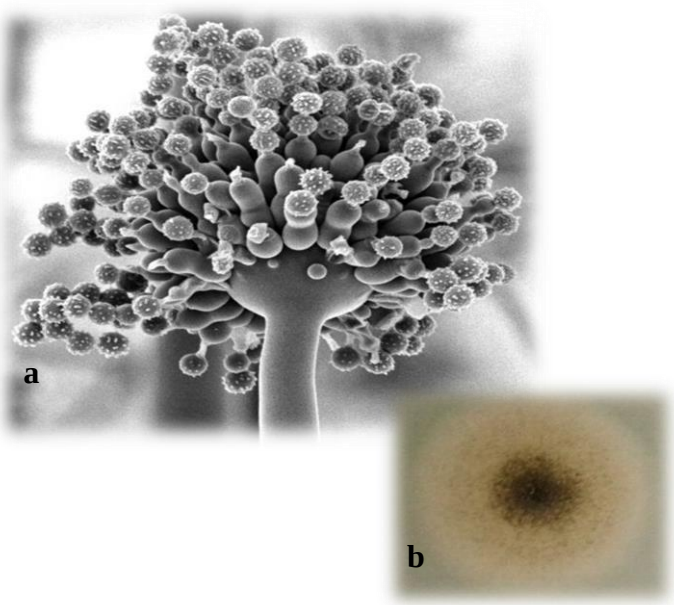

Figure 1.1.2.1 a- SEM image of asexual structures of *A.niger* (Hertz-fowler & Pain, 2007) b- *A.niger* on Potato Dextrose Agar. (3th day)

These processes lead to the formation of a true circular colony as shown in Figure 1.1.2 which appears black due to black colored conidia. Conidia is spreaded by wind, water, insects or animals to the nature.

1.2 Submerged Fermentation

Submerged fermentation (SmF) is the fermentation process in which microorganisms are grown in a liquid medium in fermenters. The substrates are used very fast so that it needs to be constantly supplemented with nutrients. During growth, the enzymes and bioactive compounds are secreted into the fermentation media (Ravichandran & R, 2012). SmF is mostly preferred for the fermentations of microorganisms such as bacteria that require high moisture content.

There is also another technique called solid state fermentation (SSF) in which microorganisms do fermentation by growing on the surface of the solid substrate. In SSF the fermentation is easy to control and to conduct. It does not need aeration or agitation during the fermentation. The separation of the product is easy since the microorganism is not discharging into the fermentation medium. Just the temperature and humidity of the fermentation chamber need to be controlled. Due to high evaporation rate of the fermentation medium from the surface the product is concentrated in the media. Therefore the expenses and losses during recovery and purification are lower.(Demain, 2000) SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content.

However, SSF has some disadvantages such as, scaling up is hard and very expensive compared to SmF and also fermentation time is long and therefore productivity is low. In SmF since the medium and all components are dispersed in liquid medium heat and mass transfer can be adjusted efficiently. Because it provides a better controlling and analysis of the process during fermentation the scaling-up process is easier in SmF. (Saranraj & Naidu, 2014)

In this study, the submerged fermentation was chosen to work with due to the advantages of the scaling up process

1.3 Amylase Enzymes

The amylases are the enzymes which catalyze the hydrolysis of starch and glycogen. The amylase was possibly the first enzyme to be revealed. in 1811 Kirchhoff was the first one to show starch is degraded by amylase. The digestive action of saliva on starch was shown by Leuchs in 1831.(Amylase Research Society of Japan., 1988a) Then a starch digestion active substance in malt was revealed and isolated by alcohol precipitation method and named as ‘’Diastase’’ (from Greek means ‘separation’) by Payen and Persoz in 1833. (Payen & Persoz, 1833) In 1878, Märker revealed that malt amylase contained two different enzymes (Märker, 1878).

In 1887 the α-amylases were firstly described and isolated from A.oryzae by J.Takamine in Tokyo. It was called taka-diastase at that time and now is also known as TAKA-amylase (Bruce et al., 2002). Later in 1925 it is formerly named as α-amylase by Kuhn, since the hydrolysis products are in the alpha configuration. In 1930, Ohlsson described the other amylase, which generated a β-mannose and named it as β-amylase. (Ohlsson, 1930).

Myrbäck & Neiimuler (1950) suggested a new classification for the amylases that was, exoamylases and endoamylases. β-amylase was listed as an exoamylase and also was called at that time as the saccharifying amylase and α-amylase was noted as an endoamylases or starch liquefying amylase (Myrbäck & Neumüler, 1950) . The principle of the amylase classification was based on its action mode. (exo = β-amylase, endo = α amylase).

On the other hand, recently it has been shown that an exo-amylase is not always a β-amylase. Moreover α-amylases which produce maltose, maltotriose, and dextrin were reported to attack starch exowise. (Amylase Research Society of Japan., 1988a)

1.3.1 α-Amylase

α-Amylase (E.C.3.2.1.1) is one of the industrially most important hydrolase enzymes that catalyzes the hydrolysis of internal α-1, 4-glycosidic bonds in starch and produce various length of oligosaccharides that have an α -configuration and α-limit dextrin (Kumar & Duhan, 2011). (Figure 1.3.4.1)

A wide variety of microorganisms, Archaea, Bacteria, Fungi express amylase enzyme (Sundarram & Murthy, 2014). Several microorganisms produce α-amylases that have different features, action patterns, different substrate specificities and big variations in thermostability, optimum working temperature and pH. Moreover recent genetic engineering techniques have enabled the production of desired amylases in desired microorganisms with desired action pattern (Gupta, Gigras, Mohapatra, Goswami, & Chauhan, 2003; J. E. Nielsen & Borchert, 2000). Table 1.3.1.1 shows the production of α-amylase from several microorganisms.

In the industry, low cost production strategies and stable enzymes with high activity are favored. This is achieved via recent genetic and protein engineering techniques through heterologous expression of recombinant proteins (Nevalainen & Peterson, 2014). Moreover thermostability is the most desired characteristic of the majority of the industrial enzymes. Thermostable organisms are perfect donors for the thermostable enzymes which are widely used in recombinant enzymes for commercial production (Sundarram & Murthy, 2014). Thermostability characteristic is preferred for alpha amylase due to its utility in liquefaction and thinning of starch (Amylase Research Society of Japan., 1988a). Furthermore, thermostable alpha amylases minimize the risk of microbial contamination and reduce the time of reaction, thus provide considerable energy saving. Moreover, since hydrolysis occurs at higher temperatures, D-glucose polymerization to iso-maltose is minimized (Pandey et al., 2000)

Table 1.3.1.1 Some microbial α-amylases, properties and their producers. (Gupta, Gigras, Mohapatra, Goswami, & Chauhan, 2003)

Source	MW (Kda)	pH / Stability	Temperature Optima / Stability	Inhibitors	Stabilizers
Fungi and Yeast					
A.oryzae	//	5.4/ 5.0-9.0	50°C (30 min)		
A.niger	58.0	4.0-5.0/ 2.0-7.0	60°C (10min)		Ca^{+2}
A.flavus LINK	52.5	6.0/ 6.0-10.0	50°C (1h)	Ag^{+2}, Hg^{+2}	Ca^{+2}
A.usami	54.0	3.0-5.5	60°C	Ag^{+2}, Hg^{+2} ,Cu^{+2}, Zn^{+2}	
Crytpcoccus S2	66.0	6.0	50-60°C		
S. cerevisae	54.1	5.0	50°C		Ca^{+2}
Trichoderma viride	42.0	5.6	65°C		
Bacteria					
B.subtilis	48.0	6.5	50°C	Fe+2 , Hg+2, Al+3	Mn, Co
Bacillus sp IMD 435	63.0	6.0-6.5			
Bacillus sp WN 11	76.0, 53.0	5.5 -9.0 (1h)	75-80°C	Fe^{+2}, Hg^{+2} , Cu^{+2}	
E.coli	48.0	6.5	50°C	Fe^{+2}, Hg^{+2} , Cu^{+2}	
L plantarum A6	50.0	5.5 /3.0-8.0	65°C	I_2, Hg^{+2}	
Streptomyces sp	47.8	5.5	60°C		Ca^{+2}
Pseudonomas stuzeri	12.5	8.0	47.0 (1h)°C		

The structure of alpha amylase of different species were extensively studied. α-amylase from *A.oryzae* (Wang et al., 2016), and a human salivary α-amylase (Lévêque, Janeček, Haye, & Belarbi, 2000) and thermophylic archeabacteria α-amylase from *Bacillus licheniformis* (BAA) (Fitter & Haber-Pohlmeier, 2004) consist of three domains in common. These domains are referred as A, B, and C domains (Figure 1.3.1). A domain is the catalytic domain and it has an amino-terminal (β/α)8-barrel structure. A domain is followed by a domain C which

consists antiparallel β-strands. And there is a small B domain between the third β-strand and the third α-helix of the (β/α)8-barrel (Fitter & Haber-Pohlmeier, 2004).

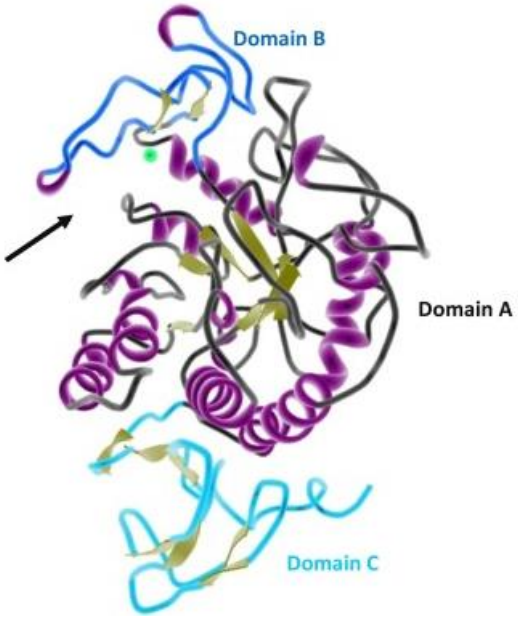

Figure 1.3.1 The green dot indicates the Ca2+-binding site. The blue and pink parts represent beta-strands and alpha-helixes, respectively. The arrow shows catalytic site. Domains A, B ,C are set apart by different colors and labelled by the matched color (Zhang, Han, & Xiao, 2017)

There is also observed an additional domain named as E domain in the structure of α-amylase of *Streptomyces limosus* (αSli) and malto-tetraohydrolase of *Pseudomonas saccharophila* (G4α) which plays role in the adsorption of raw starch (Ohdan et al., 1999).

Moreover some carbohydrate-active enzymes show an integrated assembly where catalytic modules of the enzymes targets the insoluble substrate. This assembly is noncatalytic and one or more of this could be attached to the enzyme which facilitates the activity of the enzyme. These assemblies are called *Carbon Binding Modules* (CBMs) (Fujimoto, 2013). Due to the fact that these modules were first discovered in amylases that hydrolyzed raw starch they were also called *Starch Binding Domains* (SBDs). They are found in a wide spectrum of often non-amylolytic enzymes (Machovič & Janeček, 2006). They are only observed and in

microbial enzymes related to starch metabolism where they are preserved. CBMs are categorized into numerous different families according to their amino acid sequence resemblances (Rodríguez-Sanoja, Oviedo, & Sánchez, 2005). Basic functions of CBMs are; (i) to bind raw starch, (ii) to enable enzymatic interaction with the substrate in the solution, (iii) to deliver the substrate to the catalytic site of the enzyme and (iv) to disrupt the surface of the starch grain (Boraston, Bolam, Gilbert, & Davies, 2004; Machovič & Janeček, 2006) (Figure 1.3.2 b).

Enzymes that normally do not have a CBM domain can be improved by adding a suitable Carbon Binding Domain by protein engineering techniques (Mehta & Satyanarayana, 2016; Zmasek & Godzik, 2014). Three dimensional structures of amylase (ABC domain) and CBM domain combination in the genome is shown on Figure 1.3.3a.

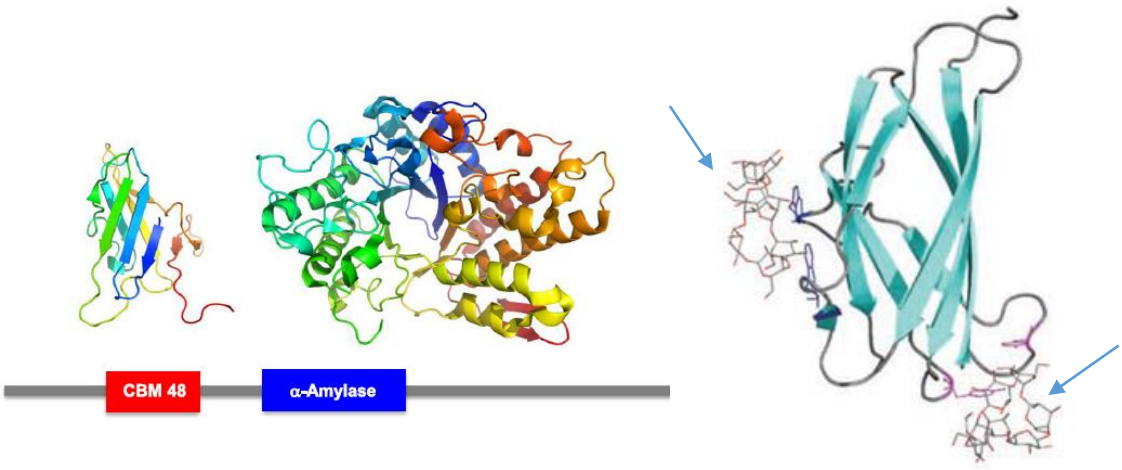

Figure 1.3.2 a) CBM48 domain placed in front of the α-amylase gene in the genome.(Zmasek & Godzik, 2014) b) α-amylase of *A.niger* improved with two CBM20 domain (indicated with the arrows.) (Machovič & Janeček, 2006)

α-Amylases are widely known to be Ca^{2+} dependent metalloenzymes. Ca^{2+} ions needed in stabilizing the architecture of the enzyme domains together and the activation of the catalytic site. Furthermore, it increases the thermostability of the enzyme (Nielsen, Pusey, Fuglsang, & Westh, 2003; Sundarram & Murthy, 2014).

However, only two extracellular secreted α-amylases do not require Ca^{2+} ions. These are archaeal *P. furiosus* and eubacterial *Thermophilus sp.* (Lévêque et al., 2000). In these enzymes rather than Ca^{2+} ions , Na^{+} ions provide the stability (Nonaka et al., 2003).

Besides α-amylase, there are also two more types of amylases which are β-Amylase and γ-Amylase.

1.3.2. β-Amylase

β-Amylase (EC 3.2.1.2) works to catalyze the non-reducing ends by hydrolysis of the second α-1,4 glycosidic bond of the dextrin, which pulls out a maltose at a time. (Figure 1.3.4.1)

They have commonly been acquired from plant sources. However, bacterial strains (*Bacillus, Pseudomonas, and Clostridium* (anaerobic) *sp)*. actinomycete strains (*Streptomyces sp.)* and fungal strains (*Rhizopus sp*.) are also reported to synthesize β-amylase. It is not found in animal tissues. It is highly active during the ripening of the fruit where starch is hydrolyzed by β-amylase into maltose, resulting in the formation of sweet flavor of the ripened fruit (Amylase Research Society of Japan., 1988b). Calcium is not required for the functioning of β-amylases and it is active at lower pH conditions than α-amylases (4.0-5.0). Moreover its action is slower than α-amylase (Ray & Nanda, 1996).

1.3.3. γ-Amylase

γ-Amylase (EC 3.2.1.3, glucoamylase) cleaves α(1–6) glycosidic bonds, along with the last α(1–4) glycosidic bonds at the nonreducing end of amylose and amylopectin, to get glucose. (Figure 1.3.4.1) The γ-amylase has the most acidic optimum pH of all amylases since it is most active around pH 3.0 (Pandey et al., 2000). It has a major importance in fermentation and food industries due to saccharification of starch and other related oligosaccharides. (Pavezzi, Gomes, & da Silva, 2008) .

1.3.4 Starch

Starch is a polysaccharide and occurs in semi crystalline granules which consist of two components; amylose and amylopectin. Amylose is a linear chain of glucose bound with α-1, 4-glycosidic bonds (up to 600 units). It forms the 20-25% of the starch. Amylopectin constitutes 75-80% of starch and includes branched chains of glucose units (up to 16 units) (Hobson, Whelan, & Peat, 1951; Ray & Nanda, 1996). Branching occurs via α-1, 6 glycosidic bonds in every 15-45 glucose units (Figure 1.3.4.1).

Hydrolysis of starch is highly dependent on the nature of the enzyme used (endo vs. exo). Earlier, starch was used to be hydrolyzed into glucose by acid hydrolysis which had lots of disadvantages like operations were carried at acidic environment accompanied with high temperatures and consequently very acidic nature of the end product for the next uses. These drawbacks were overcome with industrial production of α-amylase enzymes for the desired working conditions with optimum, safe and convenient routines (Sundarram & Murthy, 2014).

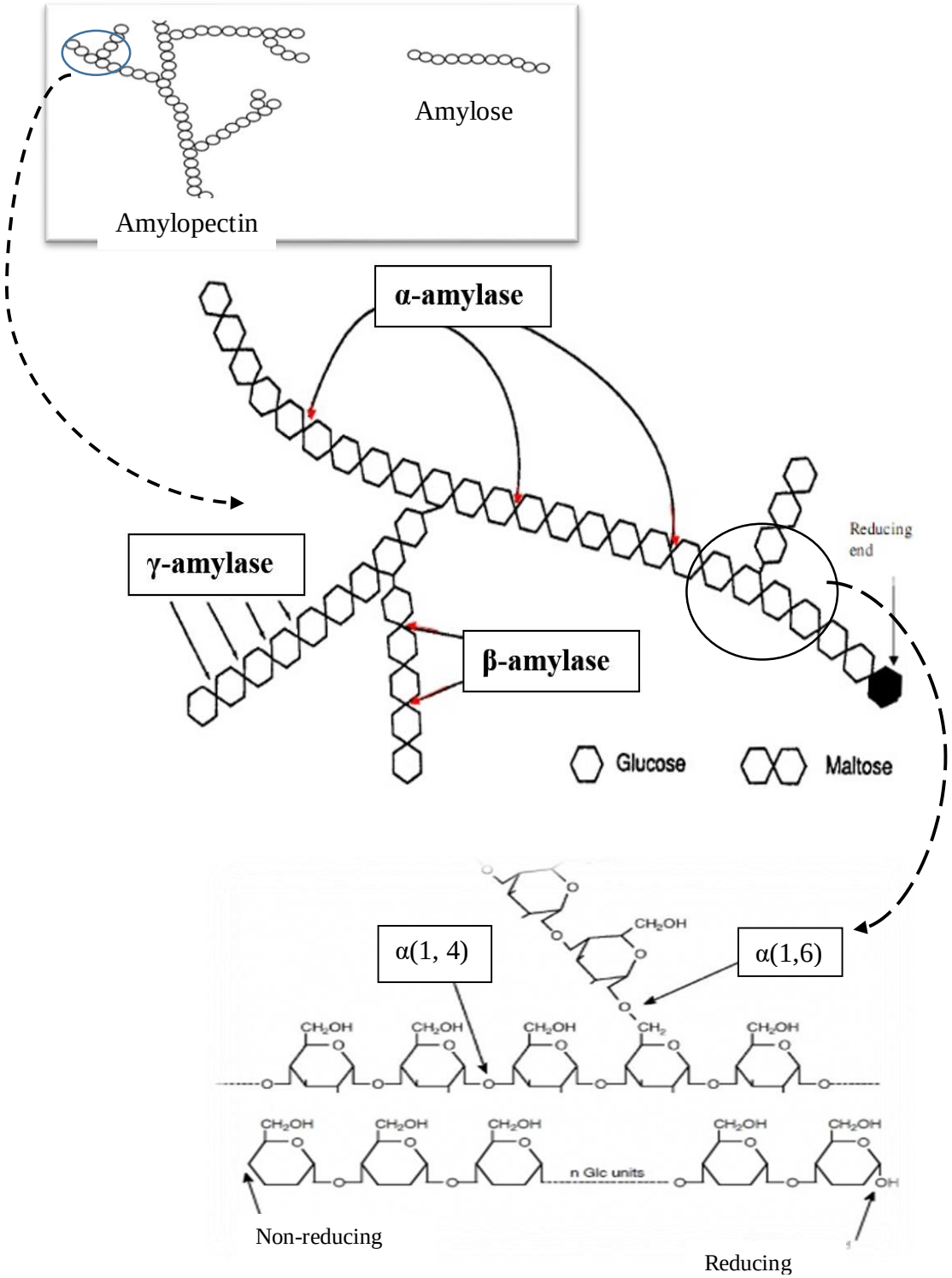

Figure1.3.4.1 Amylopectin and amylose. Action of Amylases. Reducing & nonreducing ends. (Hii, Tan, Ling, & Ariff, 2012)

1.3.5 Enzyme Activity Assays

There have been many methods developed to measure amylase activity on starch. Quantitative assays of amylases have two approaches to measure the enzyme activity. The first one is measuring the emerging, newly produced reducing ends after hydrolysis and the second one involves quantifying the amount of hydrolyzed starch from the beginning of the reaction. Both approaches are based on the use of colorimetric measurement to quantify the product or hydrolyzed substrate (Nelson, 1944; Mulimani & Lalitha, 1996).

The reducing groups could be detected by using alkaline copper, alkaline ferricyanide or 3,5 dinitrosalicylic acid (DNS). Each of these methods uses different known concentrations of maltose or glucose as a reference to estimate the reducing sugar concentration. The ferricyanide and copper methods detect only the reducing ends of the sample regardless of the length of the polymer chain. However DNS method (Figure1.3.6.1) gives increasing reducing values with increasing number of D-glucose units in the oligosaccharide chain of the substance. Moreover, calcium ions decrease the sensitivity of the DNS reagent. DNS is a simple to use and quick method but its sensitivity and accuracy is not that high but still is a good measure for quantitative estimate of the number of reducing ends produced (Hospital, 1944; Mulimani & Lalitha, 1996; One & Ki, 1996).

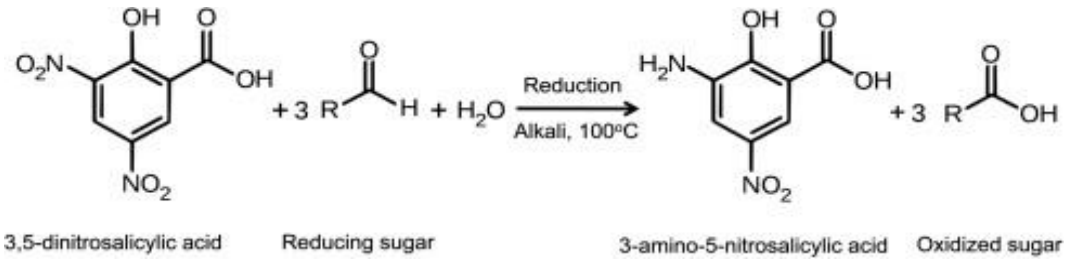

Figure 1.3.5.1 Chemical reaction of 3,5 dinitrosalicylic acid with reducing sugars.

The other method that is commonly used for quantifying enzyme activity is the starch disappearing test. In this method, hydrolyzed starch amount is measured

through the changes in blue-black color (Figure 1.3.6.2) that is observed due to the iodine-starch complex (FUWA, 1954; Mulimani & Lalitha, 1996).

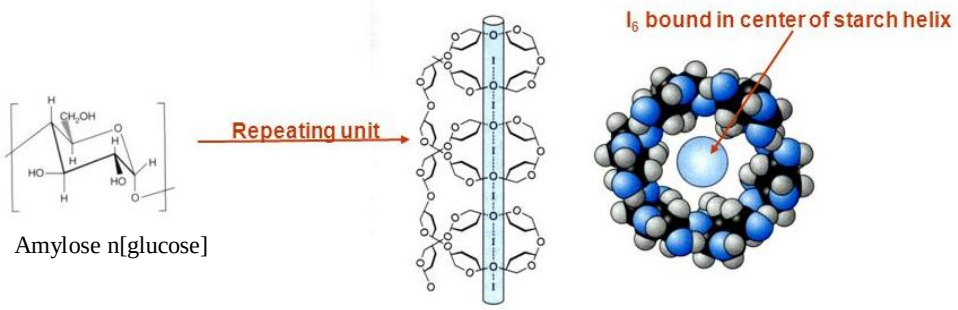

Figure1.3.5.2 The starch-iodine complex. The amylose chain built a helix structure around I6 units. (Harris, 2007)

This procedure reveals the 'endo' cleavage of the starch chains and cannot be used to analyze exo-acting amylases. The procedure is sensitive and easy to perform but not easily adoptable to quantitative determination since the minimum required iodine and starch concentrations are 2.5mM and 2.0g/L respectively (Zhang et al., 2017).

This method only measures amylase activity and the results cannot be expressed in international units that is *µmol glucose/ml enzyme/min.*

1.4. Industrial Applications of α-Amylase

Enzymes are considered to be alternative green solutions to the previously used chemical methods of hydrolysis in the industry (Crabb & Mitchinson, 1997; Guzmán-Maldonado, Paredes-López, & Biliaderis, 1995).

α-Amylases are used at baking, sugar, brewing, textile, paper industries (Mehta & Satyanarayana, 2016). In this study, the produced α-amylase was used for saccharification of starch for the use of other microbial fermentation/growth. Lactic acid from *Lactobacillus casei* and baker's yeast (*Saccharomyces cerevisiae)* was produced from the hydrolyzed starch that was used as the carbon source.

1.4.1 Lactic Acid Production

Lactic acid (LA) was first discovered as a milk component in sour milk by Swedish chemist Scheele in 1780. Then in 1789, Lavoisier named it as ‘*’acide lactique*’’. Later in 1857, Pasteur revealed that it was not a milk component, it is in fact a fermentation metabolite of some microorganisms (Ghaffar et al., 2014; Wee, Kim, & Ryu, 2006).

LA is a valuable chemical in various fields of the industry. In food industry , it is used as a preservative, acidulant and flavoring agent, in pharmaceuticals , textile and chemical industries it is a very good raw material for the production of lactate ester, propylene glycol, propionic acid , 2,3 pentanedione, acetaldehyde, acrylic acid, and dilactide (Åkerberg & Zacchi, 2000; Varadarajan & Miller, 1999). Moreover LA is a very important monomer of the biodegradable poly-lactic acid (PLA) which is known as sustainable bio-plastic material (Datta, Tsai, Bonsignore, Moon, & Frank, 1995; Litchfield, 1996). Therefore it is a worldwide demanded product that is needed roughly about over 650,000 metric tons per year (by 2010) (Ghaffar et al., 2014).

LA is produced by two groups of microorganism; bacteria and fungi. (Litchfield, 1996). Researches on the production of LA are mostly conducted on LA bacteria (anaerobically) and filamentous fungi (aerobically). Fungal species of Rhizophus sp. like *R.oryzae, R arrhizus* produe LA by utilizing starch through amylolytic activity (Ghaffar et al., 2014). Moreover, fungal fermentations of LA could take place in a low-cost medium but it needs good aeration due to being an obligate aerobe. Production yield of fungi is lower than LA bacteria and with fungal fermentation, by-products like fumaric acid and ethanol could form (Tay & Yang, 2002). Hence, production of world’s LA need is mostly provided from LA bacteria which specifically uses carbohydrates as the carbon source (glucose, sucrose, starch/maltose derived from feed-stocks such as beet sugar, molasses, whey, and barley malt). LA bacteria are studied in two groups in terms of distributing C-source into metabolites. To illustrate, using glucose to produce only LA is classified as

homolactic (some species of Lactobacilli and most species of Enterococci, Lactococci, Pediococci, Streptococci, Tetragenococci, and Vagococci) or producing by-product like CO_2 and/or ethanol is classified as heterolactic (Leuconostoc, Oenococcus, Weissella, and certain Lactobacilli) (Das & Goyal, 2012). In commercial microbial production of LA, *Lactobacillus sp* is preferred since homolactic fermentation provides the highest productivity (Baltacı, 2017).

In this study, the produced sugar from the starch hydrolysis was tested on *L.casei* for the production of LA.

1.4.5. Bakers' Yeast Production

The ''Yeast'' term generally used to define unicellular fungus, in which hundreds of species identified as. The most well-known and famous species of yeast in wellness and health is known as *Saccharomyces cerevisiae*, which is also notorious by its other common names, brewer's yeast or baker's yeast.

The aim of baker's yeast production is to collect as fast as possible the highest amount of living cell mass at the lowest cost. Firstly, a fermenter must be inoculated with the strongest and purest microbial starters. Secondly, these microorganisms must be fed under systematically controlled conditions to improve yeast biomass and gassing power, sufficient to raise dough, so that bakers will not experience variations in bread volume (Gélinas, 2016).

The most common raw material of the industry for the yeast production is molasses which is a by-product of sugar production due to its low cost and high sugar content (Liang, Wang, Zhou, & Liu, 2009).Molasses are fed to the yeast at a controlled rate after dilution, clarification and sterilization, with nutrients. Figure 1.4.5.1 shows the basic steps of baker's yeast production.

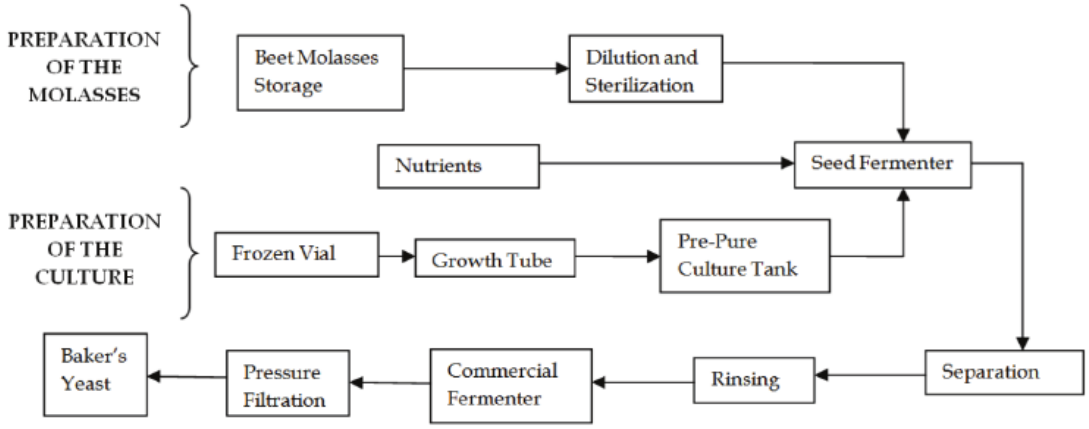

Figure 1.4.5.1 Production Steps of Baker's Yeast from molasses (Evren, Ozgun, Kaan, & Ozturk, 2011)

However in industry, the processes following the baker's yeast fermentation generate wastewater that has very high load of organic substances with high proportions of total nitrogen, trimethylglycine, sulphate, varying phosphorus content and non-biodegradable organic pollutants with dark colored water. (Liang et al., 2009) Specifically, the color is one of the most challenging parameters at the baker's yeast industry due to the melanomin present in the molasses (Büyükkamaci & Filibeli, 2002). Molasses that consists of 45-50% unprocessed sugar , 15-20% non-sugar organic materials, 10-15% minerals and 20% of water is the main contaminant of the wastewater generated from baker's yeast industry (Evren et al., 2011). Consequently extra amount of work and energy are required to treat the wastewater after yeast production (Eğilmez, Süer, Özgüner, 2012).

In the current study, working with starch hydrolysate was investigated as an alternative clean carbon source for the yeast production. The starch hydrolysate is not necessarily cheaper than molasses. However, it is easily reachable with constant and standard characteristics as the composition of molasses could vary a lot among different batches (Spigno, Fumi, & De Faveri, 2009).

1.5 Objectives of This Study

This study aims to produce the structurally improved thermophilic alpha-amylase enzyme from the fungal host: *Aspergillus sp*. within controlled fermentations. Different strains were tested for enzyme production. Following the production of the enzyme in experimental flasks, to mimic industrial conditions, scale up experiments were also conducted in large volume fermenters. Another goal of the study was to utilize this enzyme to create a fermentation/growth medium for different microorganisms. Lactic acid production from *Lactobacillus sp* and baker's yeast production (*Saccharomyces cerevisae)* experiments were performed using the hydrolyzed starch obtained. Thus the effect of a good and cheap alternative carbon source for likewise fermentations was explored.

CHAPTER 2

MATERIALS AND METHODS

2.1 Measurement of the Enzyme Activity

Since the main goal of the study was to obtain alpha amylase with the highest activity for the tested conditions and strains, *enzyme activity* was the main response in all experiments. Therefore before explaining the enzyme production ways, the activity measurements will be explained.

In the study, alpha amylase activity was measured using two methods: Dinitrosalicylic acid (DNS) and Starch Disappearing Method (SDT).

2.1.1 Dinitrosalicylicacid Method (DNS)

DNS reagent is used to estimate the concentration of reducing sugars in a sample colorimetrically.

Method was adjusted from the study of (Mohamed, Al-maliki, & Kumosani,(2009). First, 1.0 g of DNS was dissolved in 50 ml of distilled water. Thirty grams of sodium/potassium tartarate tetra hydrate (Rochelle salt) was added to the solution afterwards. Twenty ml of 2N NaOH was added to the solution which turned the solution to transparent orange yellow color. The final volume was completed to 100 ml using distilled water. The solution was kept in dark for further analysis.

For the method, D-glucose was used as the standard. A calibration curve was prepared using glucose at a concentration range of 0.03-1.5g/l. The curve is provided in Appendix B.

To determine the activity, 1 milliliter of 1% starch solution was pipetted to a 10 ml test tube and 1 ml of the spent medium (fermentation medium containing alpha amylase) was added later. The 2ml mixture was put into a 80°C heat block and kept there for 10 min (Only for *Aspergillus niger* N402 experiments, temperature was set to 35°C). Afterwards 1 ml of DNS solution was added and the final mixture was put into boiling water for 5 min. Following cooling to room temperature the sample was diluted with 9 ml of distilled water so that the absorbance values would not exceed 1.00. Absorbance of the samples was measured at 540 nm by using a spectrophotometer (UV 1202, Shimadzu, Japan).

Absorbance values were substituted to the calibration curve equation (Figure B1) to find the reducing sugar concentration (C) and the activity was calculated using the formula given below:

$$Amylase\ Activity\ (U) = \frac{C*DF}{t*MW_{glucose}*R_e} \qquad \text{(Equation 2.1)}$$

Where

- C : concentration of reducing sugar in the final hydrolysate (g/L)
- DF : Dilution Factor
- t : hydrolysis time (minute)
- $MW_{glucose}$: Molecular Weight of Glucose (µg/mole)
- R_e: Ratio of enzyme in the reaction medium (ml/ml)

Activity (U/ml) (1U = amount of glucose released (µl? mole) by 1 ml enzyme solution /1 min at 80 °C)

One unit of activity was defined as one micromole of glucose released by 1 mL of enzyme in 1 minute at 80 C*.

(*)(WT strain's amylase was determined at 35°C)

2.1.2 Starch Disappearing Method (STD)

Starch Disappearing Method is a microplate based starch–iodine assay that is used to measure amylase activity from the amount of degraded starch in the solution (Xiao, Storms, & Tsang, 2007).

Two g/L starch solution was prepared in 50 mM acetate buffer (pH 5.00). Acetate buffer of pH 5.00 was prepared by mixing 0.2M sodium acetate solution (2.72g/100ml) ($CH_3COONa \bullet 3H_2O$ 136.08 g/mol) with 0.2M acetic acid (60.05 g/mol) (1.15ml of glacial acetic acid was made up to 100ml) at 70/30 ratio and diluted at 1:4 ratio to adjust molarity to 50mM.

Another reagent in SDT test was the Lugol solution. Lugol Solution was prepared by mixing 0.17 g I_2 (253.81 g/mol) and 1.27 g KI (166.01 g/mol) in 100 ml distilled water.

Calibration curve for SDT method was prepared with starch solution at a concentration range of 0.25 -2.0 g/L. A separate calibration curve was prepared for each microplate. A representative calibration curve is provided in Appendix B (Figure B2).

Forty µl spent medium and 40 µl starch solution (2.0 g/l) was mixed in a 0.2 ml PCR tube. The mixture was incubated for 30 min at 80°C in the PCR machine (ARKTIK-ThermoScientific,Finland). Hydrolysis was stopped by adding 20 µl 1 N HCl. Afterwards 100 µl Lugol Solution was added to the mixture. Depending on the remaining starch amount in the solution color changes black to yellow. (Appendix B (Figure B3). One hundred and fifty µl iodine treated samples were transferred to flat-bottomed 96-well microplate and the absorbance values were recorded at 580nm via a microplate reader (MultiscanGo-ThermoScientific, Finland). Microplate reader measurements were conducted using the conditions given in Table 2.1

Table 2 1. Microplate reader measurement protocol.

Protocol parameters		
	Check temperature at start [C°]	No
Shake		
	Duration [hh:mm:ss]:	00:00:10
	Shaking type	Continuous
	Shaking speed	High
Absorbance		
	Wavelength [nm]	580
	Use transmittance	No
	Measurement mode	Precision
	Path length correction	No

Absorbance values were substituted to the calibration curve equation (Figure B2) to find the remaining starch amount (S_r) and the activity was calculated using the formula given below:

$$Amylase\ Activity\ (U) = \frac{(S_i - S_r) * DF}{1000 * t * C_f} * 60 \qquad \text{(Equation 2.2)}$$

Where

- S_i : initial starch amount (80 µg)
- S_r : Remaining starch amount in the final hydrolysate (µg)
- DF : Dilution Factor
- t : incubation (hydrolysis) time (minute)
- C_f: conversion factor required to calculate enzyme activity in one milliliter enzyme sample.

- 1000 and 60 denote the conversion factors of *'μg → mg'* and '*min→h*' respectively.

2.2 Fermentation

Fermentation experiments were carried out at 250 ml flasks, 1.5 L bioreactors, and fermenters of 60L and 80L. pH of the fermentation medium and the temperature was set to 5.50, 30 °C respectively. To achieve the highest level of enzyme activity effect of different factors on fermentation were investigated. Enzyme activity was measured as the main response to examine the effect of different factors.

2.2.1 Preparation of Spore Suspensions.

All spore suspensions were prepared fresh before the experiments. *Aspergillus sp* that were used in the study were kindly provided as spore samples from Leiden University, Netherlands. All spore handling procedures were conducted aseptically. The spores were inoculated on potato dextrose agar (PDA) and incubated at 30 °C for 4-5 days. Afterwards spores were harvested from the lawn culture of *Aspergillus* sp. on potato dextrose agar by flooding the culture with a sterile saline solution (0.9% NaCl, pH 6-7) containing 0.05% (v/v) Tween80. Then the spores were dislodged from the hyphae on PDA with the help of a sterile glass spreader or curved tip forceps and finally the solution with spores were transferred to sterile tubes and vortexed vigorously.

The number of spores in the spore suspension was counted using a hemocytometer (Fuchs-Rosenthal) and diluted to desired number of spores/mL to obtain a stock spore solution, and kept at 4 °C until further use.

The spore suspensions for fermenters (V> 50L) were prepared also on PDA on heat resistant glassware (A > 220mm x 220mm) and the lawn culture was harvested with saline solution as explained before.

2.2.2 Complex medium 1 (C1)

This medium was used for the growth of *Aspergillus sp*. in flask (V=250 mL) experiments (Section 3.1 and 3.2)

The medium was prepared using 1L distilled water of which the composition is listed in Table 2.2 pH of the medium was adjusted to 5.50, otherwise stated.

Table 2.1. Composition of Complex Medium 1 (adapted from Anderson & Smith, 1971)

	% (w/v)
Yeast Extract	0.10
KH_2PO_4	0.10
$MgSO_4.7H_2O$	0.05
$CaCl.2H_20$	0.01
KCl	0.50
Starch (As the carbon Source)	0.10

Before the fermentation, the medium was autoclaved at 121°C for 15 minutes. After cooling to 30°C, the spores were inoculated and the fermentation was started.

2.2.3 Complex medium 2 (C2)

This medium was used for the production of alpha amylase enzyme from *Aspergillus sp*. (Section 3.3)

Table 2.2. Composition of Complex Medium 2

	% (w/v)
Yeast Extract	0.05
KH_2PO_4	0.10
$MgSO_4.7H_2$=	0.05
Starch (As the carbon Source)	1.00
	Volume
Vishniac Solution$_{1000x}$	1 ml
Asp+N$_{50X}$	20 ml
Total Volume	1 L

For Asp+N and Vishniac solutions, study of (Vishniac & Santer, 1957) was used. The full recipe of the solutions are provided in Appendix C.

2.2.4 Investigation of the effect of several factors on the enzyme activity *(Flask Experiments)*

2.2.4.1 Effect of Carbon Source

Fermentation volume of 125ml in a 250 ml flask at a spore concentration of 10^6 spores/ml was used. Shaking incubator was set to 200 rpm at 30 ^oC. As the different carbon sources; cellulose, sucrose, glucose, fructose, maltodextrin, maltose syrup, maltose, and starch were utilized. Enzyme activity was measured using DNS method.

2.2.4.2 Effect of Medium Volume / Total Flask Volume (MV/TFV)

Considering the results obtained in the previous section, starch was selected as the best carbon source and using the same fermentation conditions, effect of *Medium*

Volume/Total Flask Volume was investigated (Table 2.2.4.2) Inoculated spore level was adjusted 10^6 for each flask. Enzyme activity was measured using DNS method.

Table 2.2.4.2 Experiment design set up to explore the effect of MV/TFV ratio on α-amylase activity

Medium Volume (MV) (ml)	**Total Flask Volume (TFV) (ml)**	**MV/TFV**
50	500	0.1
100	500	0.2
150	500	0.3
200	500	0.4
250	500	0.5
300	500	0.6
350	500	0.7
400	500	0.8

2.2.4.3 Effect of Fermentation Medium's pH

Using the conditions in the previous section and MV/TFV ratio of 0.5, experiments were carried out at pH values of: 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, and 8.0. During the experiments, pH was kept constant and adjusted with 2M NaOH or 2N HCl when necessary. Enzyme activity was measured using DNS method.

2.2.4.4 Effect of Nitrogen Source

Effect of nitrogen source was investigated using the best conditions obtained so far (Carbon source = 1% starch, T=30 ^{o}C, pH=5.50, MV/TFV=0.50, Spore Inoculum

Level=10^6 spores/ml). Ammonium sulfate $(NH_4)_2SO_4$, sodium nitrate ($NaNO_3$), urea (CH_4N_2O) and yeast extract were tested as the nitrogen sources. Enzyme activity was measured with DNS method.

2.2.4.5 Effect of Temperature

For the conditions in the previous section and using the yeast extract as the N source, effect of different temperatures (25 - 30 - 35 - 40 - 45 °C) were tested. Enzyme activity was measured using DNS method.

2.2.4.6 Spore Inoculum Level

For the conditions in the previous section and setting the fermentation temperature to 30 °C, effect of different spore numbers (10^3 , 10^4 , $5x10^4$, $2x10^5$, $2x10^5$, $5x10^5$,10^6 , $2x10^6$, $1.5x10^7$ $2x10^7$ g/ml) were tested. Enzyme activity was measured using DNS method.

2.2.4.7 Experiments with Genetically Modified Strains

For the conditions in the previous section and using a spore number 10^5, 3 different genetically modified strains of *Aspergillus niger* that were provided by Leiden University, Netherlands were tested for their alpha amylase activities. These strains were named as Am13, AmC18 and AmC28 while the original strain used in previous experiments was named as *A.niger N402*. Enzyme activity was measured using the DNS method.

2.2.4.8 Bioreactor Experiments

Bioreactor experiments were performed using a Sartorius Biostat B plus 3L (Germany) fermenter. All *Aspergillus* strains were tested at 1.5L volume using C1 media (Section 2.2.2) at %1 starch concentration. At a spore inoculum level of 10^5/ml, pH was set to 5.5, and temperature was kept at 30 °C. Agitator was adjusted to 700 rpm. After preparing the bioreactor, with the fermentation media it was autoclaved at 121°C 15 minutes. Following the cooling to 30°C the

fermentation was started by adding spores aseptically. Enzyme activity was measured using DNS method. In all bioreactor and fermenter experiments air was used as the O_2 source and it was filtrated filtered using a 0.22 μm filter (MILLIPORE Millex-FG 50mm PTF). Aeration was set to > 1L/min.

2.2.4.9 Enzyme Production Experiments Using Complete Medium

In this part of the study, C2 medium was utilized in flask experiments. Temperature was set to 30 °C pH 5.50 and carbon source was selected as the maltose syrup %1 at a 0.5 MV/TFV ratio.

Enzyme activity was measured using the STD method.

2.2.4.10 Bioreactor experiments (V=1.5L) with Amc28 strain to find the spore amount higher alpha amylase production

For the experiments in this section, C2 medium was used in a 1.5L fermenter (Biostat B plus Bioreactor). Temperature, pH and agitation rate were set to 30°C, 5.50 and 700 rpm respectively.

Spore amount was tested at a range of 10^4-10^8 spores/ml.

All the reducing sugar analysis of this section was conducted using HPLC (Agilent Technologies, USA) with a Rezex™ RFQ-Fast Acid H+ (8%) column of 100 x 7.8 mm, (Phenomenex Inc., USA) accompanied with refractive index detector (RI). The temperature of the column and the detector was set to 25 °C, 30°C, respectively. 10μl of analyte was injected automatically a 0.6 ml/min flow rate using 0.05 M H_2SO_4 as the running buffer. Enzyme activity was measured using the STD method.

2.2.4.11 Amc28 Scale up trials with 1.5 L, 60 L and 80 L Volumes

Scale up experiments were performed by increasing the volume of fermentation that was conducted in 1.5 L bioreactor with Amc28 strain to 60L and 80L. Fermentation conditions were set to: 30 °C, pH 5.50 and 700rpm. The sugar analysis was

conducted by HPLC. 1.5L bioreactor was autoclaved at 121 °C for 15min with the fermentation media. And 60L and 80L fermenters were sterilized by increasing the whole temperature of the fermenter to 109°C for 1 hour and then cooling to 30°C to initiate the fermentation. Photos of the fermenter used in the study are given in Appendix. Enzyme activity was measured using the STD method

2.2.4.12 Use of the produced enzyme in hydrolysis of starch

After fermentations ended, the remaining medium was filtrated using filter paper (for flasks and 1.5 L bioreactors) or filterpres (for 60 and 80 L fermenters) (60L and 80L)

Following the filtration, the filtrate was loaded to the fermenter to start the hydrolysis at which initial starch concentration was set to 10%. pH was adjusted to 5.00 and temperature was increased 80°C. Mixing rate was set 250 rpm the hydrolysis was monitored using HPLC.

After letting sufficient time which (2-5h) for hydrolysis of starch by alpha amylase, glucoamylase was added.

At the end of the hydrolysis, the resulting hydrolysate (mostly a glucose solution) was filtered via filterpress and sterilized at the fermenter at 100 °C for 1 hour before use.

2.2.4.13 Employment of the hydrolysis product in the production of lactic acid by *Lactobacillus casei*

Lactobacillus casei NRRL B441 was used to produce L-LA from the glucose obtained in the previous section. The strains were kept at -80°C stocked in glycerol media. To prepare the culture for fermentation, 150 µl of *L.casei* culture was added from the stock media to 5 ml of MRS broth medium for activation. It was incubated at 38°C at 160rpm for 15h. This was repeated twice.

After second cultivation, 5ml of the *L.casei* cultures were added to 100ml sterilized MRS broth medium and cultivated at 38°C at 160rpm for 15 hours. At the end of

15 h *L.casei* was added to 1.5 L of growth medium that included: 20 g/L glucose, 10 g/L peptone, 8 g/L meat extract, 4 g/L yeast extract, 2 g/L dipotassium hydrogen phosphate, 5 g/L sodium acetate trihydrate, 2 g/L triammonium citrate, 1 g/L Tween80, 0.2 g/L magnesium sulfate tetrahydrate and 0.05 g/L manganous sulfate tetrahydrate.

2.2.4.15. Baker's Yeast *(S. cerevisiae)* Production

Inoculum of *S.cerevisae* was prepared from single colony isolated from a commercial yeast of Pakmaya using the scratched YPD agar plate in 100ml of YPD broth. After letting it to grow for 12 h, it was transferred to 1.5 L of YPD broth which was prepared 1L simultaneously to start fermentation. After this stage *S. cerevisae* was transferred to 20L fermenter aseptically. Vitamin + mineral solution (Recipe is available in Appendix B) was added to *S.cerevisae* in the reactor. Nitrogen source and glucose were added slowly. Aeration was set to be higher than 1L/min.

CHAPTER 3

RESULTS AND DISCUSSIONS

In this thesis, production of thermophilic alpha amylase from *Aspergillus sp.* and its utilization for different applications were covered. Figure 3.1 shows the main steps that were conducted in the study.

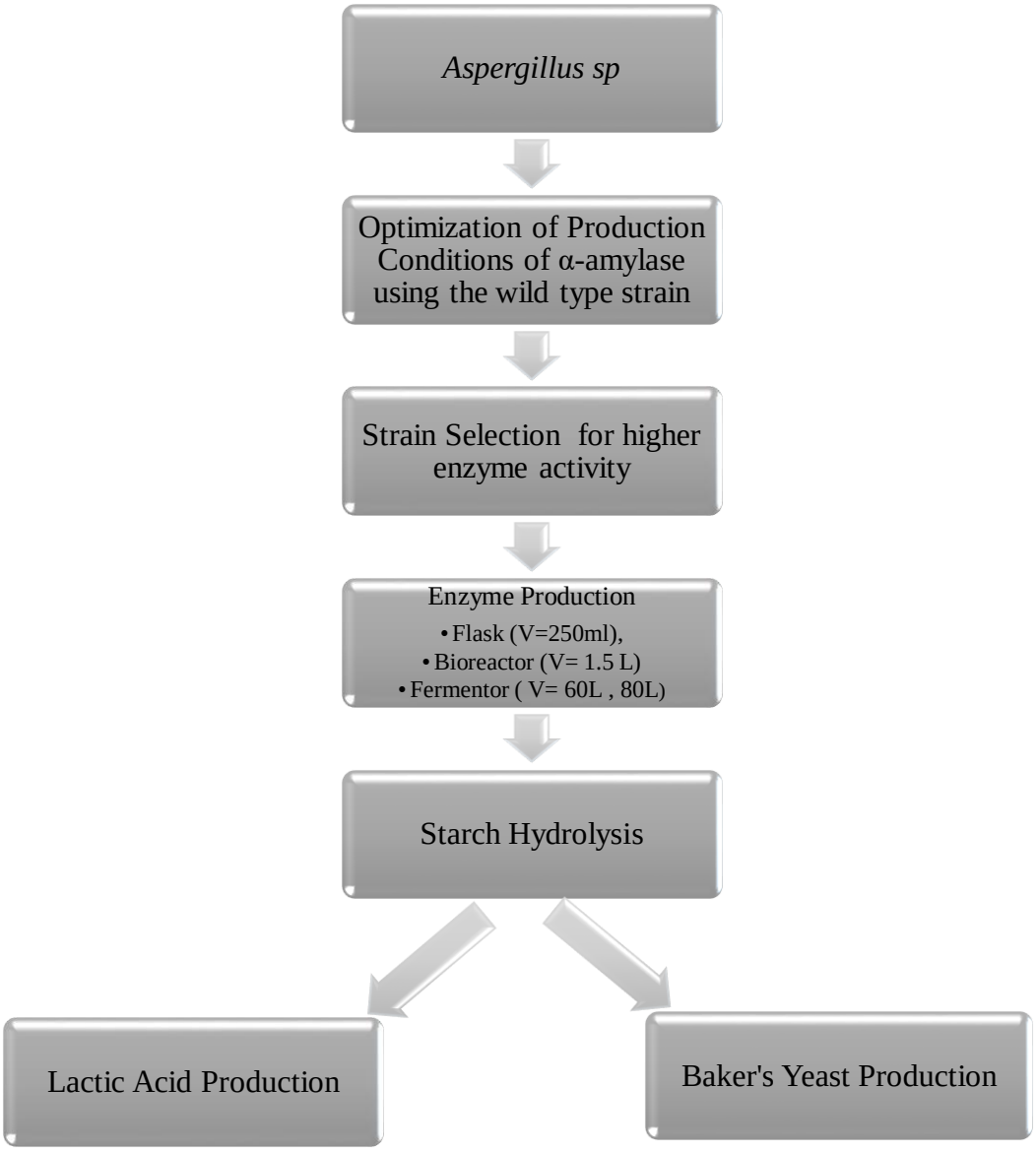

Figure 3.1 Main steps of the study covered in this thesis.

In the following section results that were obtained at each step will be discussed in more details.

3.1 Determination of the best production conditions of alpha amylase using the *Aspergillus niger* N402 (wild type) strain

Achieving the maximum possible amylase activity was the goal in the study. For that purpose, *A.niger* N402 (wild type –WT strain) was used and effect of different carbon sources, pH conditions, fermentation volumes, temperature and inoculated spore amounts were investigated. Figure 3.2 shows the schematic of the parameters investigated. Once the best conditions for the highest enzyme activity from the wild type strain were determined, genetically modified strains were tested for enzyme production.

As stated in Materials and Methods, Section 2.2.4, all experiments in this section were conducted using a 250ml flask volume. The enzyme activity was quantified using dinitrosalicylic acid (DNS) method (Miller, 1959)

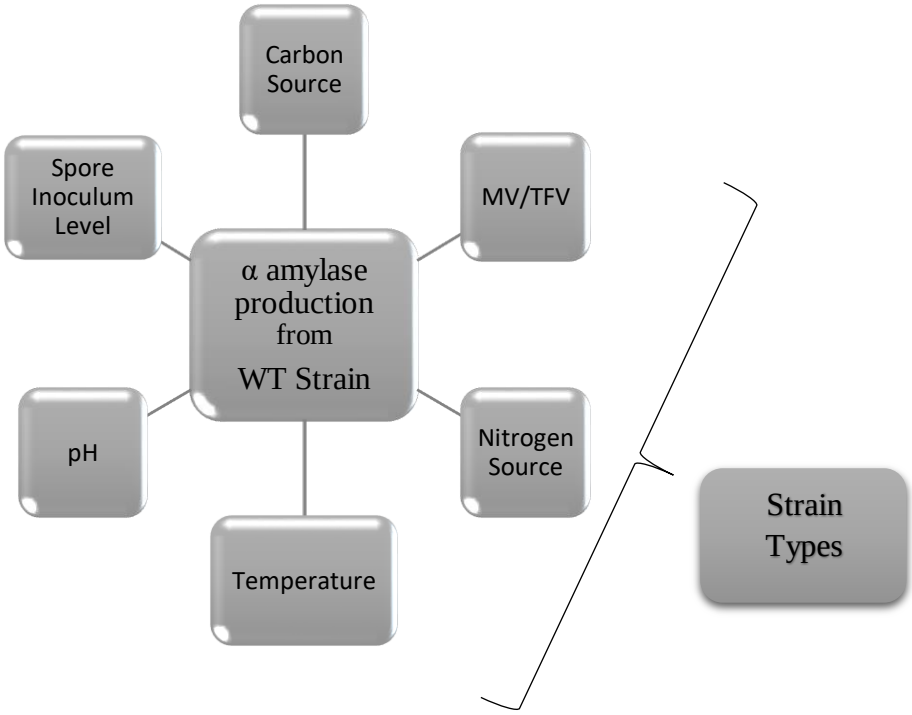

Figure 3.1 Schematic representation of parameters that were screened for better α-amylase production

3.1.1 Effect of Carbon Source on α-amylase Activity

As the carbon sources, sucrose, glucose, fructose, maltodextrin, maltose syrup, maltose, and starch were tested. Fermentation was conducted in 125ml/250ml volume flasks at 30 °C at 200 rpm. As seen in Figure 3.1.1 starch had significantly ($p \leq 0.05$) the highest alpha amylase activity. And the lowest activity values were observed for, sucrose and glucose. Considering that starch is a substrate for α-amylase and α-amylase hydrolyses the starch into low molecular weight carbohydrates and maltose, it would be reasonable for the amylase secreting organism to secrete more enzyme in the presence its own substrate (Morkeberg, Carlsen, & Nielsen, 1995).

The following flask experiments were decided to continue with starch as the carbon source.

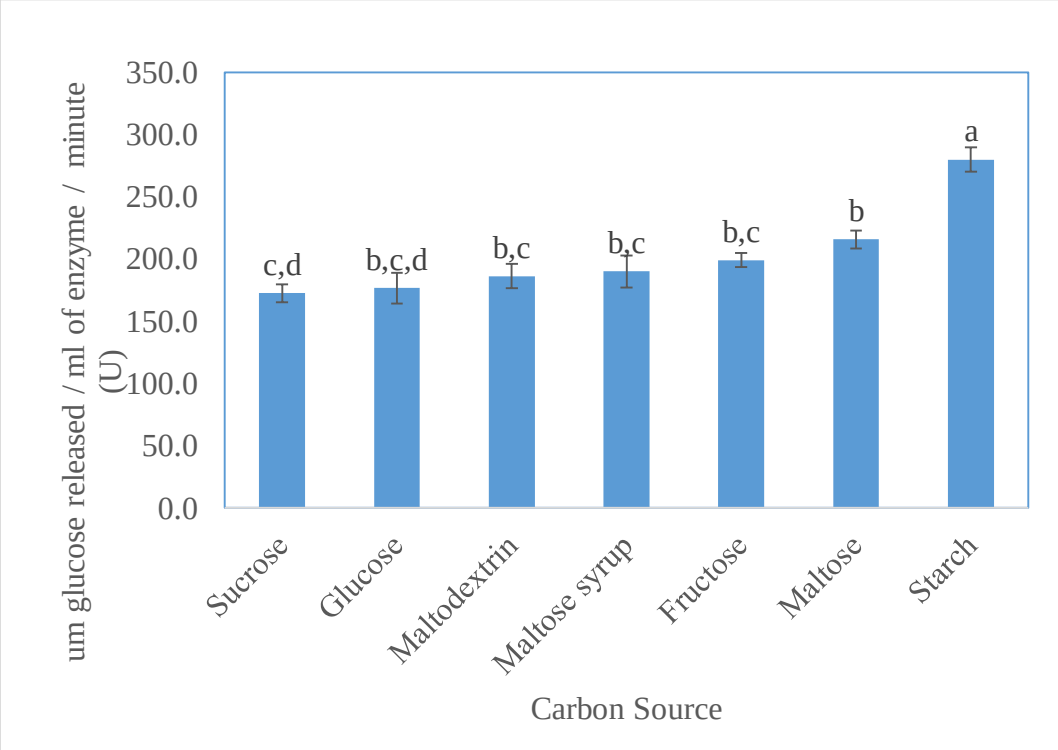

Figure 3.1.1 The effect of carbon source on alpha amylase activity

3.1.2 Effect of Medium Volume / Total Flask Volume (MV/TFV) on enzyme activity in 250 and 500 ml flasks.

Effect of MV/TFV ratio was explored to obtain higher amylase activity in flasks. Volume of the fermentation medium was adjusted according to the total flask volume.

Fermentation volume in the flask and agitation rate of the shaker affect the amount of the dissolved oxygen. In this study agitation rate of the shaker was kept constant at 200 rpm and effect of fermentation volume was sought after. It is known that as the fermentation volume increases, the amount of the dissolved oxygen decreases. Thus MV/TFV ratio could be correlated with the dissolved oxygen amount in the fermentation media (Henzler & Schedel, 1991). As seen in Figure 3.1.2 amylase activity increased linearly between 0.1 to 0.5 MV/TFV ratios. However, after 0.5 MV/TFV oxygen might have started to be a limiting factor for the fermentation. Besides, reduced medium volume may cause minor nutrient accessibility, and evaporation risk, on the other hand excess volumes might be safe against nutrient diminution however it can limit gas and heat exchange. Moreover the 250ml system gave a more significant reaction to increasing medium volume compared to the 500ml system after MV/TFV =0.6. That might be the result of dissolved oxygen amount is dramatically effecting the fungi cells in 250ml flask more than 500ml since 250ml flasks have lower dissolved oxygen values. (Collins et al., 2013)

Considering the maximum alpha amylase activity among the ratios tested, the highest MV/TFV ratio of 0.5 was selected. Next experiments were continued with using this ratio.

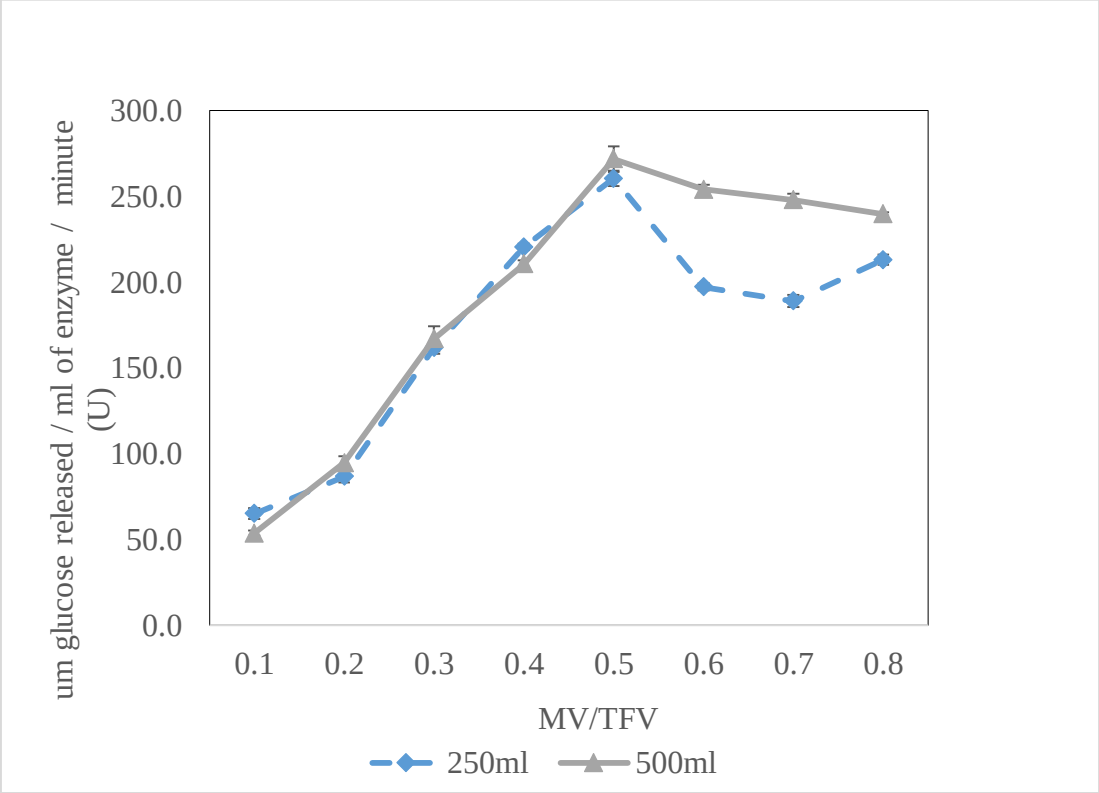

Figure 3.1 2. The effect of MV/TFV ratio on α-amylase activity

3.1.3. Effect of pH of Fermentation medium

Effect of fermentation pH on amylase activity was also explored the. pH was adjusted to 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0 and kept at the set value ± 0.1 for each flask . Fermentation was performed at 30 °C, 200 rpm. Highest amylase activity of 257.66 ±30.08 U was obtained between pH's 4-5.5 ($p \leq 0.05$). When pH was < 4 and >6 alpha amylase activity decreased dramatically. Aspergillus is an acid tolerant fungus where it can produce citric acid at very low pH of the fermentation medium.(Max et al., 2010; Papagianni & Mattey, 2006) that may explain the alkalinity of the fermentation medium is not favorable for its growth so the α-amylase activity. However ,the dramatic decrease in enzyme activity in acidic medium can also be explained with unfavorable fermentation conditions (Passamani et al., 2014). Nevertheless, in the study of (Donnell et al., 2001) It is shown that as the pH of the medium decreases (6.0 to 1.0) the proteolytic activity increased in the

fermentation medium. Consequently amylase activity might be decreased by the proteolytic activity where the pHs lower than 5.50 in this experiment. Moreover the pH was kept constant in this experiments. For the next experiments the pH would be not controlled strictly and it is experimented that the pH of the medium is steadily decreasing during the fermentation. Therefore the pH was started the best & highest value which is 5.50. Hence, the following experiments were continued at pH 5.50.

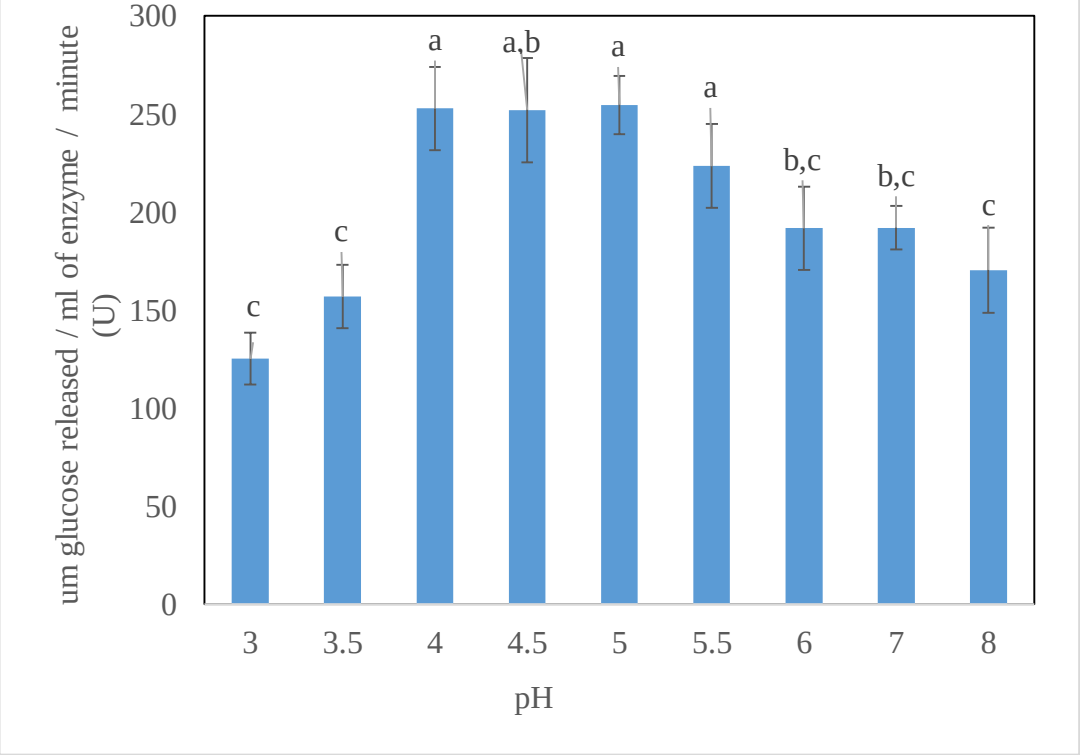

Figure 3.1 3. The effect of fermentation pH on amylase activity

3.1.4) Effect of Nitrogen Source

As another factor on for amylase production, the effect of four different nitrogen sources was explored. Fermentation was conducted at pH 5.5, 30 °C, and 200 rpm. Among ammonium sulfate $(NH_4)_2SO_4$, sodium nitrate ($NaNO_3$), urea (CH_4N_2O), and yeast extract, *Aspergillus niger* N402 gave significantly ($p \leq 0.05$) the highest activity of 236 U ±24.2 with yeast extract (George-Okafor, Tasie, & Anyamene,

2013). Following yeast extract, urea gave the second highest activity of 159.3 U ±8.4. There was no significant difference between ammonium sulfate and sodium nitrate ($p \geq 0.05$). Yeast extract is an organic nitrogen source which also include some important cellular components where promotes growth of *Aspergillus sp.* and production of enzyme. Furthermore, according to the literature of nitrogen metabolism of fungi the nitrogen sources like urea or nitrate are converted to ammonia in the cell and then consumed (Zoecklein, Fugelsang, Gump, & Nury, 1999). Naturally, it can be expected from ammonia to give higher enzyme activities than other nitrogen sources (NO_3^- , CH_4NO_2) in this case. However there has been so many researches on the effect of organic and inorganic nitrogen sources on α-amylase production/activity. It has been agreed that presence of yeast extract reduce the lag phase which promotes the growth of the microorganisms. However, among the inorganic nitrogen sources, like NH_4^+, NO_3^- could stimulate the amylase activity and growth more than urea. There is also many other studies reported differently where it was shown positive or negative effects of urea and NH_4^+ , NO_3^- ions on production and activity of amylases. (Acevedo & Gentina, 2013; Deljou & Arezi, 2016; Dharani Aiyer, 2004; Li, Liao, Zhang, Du, & Chen, 2011)

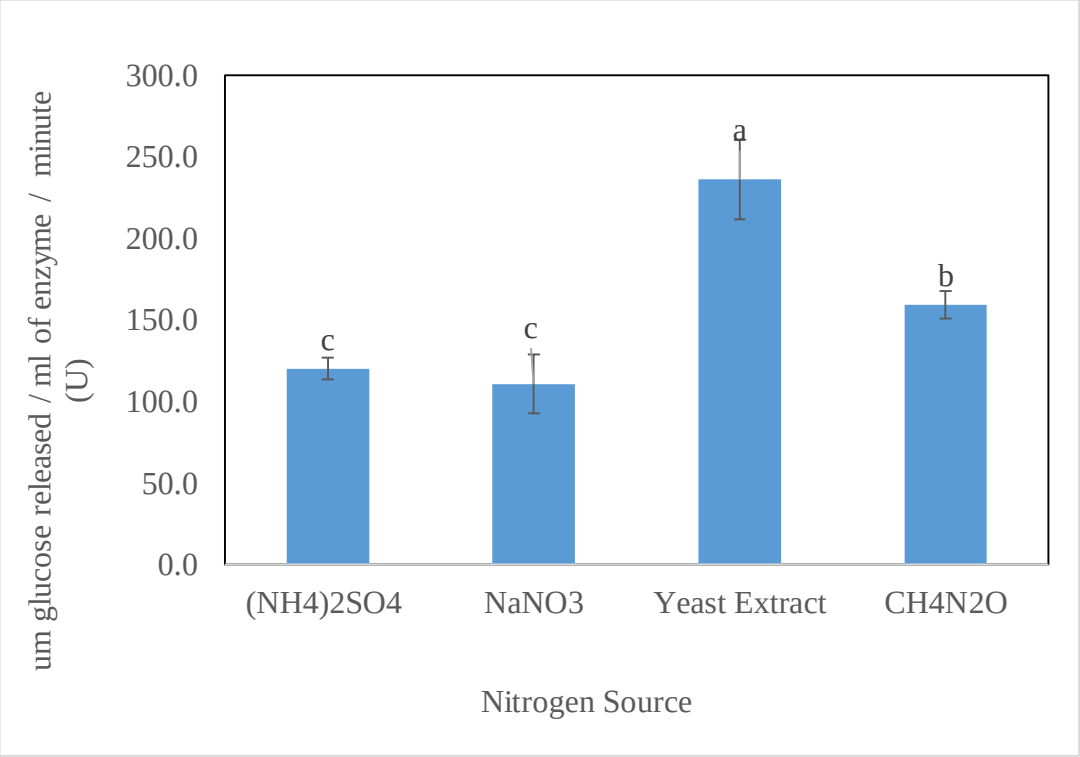

Figure 3.1 4. The effect of nitrogen source on amylase activity

The experiments were continued using yeast extract as the nitrogen source.

3.1.5) Effect of Temperature

Temperature could significantly affect the microorganism growth and thus the properties of the enzyme produced. Considering the studies in the literature (Prasad, Kurup, & Maheshwari, 1979) the tested temperature range was selected between 25-45ºC. The highest amylase activities were obtained at 30-35ºC for ($p<0.05$). The following experiments were conducted at 30°C.

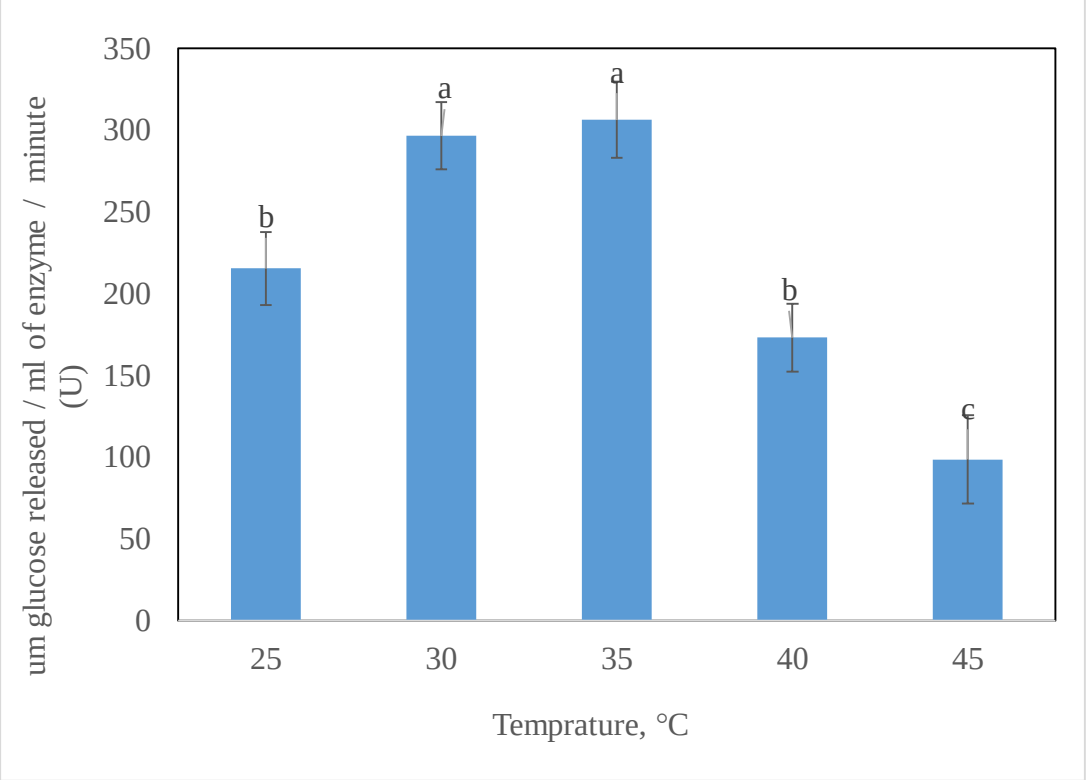

Figure 3.1 5. The effect of temperature of fermentation on α-amylase activity

3.1.6. Effect of Spore Inoculum Level

As seen in Figure 3.1.6 best activity recorded spore amount significantly between $1x10^5$ -$5x10^5$. ($p \leq 0.05$). From 10^4 to $5x10^5$ spores /ml the activity of amylase increased. However when spore inoculum level was higher than $5x10^5$ spores /ml fermentation, the amylase activity dramatically decreased. This might be due to some limiting nutrients or insufficient dissolved oxygen in the fermentation media. Since increasing spore amount creates an overpopulation condition in the flask, amount of the nutrients might have been limited and delivery of oxygen to cells might have been more difficult than less populated fermentation flasks. Therefore this unfavorable growth conditions might have affected the activity of amylase enzyme negatively (Amadi & Okolo, 2012; Sethi et al., 2016). For the following experiments, the spore inoculum level was adjusted to 10^5 spores/ml of fermentation medium.

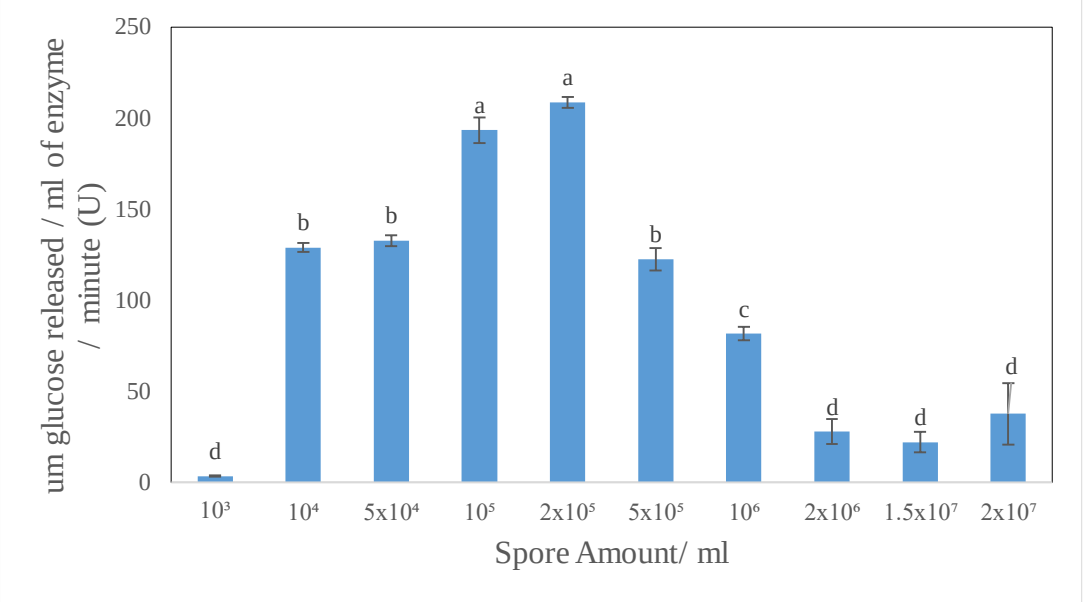

Figure 3.1 6. The Effect of Spore Inoculum Level on alpha α-amylase activity

3.1.7. Experiments with Genetically Modified Strains

Once the best production parameters (environmental factors) for the higher activity alpha amylase were determined ; these environmental factors were tested on different strains that were provided from Leiden University, Netherlands These strains were identified as *Aspergillus niger* Am13 , *Aspergillus niger* AmC18 and *Aspergillus niger* AmC28. These strains had been genetically modified through insertion of thermophilic alpha amylase gene by fungi protoplast-mediated transformation (Meyer, Ram, & Punt, 2010).

The thermophilic α-alpha amylase gene is originally from *Thermophilus aquaticus* sp., which is an extremophile that can tolerate high temperatures up to 80 °C. The metabolites and enzymes of this organism were also found to be tolerant to high temperatures as this microorganism could grow readily at high temperatures and this functionality makes this microorganism and its proteins industrially and biotechnologically highly valuable. (Pantazaki, Pritsa, & Kyriakidis, 2002) *Thermophilus aquaticus sp.* produces many industrially important enzymes of which some are thermostable DNA polymerases, starch hydrolytic enzymes, xylose

converting enzymes, glucohydrolyses etc. In this study, thermostable alpha amylase was selected to be produced by filamentous fungi *Aspergillus sp* due to its ability to secrete high amounts of the extracellular enzyme and to be tolerant to extreme cultivation conditions in industry(Meyer, Wu, & Ram, 2011).

AmC18 and AmC28 were genetically engineered to carry an additional CBM domain (Carbohydrate Binding Module) but Am13 strain did not have this domain. In this two strains CBM domain was attached to their thermophilic α-amylase enzyme to assist the amylase during enzyme activity (Fujimoto, 2013). CBM domains help enzymes in different ways. It helps enzyme to target distinct regions of its substrate like internal polysaccharide chains, reducing end, and non-reducing end depending on the action mechanism of the enzyme (endo/exo enzyme). As an example, when CBM module is used with the starch hydrolysis enzymes like amylases, it helps to loosen the substrate and the substrate exposes more to the catalytic module of the enzyme for more efficient degradation. Thus the use of CBM domains in enzyme engineering and biotechnology is considered to be a fine tuning strategy for carbohydrate recognition of the enzyme for enhanced enzyme activity. (Boraston, Bolam, Gilbert, & Davies, 2004)

Among these three, modified strains of *Aspergillus*, Amc28 showed the highest activity ($p \leq 0.05$) of 1,200U that was almost 6 times higher than *A. niger* N402. There were no significant difference between the activities of the enzymes produced by AmC18 and Am13 respectively (Figure 3.1.7).

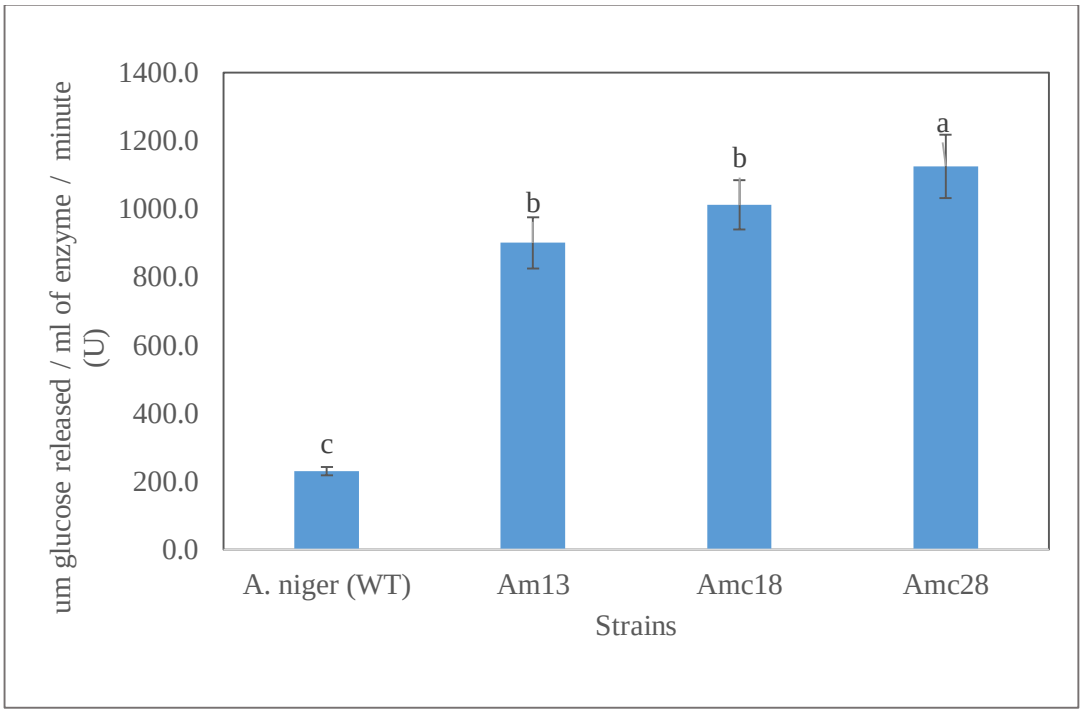

Figure 3.1 7. α-amylase activity of the Aspergillus strains (quantified using DNS method)

3.2) Bioreactor experiments

All *Aspergillus* strains were tested at 1.5L volume with C1 media (See Material &Methods Section 2.2.2). Fermentation lasted 168 hours for each strain. As seen in Figure 3.2.1, AmC28 strain showed the greatest activity for the first 4 days of fermentation. At the 4th day of fermentation, carbon source was depleted in the medium. After that fermentation was let to continue for further observation of the enzyme activity. Dramatic decrease was observed for AmC28 and Am13 strains. Enzyme activity from wild type *A. niger* and AmC18 decreased less for the last days of fermentation. Considering the first 4 days (96 hours) of the fermentation, the highest activity strain was found to be AmC28 which was also consistent with the flask experiments (Section 3.3.6). For comparison of the CBM (AmC18 and AmC28) and non-CBM possessing strains (Am13) , the enhanced enzyme activity effect of CBM domain was clearly observed when the activities in the first 4 days of fermentation was examined. The enzyme activities were found to be 1301.9

±49.9 U, 916.5 ± 89.1 U, 865.6 ±74.2 U, 225.5 ±73.8U for AmC28, AC18, Am13 and WT strain respectively (Figure 3.2.1).

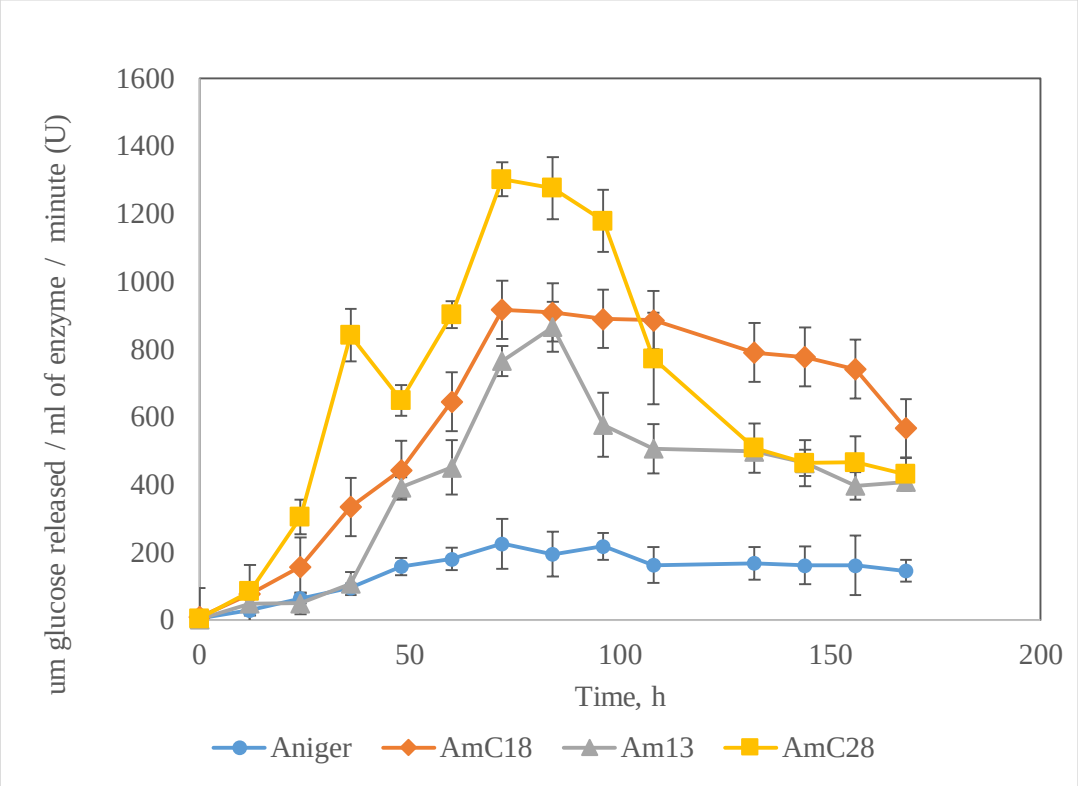

Figure 3.2 1. Bioreactor Experiments with Aspergillus Strains to investigate their α-amylase productions

Further experiments would be continued in the next sections to choose best strain to scale up production of alpha amylase.

3.3) Enzyme Production Experiments with Complete Medium

In Section 3.1 and 3.2 all fermentation experiments were conducted using C1 medium as the growth media (See Material & Method Section 2.2.2) Since for industrial enzyme production using a reduced medium is economically more feasible, most of the experiments were conducted using this medium. However, it is known in the literature that in a complete medium enzyme activity would be higher.

Thus to test the effect of a completed medium, the strains were also grown in such a medium (See Material Method Section 2.2.3).

Moreover to obtain more accurate quantitative results on enzyme, activity determination method was switched from DNS (Dinitrosalicylic Acid) (Miller, 1959) to SDT (Starch Disappearing Test) (Xiao et al., 2007). Because the fermentation media contained starch as the carbon source DNS method was proper to be used. However in the C2 media; maltose syrup were used as carbon source which contains high amount of reducing sugar. Since the DNS method measures the emerging reducing sugar from the amylase activity, the high amount of reducing sugars that came from the maltose syrup could have disturbed the DNS activity-solution by saturating it. Therefore STD method was used in the C2 media experiments. In STD method instead of measuring the emerging reducing sugars, the amount of hydrolyzed starch was taken into account to calculate the activity. Results are shown in Fig. 3.3.1. A Pearson correlation analysis was conducted on the enzyme activity results obtained with both methods and correlation coefficient was found to be greater than 0.95 ($p<0.05$).

As seen in Figure 3.3.1, the activity of the enzyme of AmC28 strain was found to be significantly different than the others and also was found to be the one with highest activity ($p \leq 0.05$). The activity of the enzymes of the strains AmC28, AmC18, Am13 and WT strains were found to be 179.8 ± 5.4U, 153.3± 9.8U, 80.0 ± 21.1 U and 0.3± 0.4U respectively. It is worth noting to mention that WT strain's enzyme showed almost no activity with this method. The reason might be that the WT strain's amylase were screened at the 35°C so far. In this method all strains' amylase activity were screened at 80°C since genetically modified strains' secreted amylase enzyme was thermophilic and the optimum working temperature was 80°C. Due to WT has no thermophilic enzyme so its enzyme might have denatured at 80°C. When WT amylase was screened at its fermentation temperature (35°C) the activity result was no higher than 1U (STD method). Since it could barely convert 1mg starch into reducing sugar.

As Amc28 strain was found to produce the highest activity enzyme in all experiments up to that point, further experiments were conducted using this strain only.

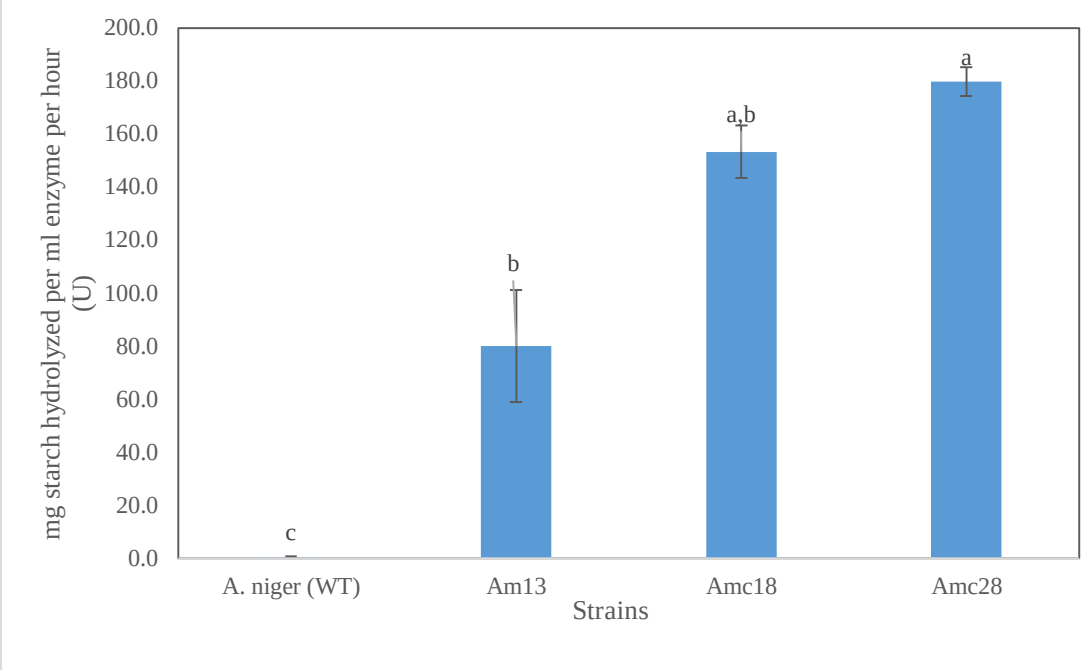

Figure 3.3.1. Amc28 with complete medium and activity measured with STD method.

3.3.1 Bioreactor Experiments with Amc28 Strain to Find Optimum Spore Amount at 1.5L Fermenter to Produce Higher Alpha Amylase Enzyme

For the experiments in this section, complete medium was used in a 1.5L fermenter (Biostat B plus Bioreactor). Temperature, pH and agitation rate were set to 30°C, 5.50 and 700 rpm respectively.

Spore amount was tested at a range of 10^4-10^8

The 1st experiment was conducted using 10^5 spore amount/ml fermentation media which was the best amount found with the flask experiments (Figure 3.1.5) using %1 maltose syrup as the carbon sources . Amylase activity and carbon source utilization were monitored daily.

As seen in Figure 3.3.1.1, the activity increased almost linearly until 12 U which was very low when compared with 179.8 ± 5.4U activity achieved with the flask experiments and after the 3rd day, the activity dropped dramatically. Figure 3.3.1.1 also shows the sugar utilization of maltose and glucose at the same time. As seen in the figure, carbon source of the fermentation was depleted after 96th hours as expected from previous experiments.

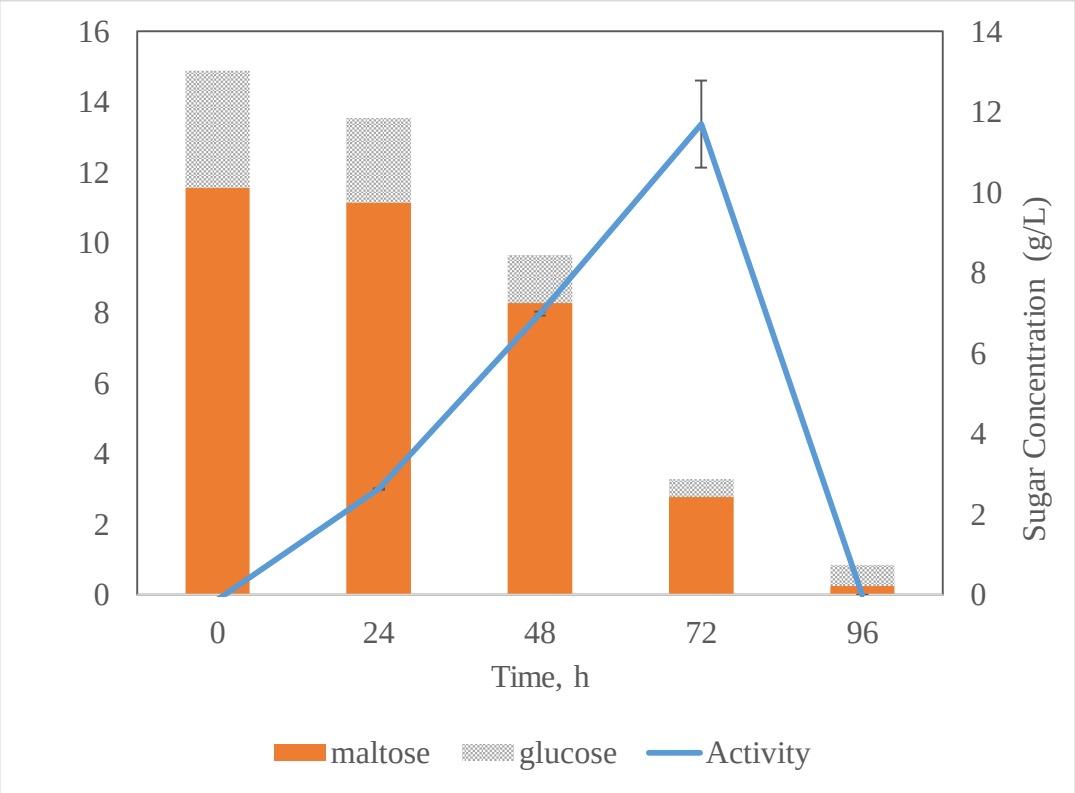

Figure 3.3.1.1. The Effect 10^5 spore amount / ml of fermentation media on α-amylase activity and Sugar Utilization of this fermentation vs Time (h)

In this trial, spore amount was chosen to be 104. The highest activity was recorded to be 15.5 ±1.0 U at the 2nd day of the fermentation. After that day, the activity dropped dramatically, even sooner than the one with 10^5 spore/ml of which the activity had been lost in the 3rd day. (Figure 3.3.1.2). Decreasing the spore amount did not help to get higher activity so it was decided to increase the spore amount for the following experiments.

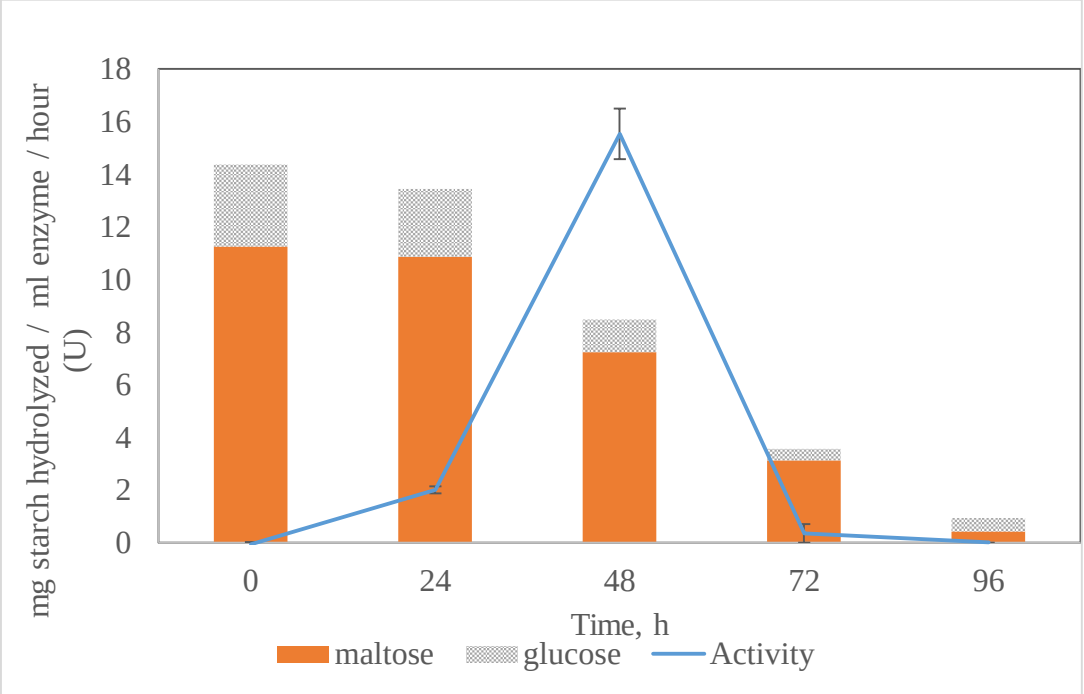

Figure 3.3.1.2 The Effect 10^4 spore amount / ml of fermentation media on α-amylase activity and Sugar Utilization of this fermentation vs Time (h)

The third experiment was done by adjusting the spore amount to 10^6 spores/ml of fermentation media. The amylase activity peaked at the 3rd day of the fermentation but after 3rd day the activity decreased. It was the highest activity in the bioreactor experiments with 42.4 ±1.17 U so far and showed that the increasing spore amount helped to achieve the higher amylase activities. (Figure 3.3.1.3)

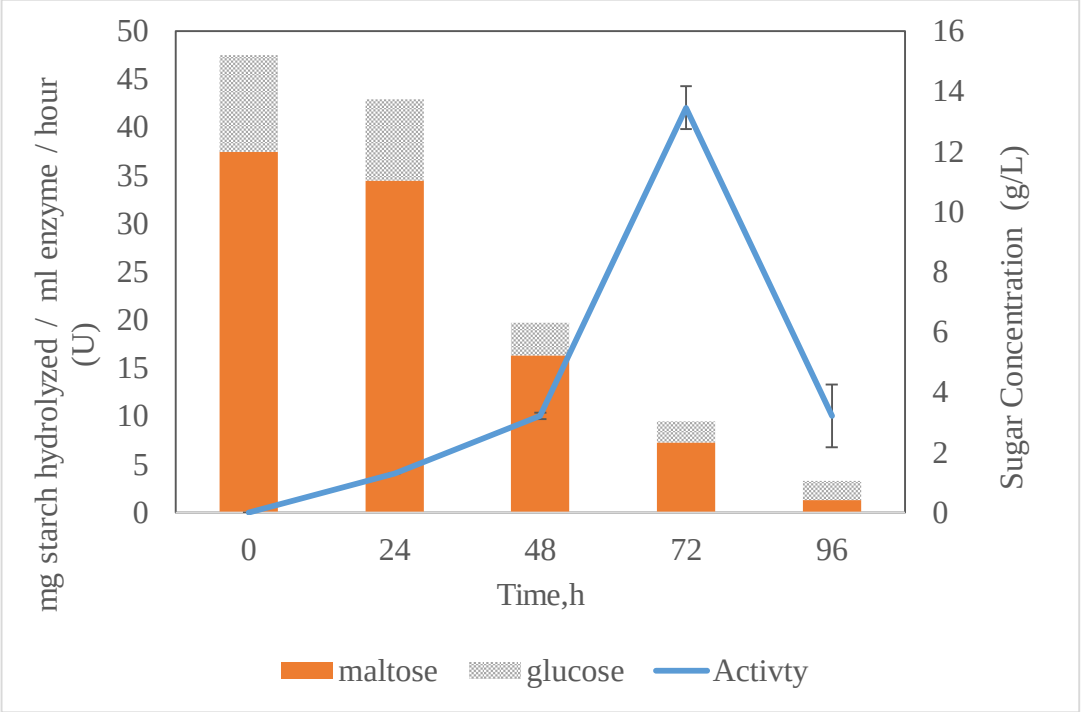

Figure 3.3.1.3. The Effect 10^6 spore amount / ml of fermentation media on α-amylase activity and Sugar Utilization of this fermentation vs Time (h)

Since the experiment of 10^6 spores/ml showed to have an increase in the activity, the next two experiments were performed using 10^7 spores/ml and 10^8 spores/ml. In the case of 10^7, the amylase activity increased until the 4^{th} day of the experiment and it was recorded as 98.0 ± 4.8 U which was higher than all other trials. After the 4^{th} day, it started to decrease again (Figure 3.3.1.4). For the spore amount of 10^8 (Figure 3.3.1.5) activity value was recorded to be 76.0 ±6.01 U at the 3^{rd} day. After that day, it decreased sharply.

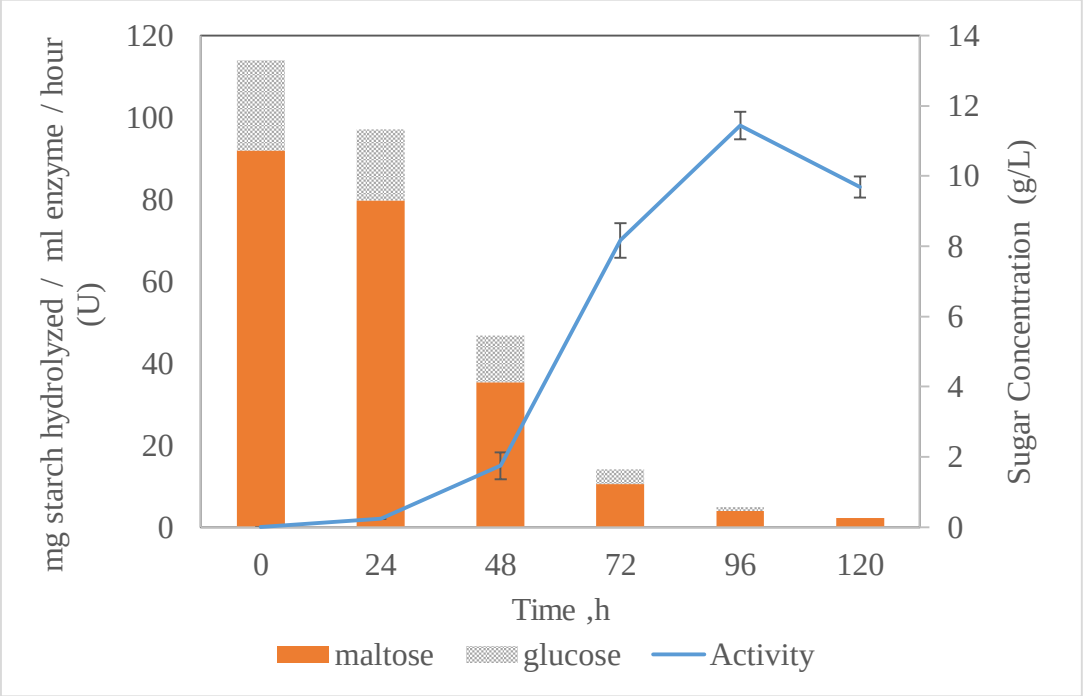

Figure 3.3.1. The Effect 10^7 spore amount / ml of fermentation media on α-amylase activity and Sugar Utilization of this fermentation vs Time (h).

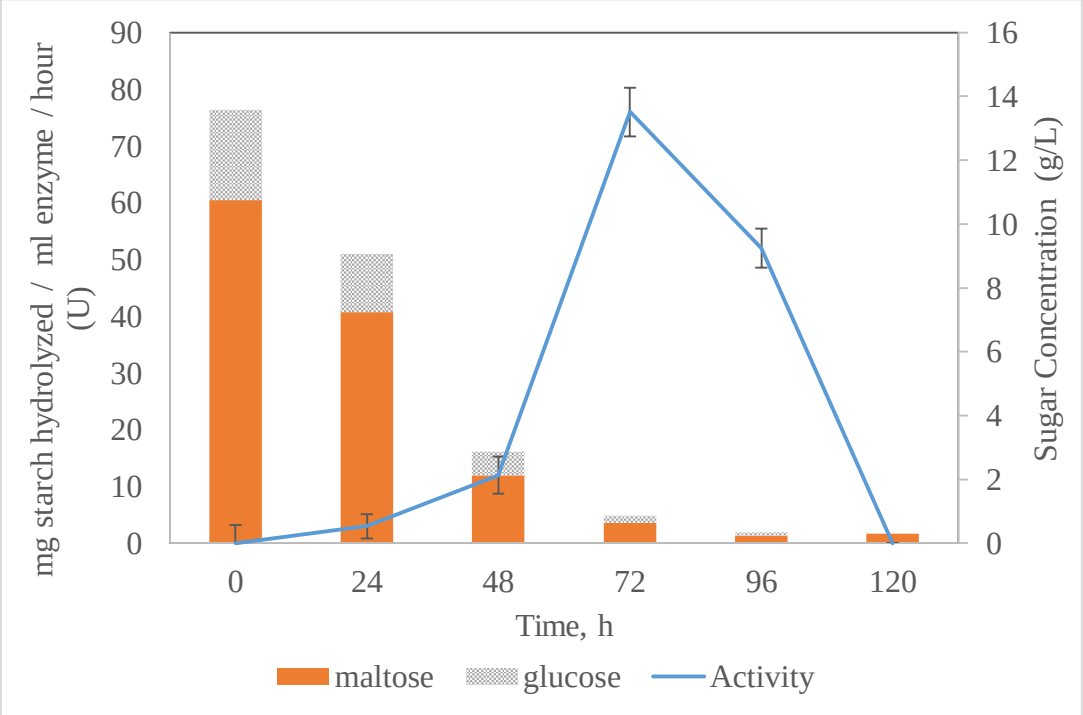

Figure 3.3.1.5. The Effect 10^8 spore amount / ml of fermentation media on α-amylase activity and Sugar Utilization of this fermentation vs Time (h).

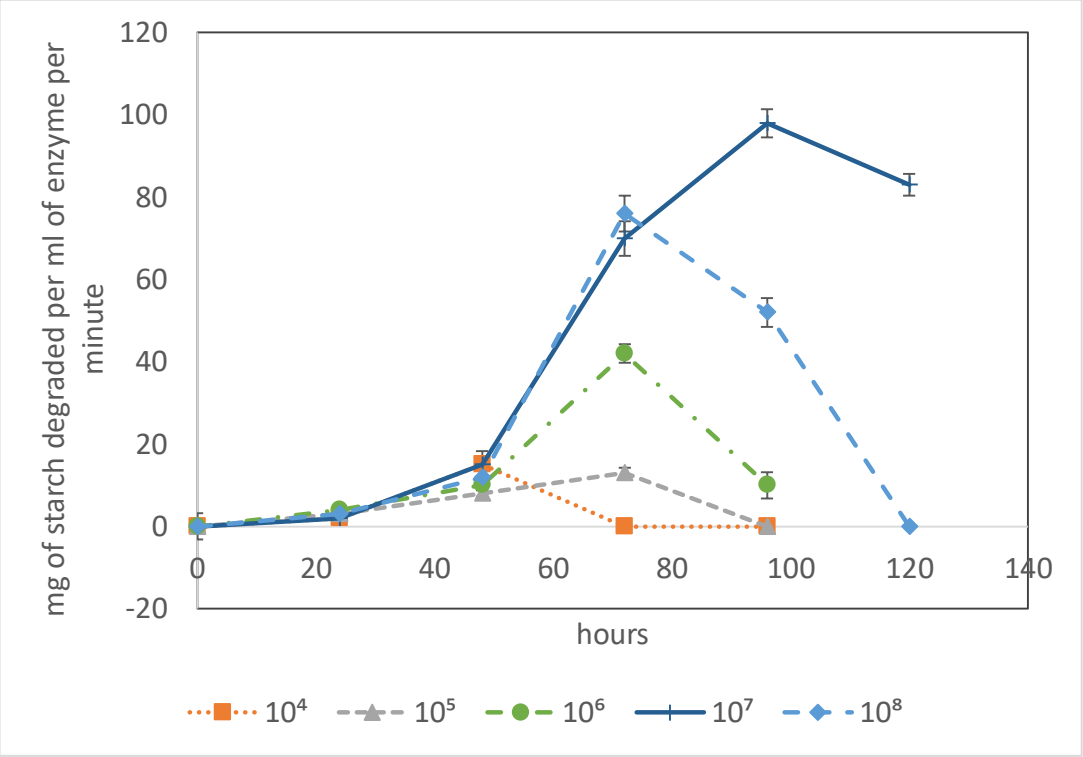

Figure 3.3.1.6 **The spore levels experimented in the reactor / ml of fermentation media on α-amylase activity and Sugar Utilization of this fermentation vs Time (h**

To sum up; In Figure 3.3.1.6 the spore amount experiments given under the same graph. According to this figure, the highest activity was recorded when the experiment was conducted using 10^7 spores/ml at the 3rd day which was also at the same time that carbon source was depleted in the fermentation media. In the other trials, the activity started to decrease before the carbon source was depleted in the medium. The decrease in the activity could be explained with the carbon source being depleted at 10^7 and 10^8 spore amounts.

An interesting observation was also noted during the experiments. In all fermentation experiments (10^4 to 10^8) the size of the fungi pellet decreased. The amount of spores in the media had an effect on the size of the pellet. The more spore present in the medium the smaller the fungi pellet would be (Papagianni & Mattey, 2006; Žnidaršič, Komel, & Pavko, 2000) The size of the pellet could be

important as it could have an effect on the amylase activity. When pellet size decreased with increasing spore amount the activity increased (at a range of 10^4-10^7).

On the other hand the reason behind the sharp decrease in the enzyme activity on the trials conducted at 10^4, 10^5, 10^6 spore amount/ml could be the relation between the pellet size and the shear stress in the reactor. As the pellet size increased it became fluffier (Figure 3.3.1.7 a-10^4) in comparison to its small pellet form (Picture 3.3.1.7 a-10^7).

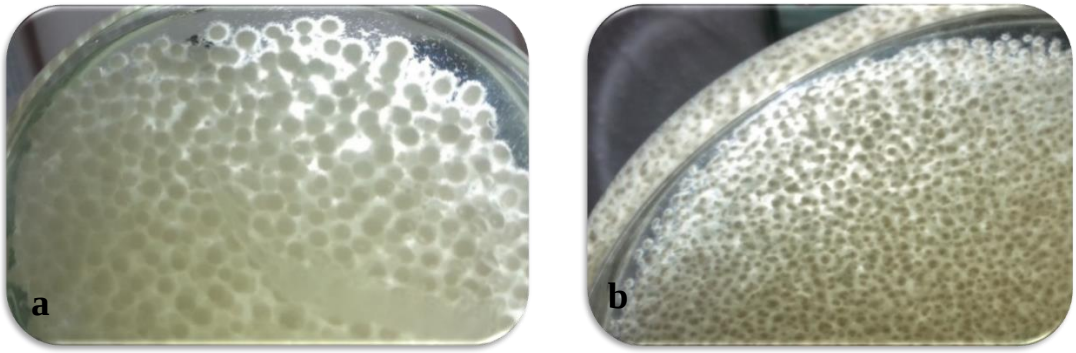

Figure 3.3.1.7. a) 10^4 spore amount /ml grown pellets of Aspergillus sp 4th day of fermentation and b) 10^7 spore amount /ml grown pellets of Aspergillus sp 4th day of fermentation

Fluffy pellets (bigger pellets) might have been affected more from shear stress than smaller pellets so that the hypeal tips might be damaged during the fermentation. This is an undesirable effect for enzyme production since the filamentous fungi secretes its extracellular proteins from just at the tips of the growing hyphae only. (Figure 3.3.1.1) (Nevalainen & Peterson, 2014; Wösten, Moukha, Sietsma, & Wessels, 1991). Therefore not only the enzyme secretion could stop but also the damaged hypeal tips could release cellular proteases in the medium that would eventually degrade the amylases produced in the fermentation medium (El-Enshasy et al., 1999).

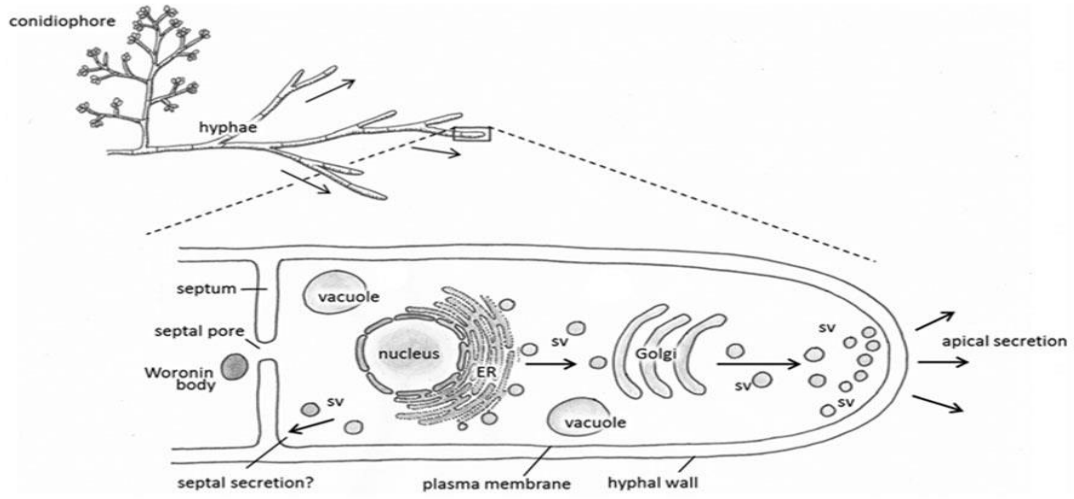

Figure 3.3.1.8. Schematic representations of morphology of *Trichoderma reesei* (filamentous fungi) and proposed pathways of protein synthesis and secretion. (Nevalainen & Peterson, 2014)

When 10^7 and 10^8 spore amounts were compared, it was seen that the activity was not significantly ($p \leq 0.05$) different before the 4th day of fermentation. However on the 4th day, activity decreased which could have been affected by the limited carbon source as the microorganism amount was higher while the carbon sources was same (%1).

3.3.2 Amc28 Scale up trials with 1.5 L, 60 L and 80 L Volumes

The experiments were scaled up using 10^7 spore amount/ml. However rather than $NaNO_3$, $(NH_4)_2SO_4$ was used as the inorganic nitrogen source due to its availability. It was confirmed with 1.5 L reactor experiments that changing the nitrogen source did not change the enzymatic activity significantly. (Figure 3.3.2.1. The temperature was set to 30 °C, pH was kept at 5.50 and as the carbon source %1.6 maltose syrup was used.

As seen in Figure 3.3.2.1 the activity reached to 109.7 ± 11.2 U at the end of 96th hour. The carbon source was depleted as expected. At that time, the fermentation was terminated and the biomass was filtered for the next step which would use the

enzyme produced for the hydrolysis of starch to get reducing sugar for further use as the carbon source.

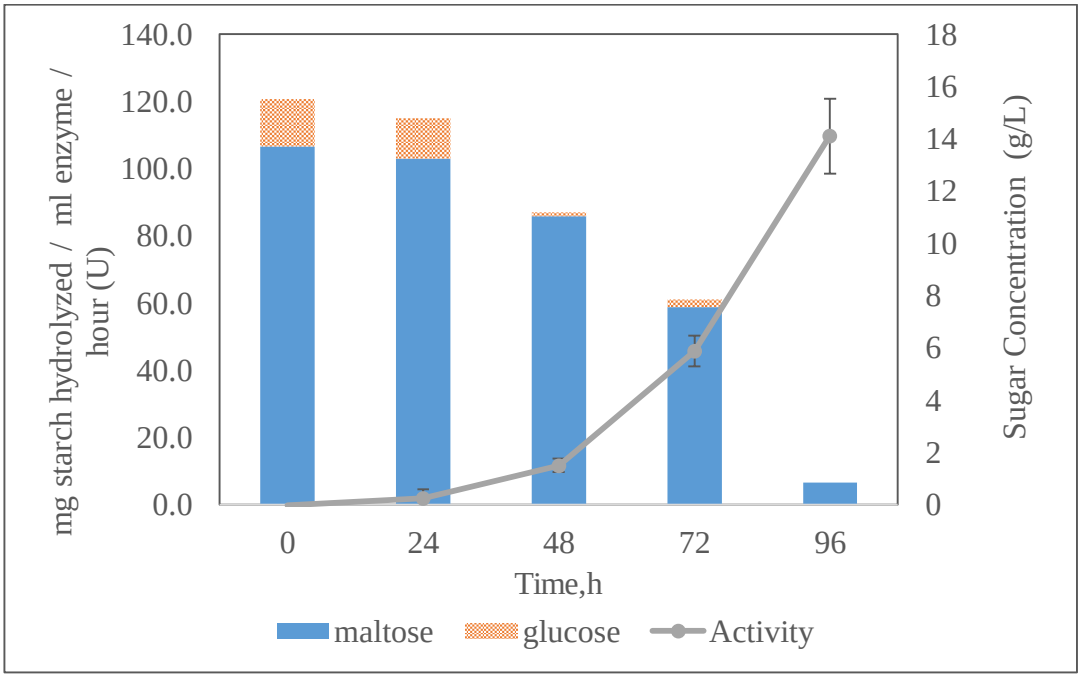

Figure 3.3.2.1 1.5L volume α-amylase production and Sugar Utilization of this fermentation vs Time (h).

As seen in Figure 3.3.2.2 the scaling up from 1.5 L to 60L was successful with activity values recorded as 112.4 7.8U at 4th day (96h). The fermentation was terminated at that point since the carbon source was depleted.

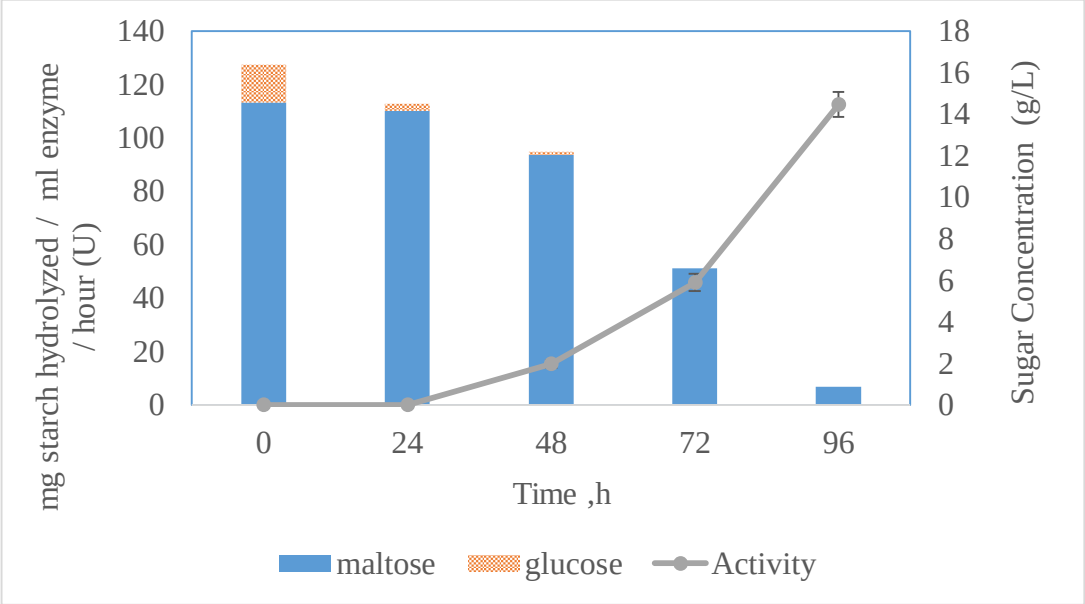

Figure 3.3.2.2 60 L volume α-amylase production and Sugar Utilization of this fermentation vs. Time (h).

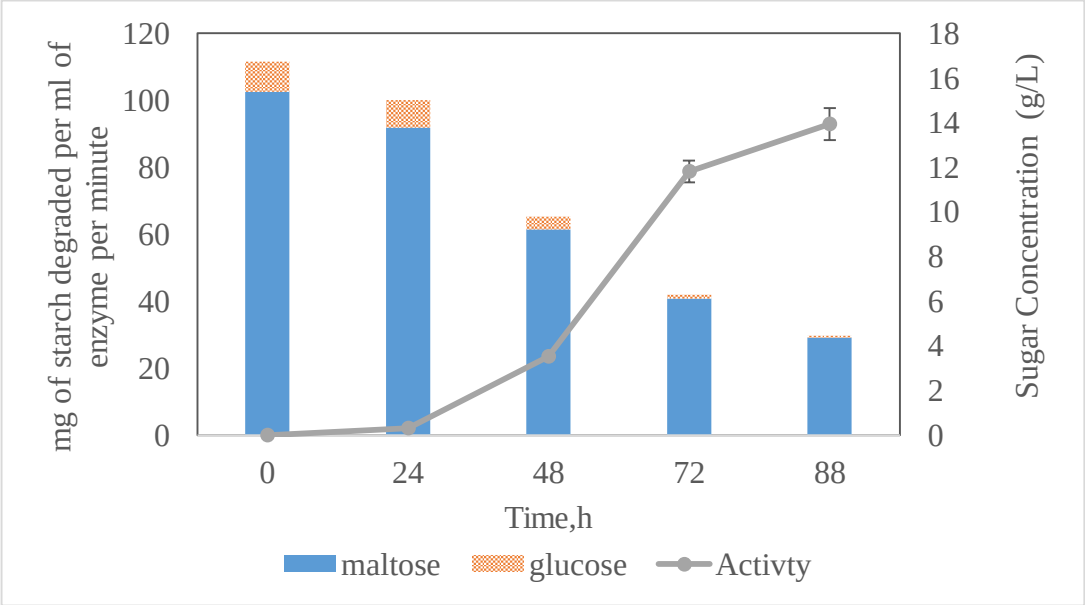

Figure 3.3.2.3. 80 L volume α-amylase production and Sugar Utilization of this fermentation vs Time (h)

The other scaling up experiment was done at 80L volume. All conditions that were set at 60L were kept constant but the volume was increased to 80L.

The activity reached to 92.9± 4.8 U at the 88th hour of the fermentation and the carbon source was 0.44% when it was terminated. After termination, biomass was filtered.

3.3.3 Use of the produced enzyme in hydrolysis of starch.

In the previous section (3.3.2), the fermentation that was conducted at 1.5 L bioreactor, which had 108 U alpha amylase activity, was used to hydrolyze starch to obtain reducing sugar for future fermentation purposes. After filtration of the biomass the spent medium (1 L) was diluted at 1:4 ratio with distilled water to be used in a 10L volume bioreactor

After dilution with distilled water and adjusting the pH to 5.0 the new activity of the spent medium was recorded as 21.8 U ± 3.6

The optimum working pH (5.00) and temperature (80 °C) of this enzyme was determined by the researchers in Leiden University, Netherlands. The hydrolysis experiment started once the medium was loaded with 10% starch and heated to 80 °C. Temperature reached to 80 °C at the 14th minute of heating (the dashed arrow on the graph). The hydrolysis was monitored via HPLC as explained in materials and methods section. Hydrolysis was allowed to continue until maltose concentration reached steady state. Maltose concentration assumed to reach steady state at 5th hour since the increase slowed down and a major increase was not observed at the 6th and 7th hour. (Figure 3.3.4.1)

Considering the calculated enzyme activity of 21,8U; 87.2g starch was expected to be hydrolyzed in an hour and 400g of starch would be expected to deplete at 4.5h.

Due to 14 min lag time in heating amylase would not be work at its optimum activity, and the overall hydrolysis would take less than 5 hours.

At 7th hour of the hydrolysis, glucoamylase (ORBA®MİL EL, 1200AGU/ml) was added to convert all reducing sugars to glucose so that it would give an idea about % hydrolysis of the starch and to obtain a fully fermentable carbon source.

The black arrow in Figure 3.3.4.1 shows the addition of glucoamylase, after which the glucose concentration was recorded to be 101.19 g/L. This indicated that all the starch that was put to be hydrolyzed (%10 of the 4L) was converted into glucose successfully.

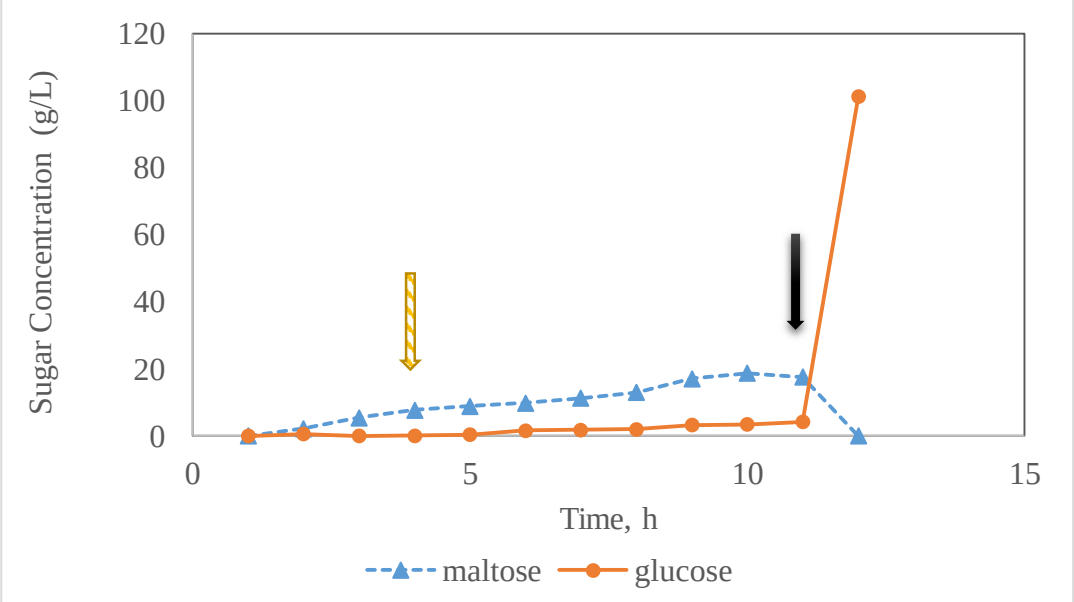

Figure 3.3.3.1. Amc28 1,5L Starch Hydrolysis with %10 solid load vs Time

The enzyme obtained from 60L fermentation (Figure 3.2.2.2) was used in a 100L fermenter. The spent medium was removed from biomass via filterpress and directly used in the hydrolysis of starch. Before hydrolysis the enzyme activity was measured as 98.2 U ± 5.65 just before the starch was added. The activity was previously 109.7 ± 11.2 U .This loss in enzyme activity could be explained by the

accidental dilution that could have occurred during filtration, charging/ unloading the bioreactor etc. Solid load was kept at 10% and enzyme hydrolysis was conducted at pH 5 and 80 °C. Temperature reached of 80 °C after 92 min of loading. Hydrolysis was allowed to last for until 9 hour and emerging maltose and glucose were monitored via HPLC

Considering the activity of 98.2 U, 5.89kg starch was expected to be depleted in 1 h and for all starch (6kg) to be depleted it should take 1.10hrs.

It took 92 minutes to achieve 80 ºC after the reactor was loaded with enzyme and starch. When this loading time was added to the theoretical hydrolysis time (1.1ohrs), steady state levels of maltose was expected to be obtained in less than 3 hours (~2.60hr)

As seen in Figure 3.3.4.2 maltose concentration reached steady state after 6h. However theoretical calculated value was 3hrs. This low working performance of the hydrolysis could be explained by the temperature fluctuations of the fermenter. Since the temperature of the fermentation could not be controlled very accurately due to hardware limitations, the performance of the enzyme might have been affected which resulted in 9 hours for hydrolysis.

After 9 hours, glucoamylase was added (black arrow in Figure 3.3.4.2.) to convert all reducing sugars into glucose. Following hydrolysis with both enzymes final glucose concentration was measured as 107.7 g/L which indicated the full conversion of %10 starch that was added.

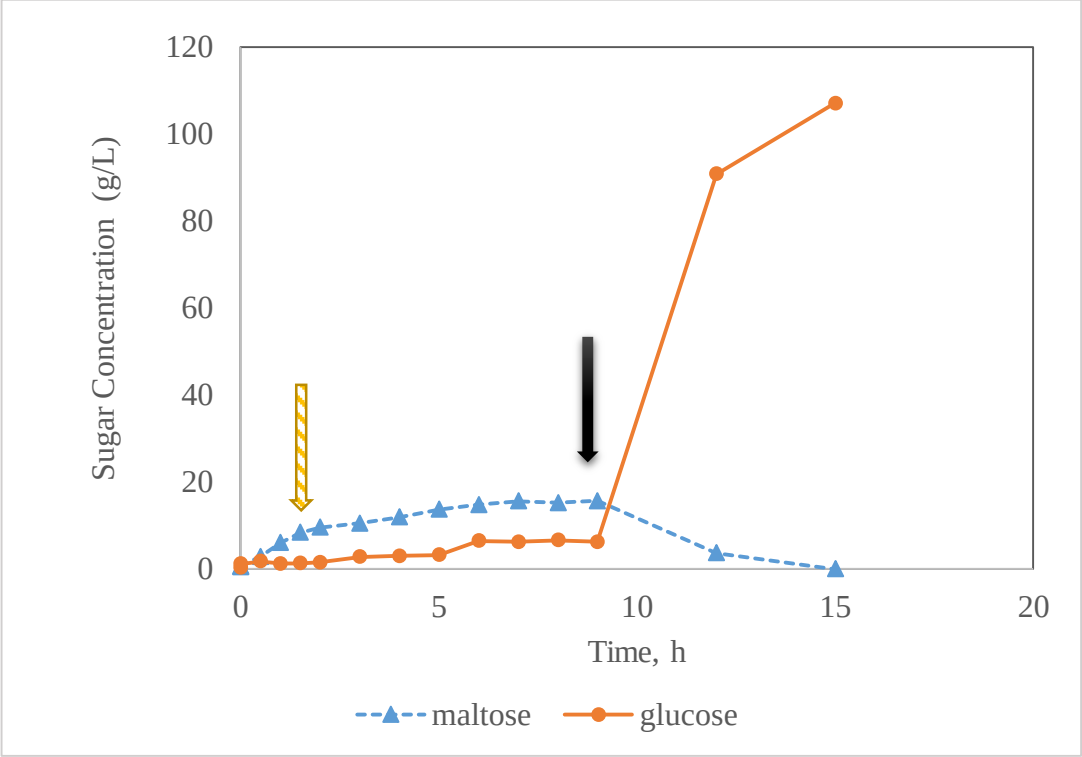

Figure 3.3.3.2. Amc28 60L Starch Hydrolysis with %10 solid load Starch Hydrolysis vs Time

Amylase obtained from 80L fermenter was also used for hydrolysis (Figure 3.3.4.3) in a 100L fermenter. The enzyme was used directly in hydrolysis without any dilution after removing biomass by filtration. Just before the starch addition activity was measured as 88.5U ±6.44 and hydrolysis was conducted at the same conditions. Reaction was allowed to continue until 7 h and emerging maltose and glucose were monitored via HPLC

Considering the activity of 88.5 U 7.08kg starch was expected to be depleted in 1 h and for all starch (8kg) to be depleted it should take 1.13hrs.

When 65 min lag time in heating was considered the overall hydrolysis was expected to be completed in less than ~ 4 hour.

Maltose concentration reached steady state after 4h as predicted. After 7th hour, glucoamylase was added (black arrow in Figure 3.3.4.3) to convert all reducing

sugars into glucose. Upon completion of the hydrolysis, final glucose and maltose concentration was measured as 94.26 ,11,94 g/L respectively making a total of 106.3 g/L reducing sugar which also indicated the full conversion of %10 starch that was added.

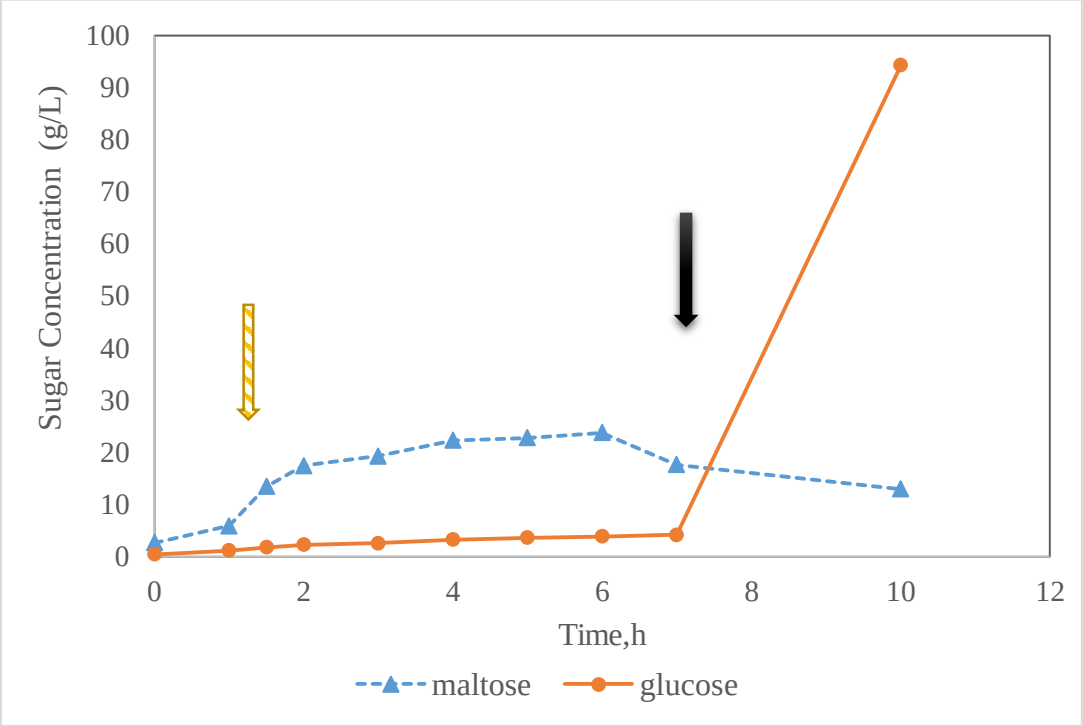

Figure 3.3.3.3. Amc28 80L Starch Hydrolysis with %10 solid load Starch Hydrolysis vs Time

3.3.4 Usage of the hydrolysis product in the production of Lactic Acid from *Lactobacillus casei*

L.casei was used for production of lactic acid from hydrolyzed starch that was explained at Section 3.3.3 from the amylase obtained through 60L (AmC28) fermentation. The aim was to observe the consumption of the reducing sugar by a different microorganism.

After producing 107.7g/L (in 60L) glucose solution, it was filtered via filter press to remove the resistant starch and other impurities to obtain a clear glucose solution

for better fermentation purposes. After filtration of glucose solution of 37L, this was used for lactic acid production after the necessary fermentation ingredients were added (See Material Method Section- Lactic Acid Medium…) and sterilized.

As seen in Figure 3.3.4.1 the concentration of the sugar started with 75.24 g/L glucose where the stock was 107.7 g/L. The reason of the lower glucose value might be the dilution during the filtration and sterilization and inoculation of *L.casei*. Fermentation lasted until the glucose was depleted in the medium which lasted 72 h in total.

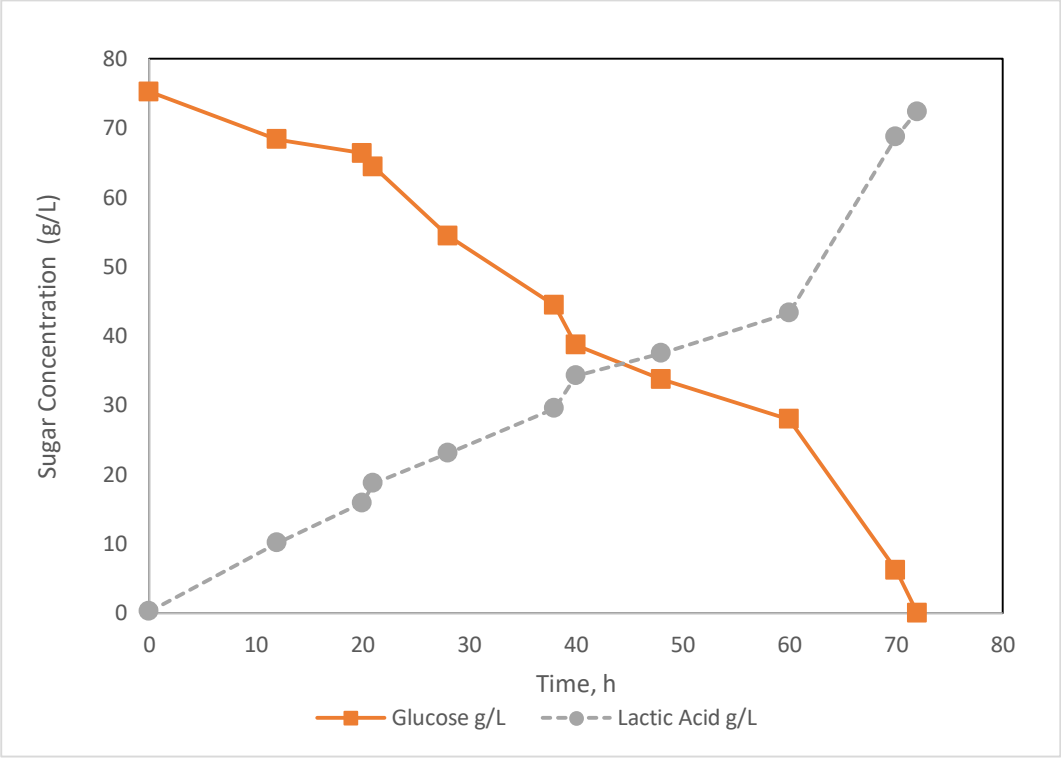

Figure 3.3.4.1. Lactic Acid Production from *L. casei*

3.3.5 Usage of the hydrolysis product in production of *Sacharromyces cerevisae*

S. cerevisiae was also grown using the hydrolyzed starch that was produced at Section 3.3.3 from the amylase was obtained through 80L AmC28 fermentation. The fermentation was conducted in a 10L fermenter and started with 5L of 1.5 g/L concentrated yeast cell. The fermentation was achieved using a fed batch procedure

to prevent ethanol production due to the possibility of *Crabtree effect metabolism* on the presence of high glucose concentration (Rodrigues, Ludovico, & Leão, 2006). In aerobic conditions and at high external glucose concentrations, rather than producing biomass via the tricarboxylic acid (TCA) cycle which is the usual pathway followed by most yeasts, ethanol production could be favored (De Deken, 1966). This is known as the *Crabtree effect metabolism* . Therefore, fermentation was monitored via HPLC and glucose and maltose concentration was kept under %0.2 during the fed batch fermentation.

Fermentation was continued until the 5L of %10 glucose was finished.

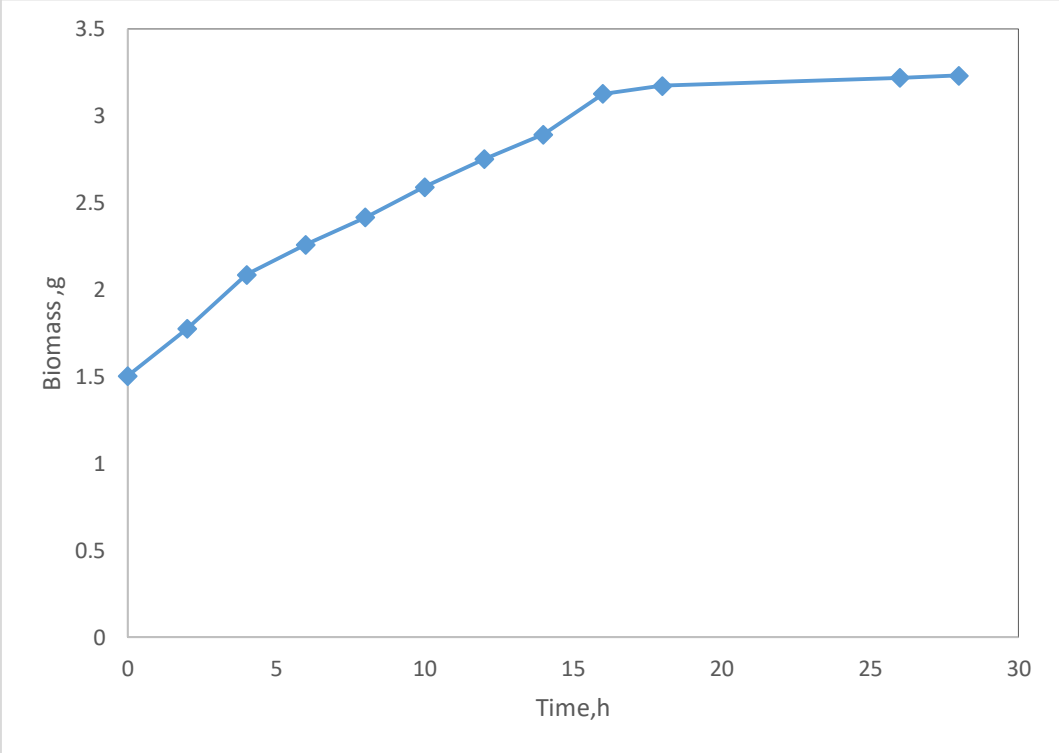

Figure 3.3.5.1. Production S. cerevisae from Glucose solution produced from the 80L volume fermentation & hydrolysis

CHAPTER 4

CONCLUSIONS

In this work using wild type strain best conditions of alpha amylase activity was determined and used to select the best strain for future enzyme production studies. In this study AmC28 was chosen among other three genetically modified strains (Am13, AmC18 and WT) with the help of flask experiments. The parameters were carbon source, nitrogen source, pH, temperature, fermentation volume: total flask volume and inoculated spore level. Within these parameter best conditions were recorded as starch and maltose syrup as the carbon source and yeast extract as the nitrogen source, temperature was set to 30 °C at pH 5.50 with 105 spores/ml and with 0.5 FV/TFV ratio at a 250 mililiter volume flask). In bioreactor –scale up experiments spore amount was an important parameter on enzyme activity.Optimum spore amount was determined as 107 spores/ml fermentation medium. One milliliter of produced amylase was capable of hydrolyzing around 100mg starch/ml enzyme/ hour activity in scale up experiments at 80 °C at pH of 5.0. Following the production of the enzyme at large scale, saccharification of raw starch (%10 solids load) was accomplished using the α-amylase produced. The produced fermentable sugar was used to grow Baker's yeast and to produce lactic acid from L.casei

For future studies of this work it is recommended that the flask experiments done for determining the parameters for highest α-amylase activity to select highest strain

can be repeated for the strain that was selected as best enzyme producer which is AmC28. Moreover, for the selection of best inoculated spore amount in bioreactor/scale up production of α-amylase enzyme can be monitored in the next experiments via electron microscopy techniques for analysing the filament conditions in the case of decreasing and increasing inoculated spore amount. This might help to see better if the tips of the Aspergillus filaments secreting enzyme of interest are damaged or not in the case of low spore amount levels (fluffy pellet conditions).

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APPENDIX A

CHEMICAL USED AND PRODUCERS

Table A1 Chemical used and Producers

Chemicals	Producers
Agar, Bacteriological Grade	Sigma-Aldrich
Ammonium Molybdate, Tetrahydrate	Sigma-Aldrich
Ammonium Sulfate	Sigma-Aldrich
Aneurine Hydrochloride Thiamine	Sigma-Aldrich
Bactopeptone	Difco
Biotin	Sigma-Aldrich
Boric Acid	Sigma-Aldrich
Calcium Carbonate	Merck
Casamino Acids	Difco
Choline, Chloride Salt	Sigma-Aldrich
Citric Acid Monohydrate	Merck
Cobaltous Chloride, Hexahydrate	Sigma-Aldrich
Cupric Sulfate, Pentahydrate	Sigma-Aldrich
D-Fructose	Merck
D-Glucose Monohydrate	Sigma-Aldrich
Ferrous Sulfate, Heptahydrate	Sigma-Aldrich
Glacial Acetic Acid	Merck
Glucoamylase	ORBA
Glucose, L-Lactic Acid Standard	YSI Incorporates
Hydrochloric Acid	Sigma-Aldrich
Iodine Crystals	Sigma-Aldrich
Magnesium Sulfate Heptahydrate	Merck
Magnesium Sulfate Heptahydrate	Merck

Table A1 Continued

Manganese II) Sulfate	Horosan Kimya
Manganous Chloride, Tetrahydrate	Sigma-Aldrich
MRS Broth	Merck
Nicotinic Acid	Sigma-Aldrich
P-Aminobenzoic Acid	Sigma-Aldrich
Peptone From Meat	Merck
Potassium Iodide Extra Pure:	Sigma-Aldrich
Potassium Chloride	Sigma-Aldrich
Potassium Phosphate, Dibasic	Sigma-Aldrich
Potassium Phosphate, Monobasic	Sigma-Aldrich
Pyridoxine-HCL	Sigma-Aldrich
Riboflavin	Sigma-Aldrich
Sodium Acetate Trihydrate	Sigma-Aldrich
Sodium Hydroxide	Merck
Sodium Nitrate	Sigma-Aldrich
Sodium Nitrate	Fisher
Starch (Raw)	Cargill, Ankara
Starch: (Soluble Extra Pure Starch)	Merck
Sucrose	Merck
Tri-Sodium Citrate Dihydrate	Merck
Tween 80	Merck
Urea	Sigma-Aldrich
Yeast Extract	Merck
Zinc Sulfate, Heptahydrate	Sigma-Aldrich

APPENDIX B

CALIBRATION CURVES

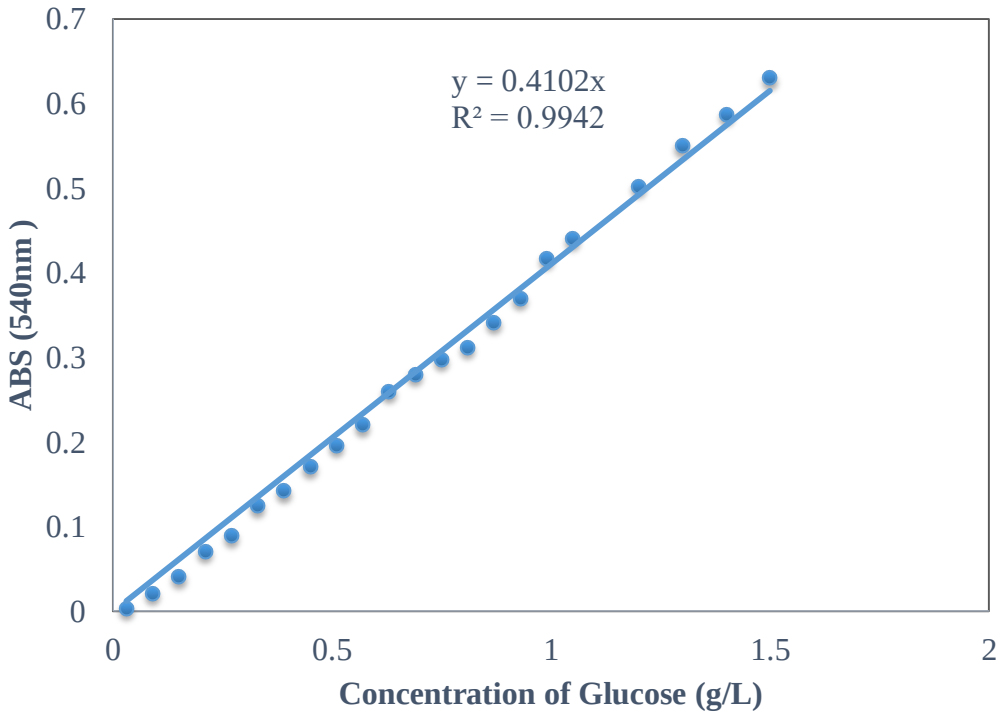

Figure B 1. Representative calibration curve* for the DNS method.

* : For each freshly prepeared DNS solution a new calibration curve was established.

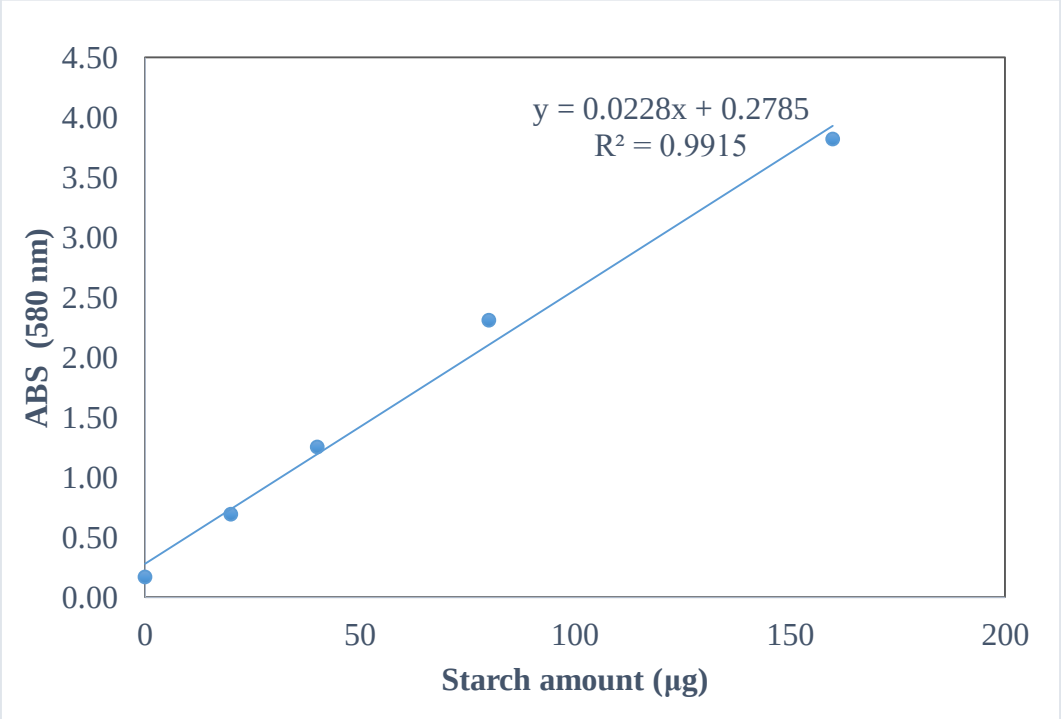

Figure B 2. Representative calibration curve* for the SDT method.

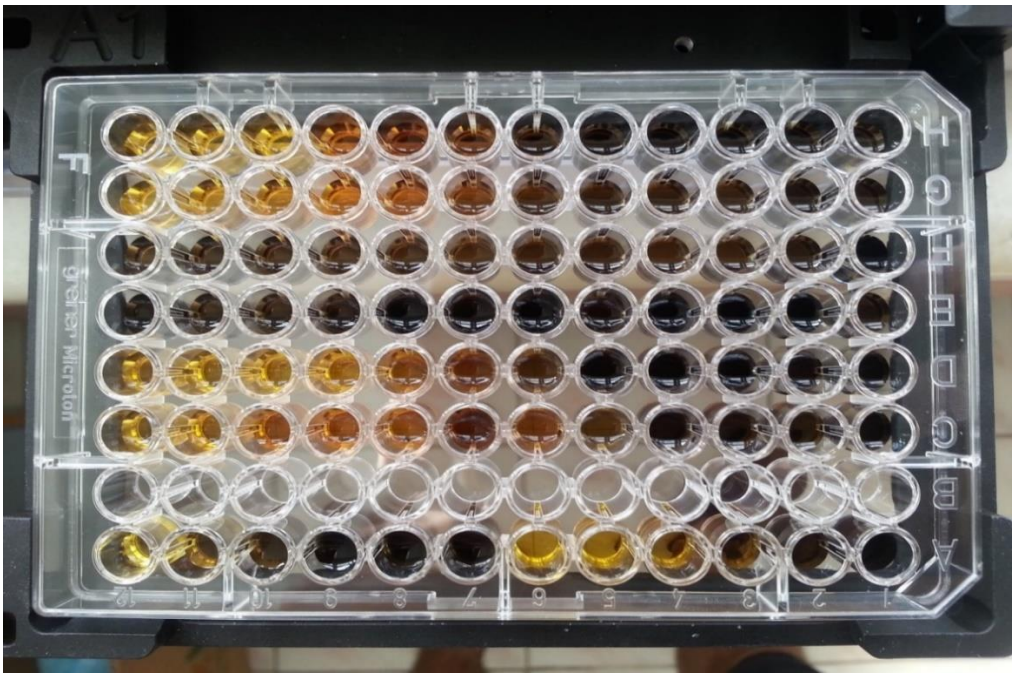

Figure B 3 Representative 96-well plate samples

*** : For each 96-well plate measurement a new calibration curve was established.**

APPENDIX C

C1 INGREDIENTS OF USED SOLUTIONS

Table C 1 Vishniac Solution Ingredients

Vishniac Solution (1000X)(Vishniac and Santer, 1957)	
EDTA	10 g
ZnSO4.7H2O	4.4 g
MnCl2.4H2O	1.0 g
CoCl2.6H2O	0.32 g
CuSO4.5H2O	0.32 g
(NH4)6Mo7O24.4H20	0.22 g
CaCl2.2H2O	1.47 g
FeSO4.7H2O	1.0 g
Adjust pH to 4.0 (use 2N HCl)	
Complete to 1000ml with distilled water	

Table C2 : Asp+N Solution Ingredients

50X Asp+N Solution	
KCL	26.1 g
KH2PO4	74.8 g
NaNO3	297.5 g
Adjust pH to 5.5 (use 5M KOH)	
Complete to 1000ml with distilled water	

Table C3 : YPD Broth/ Agar Ingredients

YPD Broth / Agar	
Peptone	20.0 g
Yeast Extract	10.0 g
Glucose	20.0 g
Complete to 1000ml with distilled water	
For YPDA add 15g Agar	
pH 6.5 (use 1M KOH)	
Sterlize by autoclaving at 121°C 15min	

Table C4: Trace Mineral Ingredients for *S. Cerevisae* production

Trace Elements	
EDTA	1.5 g
ZnSO4	0.45 g
CoCl2	0.03 g
MnCl2	0.1 g
CuSO4	0.03 g
CaCL2	0.45 g
FeSO4	0.3 g
NaMoO4	0.04 g
HBO3	0.1 g
KCl	0.01 g
KI	0.01 g
Comlete to 1000ml and use 10ml for 100L of *S.cerevisae* medium	

Table C5 : Mineral Solution Ingredients for *S. cerevisae* Production

Minerals	
MAP	400 g
NH4SO4	1600 g
PO4SO4	200 g
MgSO4	100 g
Add directy to 100L of strelizing *S.cerevisae* medium	

Table C6 : Vitamin Solution Ingredients for *S. erevisae* for *100L*

Vitamins	
Thiamine	150 mg
Riboflavine	7.5 mg
Niasine	240 mg
Biotine	3 mg
Ca-panthonate	570 mg
Inositol	8.64 mg
Para aminobenzoic acid	33.6 mg
Pyrodoxine	72 mg
Dissolve in 100ml distilled water and filtrate with 0,45µm filter	

Table C7 MRS Broth/ Agar for *L.casei* growth

MRS Broth /Agar	
Glucose	20 g
Peptone	10 g
Yeast Extract	4 g
Sodium Acetate Trihydrate	5 g
Triammonium Citrate	2 g
Dipotassium Hydrogen Phosphate	2 g
Magnesium Sulfate Tetrahydrate	0.2 g
Manganous Sulfate Tetrahydrate	0.05 g
Tween 80	1 g
Complete to 1000ml with distilled water	
For MRS Agar add 15g Agar	
pH 5.5 (use excess amount CaCO3)	
Sterlize by autoclaving at 121°C 15min	

APPENDIX D

STATICTICAL ANALYSIS OF DATA

D1 One-way ANOVA: Activity versus C source

```
Source    DF     SS    MS      F      P
C source   6  23442  3349  29,78  0,000
Error      8    900   112
Total     15  24342

S = 10,60   R-Sq = 96,30%   R-Sq(adj) = 93,07%

Grouping Information Using Tukey Method

Pooled StDev = 10,60

Grouping Information Using Tukey Method

C source       N    Mean  Grouping
Starch         2  279,65  A
Maltose        2  215,75    B
Fructose       2  198,87    B C
Maltose Syrup  2  190,02    B C
Maltodextrin   2  186,22    B C
Glucose        2  176,52    B C D
Sucrose        2  172,51      C D

Means that do not share a letter are significantly different.
```

D2 One-way ANOVA MS250; MS500 versus UV/TV

```
Source  DF   Seq SS   Adj SS  Adj MS       F      P
UV/TV    7  11288,8  11288,8  1612,7  148,76  0,000
Error    8     86,7     86,7    10,8
Total   15  11375,5


S = 3,29259   R-Sq = 99,24%   R-Sq(adj) = 98,57%

Factor  Type   Levels  Values
```

```
UV/TV   fixed       8  0,1; 0,2; 0,3; 0,4; 0,5; 0,6; 0,7; 0,8


Grouping Information Using Tukey Method and 95,0% Confidence for UMS250

UV/TV  N   Mean  Grouping
0,5    2  260,5  A
0,4    2  220,6    B
0,8    2  213,0    B
0,6    2  197,2      C
0,7    2  189,0      C
0,3    2  161,8        D
0,2    2   86,7          E
0,1    2   65,2            F

Means that do not share a letter are significantly different
```

D3 One-way ANOVA: Activity versus N Source

```
Source    DF     SS     MS      F      P
N Source   3  39026  13009  50,85  0,000
Error     12   3070    256
Total     15  42096

S = 15,99   R-Sq = 92,71%   R-Sq(adj) = 90,88%

Grouping Information Using Tukey Method

N Source          N    Mean  Grouping
Yeast extract     4  236,10  A
Urea              4  159,33    B
Ammonium sulfate  4  120,21      C
Sodium acetate    4  110,72      C

Means that do not share a letter are significantly different
```

D4 One-way ANOVA: Temperature

```
Source  DF      SS     MS      F      P
C1       4  120929  30232  55,99  0,000
Error   15    8099    540
Total   19  129027

S = 23,24   R-Sq = 93,72%   R-Sq(adj) = 92,05%

Grouping Information Using Tukey Method

C1  N    Mean  Grouping
35  4  306,16  A
30  4  296,35  A
25  4  215,32    B
40  4  173,04    B
45  4   98,44      C

Means that do not share a letter are significantly different
```

D5 One-way ANOVA: pH

Source	DF	SS	MS	F	P
pH	8	69964	8746	13.02	0.000
Error	25	16789	672		
Total	33	86753			

S = 25.91 R-Sq = 90.65% R-Sq(adj) = 94.45%

Grouping Information Using Tukey Method

pH	N	Mean	Grouping
5.5	2	286.82	A
5.0	6	254.62	A
4.0	6	252.86	A
4.5	2	252.02	A B
7.0	4	192.02	B C
6.0	4	191.81	B C
8.0	6	170.47	C
3.5	2	156.91	C
3.0	2	125.27	C

Means that do not share a letter are significantly different

D6 One-way ANOVA: Activity versus Spore amount

Source	DF	SS	MS	F	P
Spore amount	9	97230,0	10803,3	220,10	0,000
Error	10	490,8	49,1		
Total	19	97720,9			

S = 7,006 R-Sq = 99,50% R-Sq(adj) = 99,05%

Grouping Information Using Tukey Method

Spore amount	N	Mean	Grouping
2x10^5	2	208,79	A
1x10^5	2	193,39	A
5x10^4	2	132,86	B
10^4	2	129,07	B
5x10^5	2	122,53	B
1x10^6	2	81,83	C
2x10^7	2	29,31	D
2x10^6	2	28,05	D
1,5x10^7	2	22,14	D
10^3	2	3,59	D

Means that do not share a letter are significantly different.

D7 One-way ANOVA: Strains- dns

Source	DF	SS	MS	F	P
C1	3	2588215	862738	176,74	0,000
Error	12	58578	4881		
Total	15	2646792			

```
S = 69,87   R-Sq = 97,79%   R-Sq(adj) = 97,23%

Grouping Information Using Tukey Method

C1      N    Mean  Grouping
AmC28   4  1335,5  A
AmC18   4  1011,3    B
Am13    4   900,1    B
Aniger  4   230,1      C

Means that do not share a letter are significantly different.
```

D8 One-way ANOVA: strains std

```
Source   DF     SS     MS       F      P
strains   3  38277  12759  104.12  0.000
Error     4    490    123
Total     7  38767

S = 11.07   R-Sq = 98.74%   R-Sq(adj) = 97.79%

strains  N    Mean  Grouping
AmC28    2  179.79  A
AmC18    2  150.56  A
Am13     2   79.97    B
Aniger   2    0.78      C

Means that do not share a letter are significantly different
```

D8 Correlations: std; dns

```
Pearson correlation of std and dns = 0,946
P-Value = 0,054
```