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Quang D. Nguyen

Studies on some carbohydrolases with nutrition potential

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....

Head of PhD School **Supervisor**

To my parents, wife and daughter

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1. Introduction

1.1 General introduction

In recent years, consumers worldwide have met so-called functional food products, which are claimed to promote health and well-being. They are known as pro-, pre- and symbiotics in human nutrition. Among them prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in colon. Fructooligosaccharides (FOS), which selectively stimulate the growth of bifidobacteria, are considered to be prebiotics. The market of FOS grows dynamically from the middle of the eighties, especially in Japan, but in the United States of America too, because FOS are now listed as "Generally Recognised as Safe" (GRAS) by Food and Drug Administration (FDA) of USA. In European countries consumers also find them more and more in fermented dairy products. Nowadays, FOS are produced commercially by applying transfructosylating enzymes from the filamentous fungus *Aspergillus niger*. Numerous studies are published dealing with enzymes that have transfructosylating activity, but some questions are still disputed. The yields of FOS production by β-fructofuranosidase remain low, thus the cost of production is relatively high. Another aspect is that the commercially available FOS products contain only 50-60 % of FOS, the rest is made up of 20-30 % of glucose, 10-15 % of unconverted sucrose and small amount of other compounds. The primary sequences of some β-fructofuranosidase enzymes are available in protein databases, but the three-dimensional structure with real conformation is unpublished yet. *Aspergillus niger* which produces numerous enzymes with GRAS status should be an ideal source to study β-fructofuranosidase. The FOS produced by this enzyme can be readily used in food as prebiotics.

Starch is the second biggest polymer, found in the plant kingdom, after cellulose. This is one of the renewable and environmental friendly energy sources available for the humanity in all over the world. The demand for modified and/or converted starch products have increased more and more over native starch, especially in the industrial production of bioethanol, high fructose and high maltose syrups as well as thiosugars. The latter ones are also claimed to promote health and well-being. Nowadays, microbial amylolytic enzymes have replaced the acid hydrolysis of starch in industry with great success. Most of them are produced commercially by *Bacillus*, *Aspergillus* and *Rhizopus* species. α-Amylases from *Bacillus* species are stable and active between 90-95oC and are used widely in food industry and for other purposes. The main drawbacks of these enzymes are the need for Ca++ ion and that they work at relatively high and in narrow range of pH (6.2-6.5). Currently, the glucoamylase from *Aspergillus* is used in industrial scale, but this enzyme is stable and active only up to 55-60 ^{o}C and between pH 4.0-6.0. Due to the differences in the properties of the two important amylolytic enzymes, the starch bioconversion technology takes a long time (15-95 h) with various technological

steps. The "one step" hydrolysis technology would be realisable, if two main amylolytic enzymes were able to work under approximately the same conditions. Taking into consideration that bacteria do not produce adequate amounts of glucoamylase, the thermophilic fungus *Thermomyces lanuginosus*, which is reported to produce extracellularly thermostable α-amylase and glucoamylase appeared to be an ideal source to develop an economically attractive procedure for the industrial starch processing. Other applications of amylases are also described in the production of some oligosaccharides and cyclodextrins. Due to the promising biological properties of these oligosaccharides, in the past years they have gained substantial attention in the fields of medicine and analytical chemistry. New developments, especially in the synthesis and medical chemistry of thiosugars have become important for carbohydrate drug design.

In the present thesis on the one hand *Aspergillus niger* was selected to investigate the properties of β-fructofuranosidase as well as the production and purification of fructooligosaccharides synthesised by this enzyme. On the other hand thermophilic fungus *Thermomyces lanuginosus* was selected for a research dealing with thermostable amylolytic enzymes. Moreover, the understanding of these enzymes will help us in the future to develop a new type of fungal α-amylase or more thermostable glucoamylase as well as complex enzyme preparations containing both amylolytic enzymes that work at the same conditions with synergistic effect. Using these enzyme preparations, the “one step” technology of starch biodegradation can be realised.

1.2 The aims of the thesis

Mesophilic fungus *Aspergillus niger* which produces a lot of enzyme and food additive compounds with GRAS status, belongs to the non-pathogenic micro-organisms. Thus, production of fructooligosaccharides by applying enzyme synthesised by *A. niger* may be readily to use for human nutrition. *T. lanuginosus* is not recognised as a source of enzyme with GRAS status yet, but because it is a real thermophilic fungus, it indicates that this fungus secretes thermostable enzymes. The objectives of the PhD study were to gain insights on the properties of β-fructofuranosidase from *Aspergillus niger* as well as the properties of amylolytic enzymes from *Thermomyces lanuginosus*. The aims of the thesis were:

β-Fructofuranosidase from Aspergillus niger

- Development of submerse fermentation technology for the production of β-fructofuranosidase by filamentous fungus *Aspergillus niger*.
- Purification and characterisation of β-fructofuranosidase from *Aspergillus niger*.

Fructooligosaccharides

- Production of fructooligosaccharides applying β-fructofuranosidase from *Aspergillus niger*.
- Development of a method for the purification of fructooligosaccharides.

Thermostable amylolytic enzymes from Thermomyces lanuginosus

- Screening of *Thermomyces lanuginosus* strains for the production of amylolytic enzymes.
- Optimisation of medium composition for the production of amylolytic enzymes.
- Purification of amylolytic enzyme in mg quantity.
- Determination of the physicochemical properties of amylolytic enzymes as well as the effects of pH, temperature, metal ions and other compounds on the activity and stability of amylolytic enzymes.
- Determination of kinetic parameters of amylolytic enzymes on different substrates.
- Analysis of the amino acid sequence of amylolytic enzymes from *Thermomyces lanuginosus*.

2. Background of the thesis

2.1 Starch and its derivatives

Starch is the principal storage carbohydrate of plants and the major energy source for non-photosynthetic microorganisms and animals as well as for human nutrition. Its major commercial source is corn, but wheat-, potato starch and starches of other cereal grains are also produced commercially (Holló & Hoschke, 1993). Starch is composed of two distinct polysaccharides amylose and amylopectin. Amylose is essentially a linear polymer consisting of up to 6000 glucose units with α(1→4) glycosidic bounds and amylopectin is a highly branched polymer consisting of amylose chains linked at branch points by α(1→6) bonds (Holló & Hoschke, 1993; van der Maarel *et al.*, 2002). The average number of branching points in amylopectin is 5 %, but it varies with the botanical origin. The complete amylopectin molecule contains on the average about 2 million glucose units (van der Maarel *et al.*, 2002).

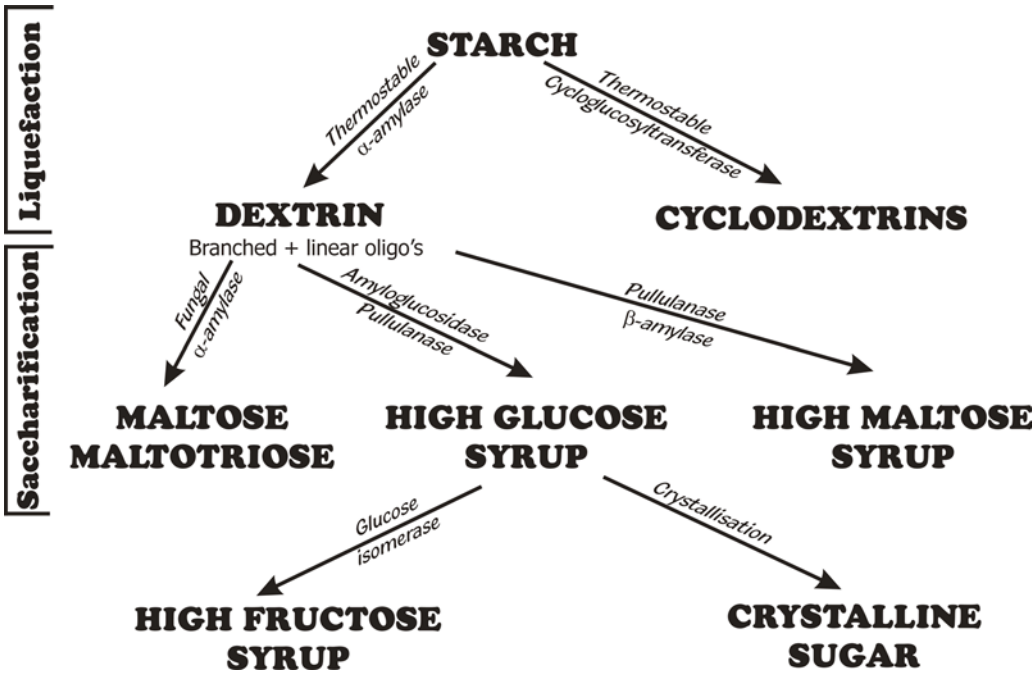


Figure 1 Overview of industrial starch processing

Nowadays, the application of modified and/or hydrolysed starches came to the front because of several advantages. The ratios of total used amount are 40 % of hydrolysed, 40 % of modified and 20 % of native starch. The main applications are in brewing and fermentation industry, in soft drinks and canned fruits. The properties and industrial application of hydrolysed starch are summarised in Table 1.

Table 1 Properties and industrial application of hydrolysed starch products
(Holló & Hoschke, 1993)

Type of syrup	DE*	Composition (%)	Properties	Application
Low DE maltodextrin	15-30	1-20 D-glucose 4-13 maltose 6-22 maltotriose 50-80 higher oligomers	Low osmolarity	Clinical feed formulations; materials for enzymic saccharification; thickeners; fillers stabilizers; glues, pastes confectionary, soft drinks, brewing and fermentation,
Maltose syrups	40-45	16-20 D-glucose 41-44 maltose 36-43 higher oligomers	high viscosity reduced crystallization moderately sweet	jams, jellies, ice cream, conserve, sauces
High maltose syrups	48-55	2-9 D-glucose 48-55 maltose 15-16 maltotriose	increased maltose content	hard confectionary, brewing and fermentation
High DE syrups	56-68	25-35 D-glucose 40-48 maltose	increased moisture holding, increased sweetness, reduced content of higher sugars, reduced viscosity, higher ferment ability	confectionary, soft drinks, brewing and fermentation, jams, conserves, sauces
Glucose syrups	96-98	95-98 D-glucose 1-2 maltose 0,5-2 isomaltose	Commercial liquid "dextrose"	Soft drinks, caramel, baking, brewing and fermentation, raw material soft drinks, conserves, sauces, yoghurts, canned fruits
Fructose syrups	98	48 D-glucose 52 D-fructose	Alternative industrial sweeteners to sucrose	

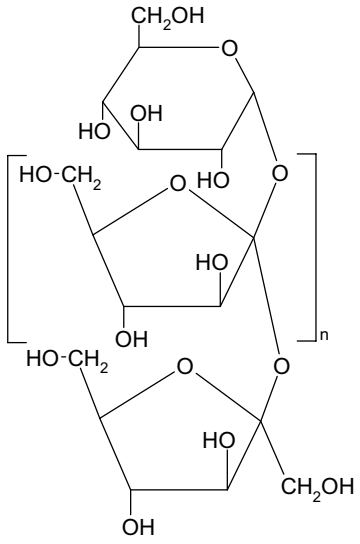
* Dextrose equivalent

Recently the acid hydrolysis method for the production glucose syrup has been replaced by enzymatic treatment with three or four different enzymes (Holló & Hoschke, 1993; Crabb & Mitchinson, 1997; Crabb & Shetty, 1999). Bacterial α-amylases and fungal glucoamylases are predominant in starch bioconversion (Figure 1). In the first step (liquefaction) the α-amylase from *Bacillus licheniformis* or *Bacillus amyloliquefaction* or *Bacillus subtilis* was applied at pH 6.0 and 95-105 ^{o}C for 90 min. Also about 50 ppm of Ca^{++} ions is needed, because the α-amylase from bacteria requires Ca^{++} for operating at high temperature (Saha & Bothast, 2000). The final DE (dextrose equivalent) value after this step is between 8 and 10. The next step is the saccharification of the starch-hydrolysate to high concentration glucose syrup with more than 95 % (w/w) glucose. This is carried out by using glucoamylase from *Aspergillus*

niger or *Aspergillus oryzae* or *Rhizopus oryzae*. To run efficient saccharification, the pH of hydrolysate has to be adjusted to 4.5 by hydrochloric acid, because fungal glucoamylase has a pH optimum of 4.2 and is stable at 60 ^{o}C. Depending on the specification of the product, this step can be accomplished at 60-62 ^{o}C in 12-96 h. In the recent years, the fungal α-amylases came to the front in liquefaction, because they have numerous advantages. Some α-amylases from fungal are thermostable (70-75 ^{o}C) and relatively independent of Ca^{++}. Some intermediate products can be produced only by fungal α-amylases.

2.2 Fructooligosaccharides

Fructooligosaccharides (FOS) are found in higher plants applied in human nutrition such as onions, asparagus, artichokes and tomatoes (Bornet, 1994; Crittenden & Playne, 1996). FOS is a common name of fructose oligomers that are mainly composed of 1-kestose (GF_2), nystose (GF_3) and 1^{F}-fructosylfuranosyl nystose (GF_4) in which fructose residues are bounded at the β(2→1) position of sucrose, respectively (Figure 2). These types of fructooligosaccharides are distinguished from other kinds of fructose oligomers by many authors, because they have been muddled with fructan (Nagamatsu *et al.*, 1990; Henry & Darbyshire, 1980), glucofructosan (Gupta & Bhatia, 1982) and inulin-type oligosaccharides (Kaur *et al.*, 1992).



n = 0: 1-kestose
n = 1: nystose
n = 2: 1^{F}-fructofuranosyl nystose

Figure 2 Chemical structure of fructooligosaccharides

The beneficial properties of some oligosaccharides were recognised very early. In 1900, Moro noted that human milk contained intestinal flora growth factors that were unknown for many

years (Kuhn, 1958). The human milk contains about 7 % (w/v) of carbohydrates (90 % of these is lactose) with about 130 forms of oligosaccharides that play an important role in an infant's immune system (Miller *et al.*, 1994). The recognising and utilisation of functional properties of synthesed fructooligosaccharides in human nutrition just started in the 1980's (Yun, 1996). Since then the demand of customers for healthier and calorie-controlled foods, a number of so-called alternative sweeteners such as fructooligosaccharides are increasing from years to years. Several studies were published dealing with the potential beneficial effects of FOS such as a decrease in colon and rectal carcinogenesis (Shimuhara, 1987; Simon & Gorbash, 1987), decrease an ageing processes and adult illnesses including cystitis and cancer as well as a decrease in the immune system response (Roberfroid, 2000; Mizutani, 1992; Mitsouka, 1990). From the digestion point of view, the human body lacks the enzymes that are able to cleave the beta links occurring in type of oligosaccharides like FOS and galactooligosaccharides (GOS). That means the FOS are resistant to the action of intestinal and pancreatic enzyme in the human small intestine (Anderson *et al.*, 1999; Spiegel *et al.*, 1994; Molis *et al.*, 1996). Most of them reach the large intestine in an intact form, where they serve as substrates for bacterial microbiota such as *Bifidobacterium* spp and *Bacteroides* spp (Engfer *et al.*, 2000; Damian *et al.*, 1999). Their capacity to retain water gives them also an osmotic effect. Interestingly and conversely, pathogenic and putrefactive bacteria including *Escherichia coli*, *Clostridium perfingens*, *Clostridium difficle* and others have been shown unable to utilise the FOS (Wang & Gibson, 1993; Gibson *et al.*, 1995; McBain & Macfarlane, 1997). The bacterial digestion of FOS occurs in two stages. In the first stage, FOS are hydrolysed to monomers by bacterial β-oxidases. In the second stage, the liberated monomers are fermented anaearobically producing short-chain fatty acids such as acetate, propionate and butyrate acids (Hidaka *et al.*, 1986) and gases (H_2, CO_2, CH_4). In addition to its role as an energy provider, butyrate has important effects on the proliferation and differentiation of cells in the intestinal flora (Rivero-Urgell & Santamaria-Orleans, 2001). Acetic acid and lactic acid cause the decrease of pH in colon (Hammes & Tichackez, 1994), which in turn restricts the growth of pathogenic bacteria. FOS are considered to behave as soluble alimentary fibres, since from a physiological point of view they fit the definition of fibre: they arrive to the large intestine where they are fermented by colonic flora (Tsuji *et al.*, 1986; Miller *et al.*, 1994). Summarisingly, FOS have numerous potential beneficial favourable functional properties including (i) improve the bifidus-type, (ii) decrease *Clostridium perfingens* in intestinal microflora (Hidaka *et al.*, 1988), (iii) reduction of toxic metabolites and undesirable enzymes, (iv) increase the absortion of different minerals in the intestine (van den Heuvel *et al.*, 1999; Takahara *et al.*, 2000), (v) relieve the constipation, (vi) decrease the total cholesterol and lipid in serum (Yamashita *et al.*, 1984), (vii) the promotion of animal growth and (viii) they are low calorie non-carcinogenic sweeteners (Yun, 1996). FOS are now produced commercially by transfructosylation activity enzymes from *Aspergillus niger* (Hayashi *et al.*, 1992a), *Bacillus* sp. and *Aureobasidium pullulans* (Yun *et al.*, 1992).

2.3 α-Amylase

2.3.1 Occurrence and general properties

α-Amylase (EC 3.2.1.1) with official name α(1→4)-D-glucan glycanohydrolase is widely distributed in plants, animals and micro-organisms. The enzymes from microbial sources generally meet industrial demands (Pandey *et al.*, 2000). Reportedly, both bacteria and fungi tend to secrete extracellular α-amylases. The most industrially applied α-amylases are from *Bacillus amyloliquefaciens* (Morcel *et al.*, 1995; Mei & Yu, 1997; Milner *et al.*, 1997), *B. licheniformis* (Declerck *et al.*, 1995; Dobreva *et al.*, 1998), *B. stearothermophilus* (Srivastava, 1984; Brumm *et al.*, 1989), *B. subtilis* (Stephenson *et al.*, 1998) and *B. subtilis* var *amylosacchariticus* (Fujimori *et al.*, 1978). Some other bacteria were also reported to produce α-amylases (Nakamura & Crowell, 1979). In the last few decades the α-amylases from fungi have also been studied intensively, because of some advantageous properties of theirs in starch-conversion application and other industries. Numerous fungi such as *Aspergillus awamori, A. niger*, *A. oryzae*, *Thermonospora* spp., *Mucor* spp. have become the study subjects of various research groups (Oteng-Gyang *et al.*, 1981; Schmidt *et al.*, 1998).

α-Amylase enzymes catalyse the hydrolysis of α(1→4)-glycosidic linkages of glucose polymers such as starch. Substrate molecules are attacked at most internal bonds with random mode, thus α-amylases are classified as endo-enzymes. The fact that starch and related polymers are universal sources of dietary carbon throughout the animal and plant kingdoms accounts for the ubiquitous presence of these enzymes.

The molecular masses of α-amylases are in the range from 45-75 kDa (**Table 2**; 3) that contain about 500-700 amino acid. The α-amylase from filamentous fungi have a molecular mass of 50–76 kDa (Table 3), but amylase from bacteria have a wider range from 40 up to 110 kDa (**Table 2**). Some special α-amylases with smaller or bigger molecular mass have been reported. Saito (1973) isolated α-amylase from *B. licheniformis* and found that the molecular weight was 22.5 kDa, Grootegoed and co-workers (1973) isolated from *B. caldolyticus* with a molecular weight of only 10 kDa. DePinto and Campbell (1968) reported the one from *B. macerans* have a molecular mass of 139 kDa. The biggest one that is reported so far, was published by Ratanakhanokchai and co-workers (1992) from *Chloroflexus aurantiacus* with a molecular mass of 210 kDa. Some α-amylases are capable to achieve monomer-dimer transformation. This hypothesis was described first by Menzi and co-workers (1957), when they reported the properties of α-amylase from *B. subtilis*. They also reported that zinc plays important role in dimmer-monomer transformation. The dimmer of α-amylase from *B. subtilis* contains about 1 gram of zinc atom.

Table 2 Some properties of bacterial α-amylase

Source	Molecular mass [kDa]	Optimum pH	Optimum Temperature [°C]	References
Alicyclobacillus cacidocaldarius	160	3.0	75	Schwermann *et al.,* 1994
Alteromonas haloplanctis	49.3	-	-	Feller *et al.,* 1992
Bacillus acidocaldurius	68	3-5	75	Buonocore *et al.*, 1976
Bacillus amyloliquefeciens	49	5-9	65-70	Welker & Campbell, 1967
	60	5,5	50-70	Borgia & Campbell, 1978
Bacillus coagulans	-	7,5-8,5	85	Medda & Chandra, 1980
Bacillus flavothermus	-	5.5-6.0	60	Bolton *et al*., 1997
Bacillus licheniformis	62	5-10	90	Morgan & Priest, 1981
	58	6.0-6.5	90	Ivanova *et al.*, 1993
Bacillus lentus	42	6.1	70	Elaassar *et al.,* 1992
Bacillus megaterium	59	-	-	Brumm *et al*., 1991
Bacillus stearothermophilus	48	5-6	65	Ogasahara *et al.*, 1970
Bacillus subtilis	54	5.6	80	Uguru *et al*., 1997
Bacillus sp.	42	9.0	70	Lin *et al.*, 1998
Bifidobacterium adolescentis	66	5.5	50	Lee *et al*., 1997
Chloroflexus aurantiacus	210	7.5	71	Ratanakhanokchai *et al*., 1992
Clostridium acetobutylicum	84	5.6	45	Paquet *et al*., 1991
Clostridium perfringens	76	6.5	30	Shih and Labbe, 1995
Lactobacillus brevis	75.9	6.5	55	Ilori *et al*., 1997
Lactobacillus plantarum	50	5.5	65	Giraud *et al.*, 1993
Micrococcus luteus	56	6.0	30	Ilori *et al*., 1995
M. varians	14-56	7.0	45	Adeleye, 1990
Myxococcus coralloides	22.5	8.0	45	Farezvidal *et al*., 1995
Nocardia asteroides	56	6.9	50	Stevens *et al*., 1994
Pyrococcus furiosus	66	-	100	Laderman *et al*., 1993
Pyrococcus woesei	70	-	130[b]	Koch *et al*., 1991
Streptococcus bovis	77	5.0-6.0	50	Freer, 1993
Thermococcus profundus	42	5.5-6.0	80	Chung *et al*., 1995
Thermotoga filiformis	60	5.5-6.0	95	Egas *et al*., 1998
Thermotoga maritima [a]	61	7.0	85-90	Liebl *et al*., 1997
Thermus sp.	59	5.5-6.5	70	Shaw *et al*., 1995

Calcium atom plays an important role in α-amylase activity and stability. They do not contain co-enzymes but they are calcium metallo-enzymes (Fischer & Stein, 1960) with at least one atom per molecule (Priest, 2000). In the presence of calcium ion α-amylases are quite resistant to extremes of temperature, pH, and even to treatment with some proteases like pepsin, trypsin and subtilisin (Stein & Fisher, 1958). Many authors (Arai *et al.*; 1969) concurrently reported that α-amylases required at least 1 calcium atom per molecule of enzyme for full activity. The one atom of tightly bond calcium could be replaced by other divalent metals such as strontium, barium and magnesium without an appreciable loss in activity (Toda & Narita, 1968). These metal ions can only replace the calcium when the original conformation of the molecule is retained and the active site has not been altered (Fogarty, 1983). The X-ray structures are available for both the calcium-depleted wild type and for a threefold mutant of this enzyme (Machius *et al.*, 1995; 1998). This has allowed them to propose a disorder→order transition that should supposedly occur when Ca^{++} ion is bound to calcium-depleted form of the enzyme. It is unknown, however, whether the calcium-depleted α-amylase can be reactivated by the addition

of calcium, thus making the disorder→order transition theory hypothetical. Heavy metals such as copper, mercury, silver and lead inhibit α-amylases (Hoschke, 1991; Jensen & Olsen, 1992). Generally the amylases from bacteria are not glycoproteins, while most amylases from fungi reported so far, are glycoproteins (Priest, 2000). The carbohydrate contents vary from 1-2% up to 15-20% (Mishra & Maheshwari, 1996; Li *et al.*, 1998b). The majority of monosaccharides are glucose, galactose, glucosamine and galactosamine.

Table 3 Some properties of fungal α-amylases

Source	Molecular mass [kDa]	Optimum pH	Optimum temperature [°C]	Reference
Aspergillus sp.	56	5.5	40	Park *et al*., 1995
A. flavus	52.5	6.0	55	Khoo *et al*., 1994
A. fumigatus	65	5.5	40	Planchot & Colonna, 1995
A. niger	56	6	55	Thenawdjaja *et al.*, 1990;
A. oryzae	52	4-5	50	Chang *et al*., 1995
Cryptococcus sp.	66	-	-	Iefuji *et al*., 1996
Filobasidium capsuligenum	56	5.6	45	Tsiomenko *et al*., 1992
Lipomyces kononenkoae	76	4.5-5.0	70	Prieto *et al*., 1995
Rhizopus sp.	64	4.0-5.6	60-65	Siqueira *et al*., 1997
Scytalidium sp.	87	6.5	50	Odibo *et al.,* 1992
Thermomonospora curvata	61-62	6.0	65	Collins *et al.*, 1993
Thermomyces lanuginosus	45	4.6-5.2	60	Jensen & Olsen, 1992

The optimum activity of α-amylases generally occurs between pH 4.8 and 6.5 (**Table 2**; Table 3), but it varies depending on the source of enzyme, even from strain to strain, and on the presence or absence of Ca^{++}. Most of α-amylases loose activities at the pH lower than 4.0. Very few micro-organisms are able to produce acid-stable α-amylase. Buonocore and co-workers (1976) reported the α-amylase from *B. acidocaldarius* have pH optimum at 3.5. In the case of fungal α-amylase, *Mucor pusillus* and *Lipomyces starkeyi* are able to secrete acid-stable α-amylase. Their pH optima were determined to be in the range 3.5-4.0 and 3.0-4.0, respectively (Moulin & Galzy, 1979). The effects of the pH on stability of α-amylases also were investigated. α-Amylases are generally stable in the pH range from 5.5 to 8.0 and to extremes of pH in the presence of full complement of calcium (Fogarty, 1983). The α-amylase from *B. licheniformis* was reported to be stable at 76-95 °C and pH in the range from 5.0 up to 9.5 (Medda & Chandra, 1980; Morgan & Priest, 1981). The most extensively studied α-amylases are from *B. amyloliquefaciens* and *B. stearothermophilus* (Borgia & Campbell, 1978) and they are stable in the pH range from 5.5 to 9.5. The α-amylase from *A. niger* is stable in the pH range from 4.5 to 8.5 (Luo *et al.*, 1994).

From the temperature point of view, the optimum activity of α-amylases varies in very large range from 30 °C up to 110 °C. Certainly the presence of calcium ion plays an important role in this case too. Generally α-amylases from bacteria more stable and have higher temperature optimum that those from fungi. The temperature optimum of α-amylase enzyme from *B. amyloliquefaciens* is 70 °C but in industrial processes using high substrate concentration about 30-40 % (w/v) the maximum operating temperature is 85-95 °C. In the case of α-amylase

from *B. licheniformis* the optimum temperature is 90 ^{o}C and it may be used industrially at temperatures in above 100 ^{o}C, even up to 125 ^{o}C. Of course, this enzyme requires more calcium when operating at temperature higher than 100 ^{o}C. For example at 105 ^{o}C stability can be maintained using 50 ppm of calcium ion (Norman, 1979).

The fungal α-amylases have optimal temperatures in the range from 38-70 ^{o}C. Some α-amylases from thermophilic fungi may be stable at a temperature higher than 60 ^{o}C. Somkuti and Steinberg (1980) reported that optimum temperature of α-amylase from *Mucor pusillus* is in the range from 65-70 ^{o}C. The optimum temperature of α-amylase from *Thermomyces lanuginosus* was also reported at 70 ^{o}C (Odibo & Ulbrich-Hofmann, 2001; Mishra & Maheshwari, 1996).

2.3.2 Structure

Information on the structure and function of different types of α-amylase now can be found in protein data bank (http://www.rcsb.org/pdb/; http://www.expasy.ch/). Presently, 73 X-ray structures are also available for α-amylases. To understand the mechanism of substrate binding the author will focus only on "Taka amylase A" (TAA) enzyme from *Aspergillus* sp.. The three-dimensional structure of TAA that is the first protein of α-amylase family, have been determined by X-ray crystallography at 3 Å resolution (Matsuura *et al.*, 1984). The primary and secondary structures of this α-amylase were demonstrated in Figure 3. Based on this structure, TAA consists of 476 amino acid residues.

Studies of secondary structure show that TAA have five β-sheets (A, B, C, D and E) each consisting of 20 β-chains (Branden & Tooze, 1991). In the protein structure there are 24 α-helices, 5 β-barrels immobilised by hydrogen bonds and 4 disulphide linkages. Disulphide bonds link the cystein residues found in the following positions: 30 and 38, 150 and 164, 240 and 283, 440 and 475, respectively. These disulphide linkages play an important role in enzyme stability. Based on tetriary structure, TAA contains 2 domains called A and B, and the main one contains the first 374 *N*-terminal amino acid residues. A central $(\alpha/\beta)_8$ TIM-barrel (domain A) forms the core of the protein molecule and contains the catalytic site. In the catalytic site the following amino acid residues occur: Glu156, Tyr135, His210, Asp206, His122, Trp83, Asp168, Tyr24, Asp233, Lys209, Leu232, Val231, Glu230, Asp297, His296, Arg344, Asp340, Tyr79 and Glu35. Domain B is formed by a protrusion between the third strand and the third helix of the TIM-barrel. Doamin B has a rather irregular β-rich structure, and varies substantially in size and structure among the α-amylases (Janecek *et al.*, 1997). The active site cleft is located on the interface between domain A and domain B, and is found at the C-terminal end of the β-strands in the TIM barrel, meanwhile the substrate binding cleft is located in domain B.

The analysis of X-ray diffraction showed that TAA have two calcium binding site (Boel *et al.*, 1990). The first site where the Ca^{++} ion is fixed by 8 ligands (Asp175 O-Δ_1 and O-Δ_2, Asn121 O-Δ_1, Glu162 and Glu210, oxygen atom of the main chain's carbonyl groups, and three water

molecules) plays a very important role in the formation of active centre and stabilising of enzyme. This Ca^{++} ion is located at the interface between two domains A and B (Figure 4). The second Ca^{++} binding site can be found at the bottom of substrate binding site. The Asp206 and Glu236 at the active site also have a role in Ca^{++} ion binding. This causes the Ca^{++} repression effect when the Ca^{++} concentration is relatively high in reactions mixture.

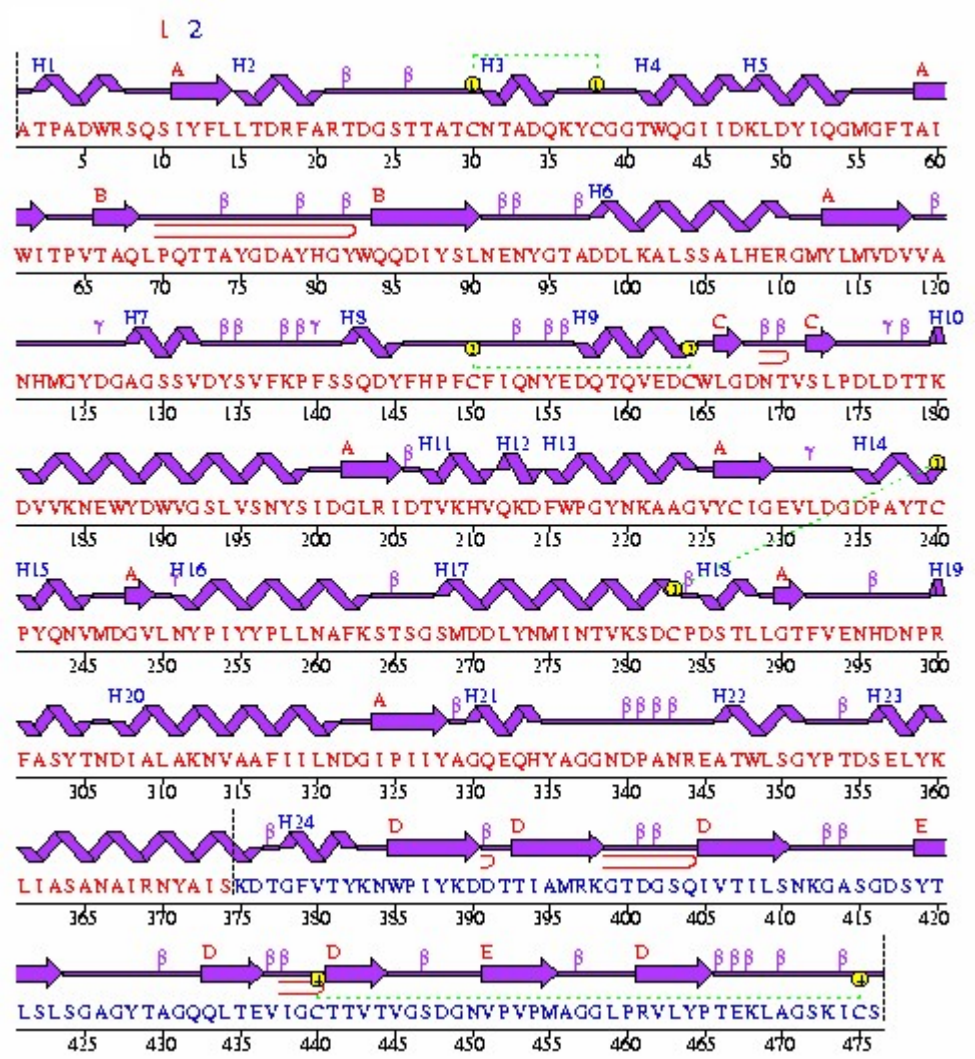


Figure 3 Primary and secondary structure of Taka-amylase A
Domain A: red letters, Domain B: blue letters
(http://www.biochem.ucl.ac.uk/bsm/6taa/mail.html)

Several α-amylases, mainly mammalian α-amylases (Brayer *et al.*, 1995; Ramasubbu *et al.*, 1996) contain a chloride ion in the active site which has been shown to enhance the catalytic efficiently of the enzyme, presumable by elevating the pK_a of the hydrogen-donating residue in

the active site (Levitsky & Steer, 1974; Feller *et al.*, 1996). These α-amylases consist of three domains namely A, B and C. A puzzling feature of chloride containing α-amylases is a serine protease-like Glu-His-Ser triad in the interface between domains A and C (Nielsen & Borchert, 2000). Aghajari and co-workers (1998) proposed that this triad is capable of performing an autoproteolytic cleavage. It has been observed that the affinity for the conserved calcium ion increases dramatically upon chloride binding (Levitsky & Steer, 1974), and it is therefore conceivable that chloride ion binding also induces conformational changes around the active site. More studies are needed to make the role of chloride ion playing in α-amylases clear.

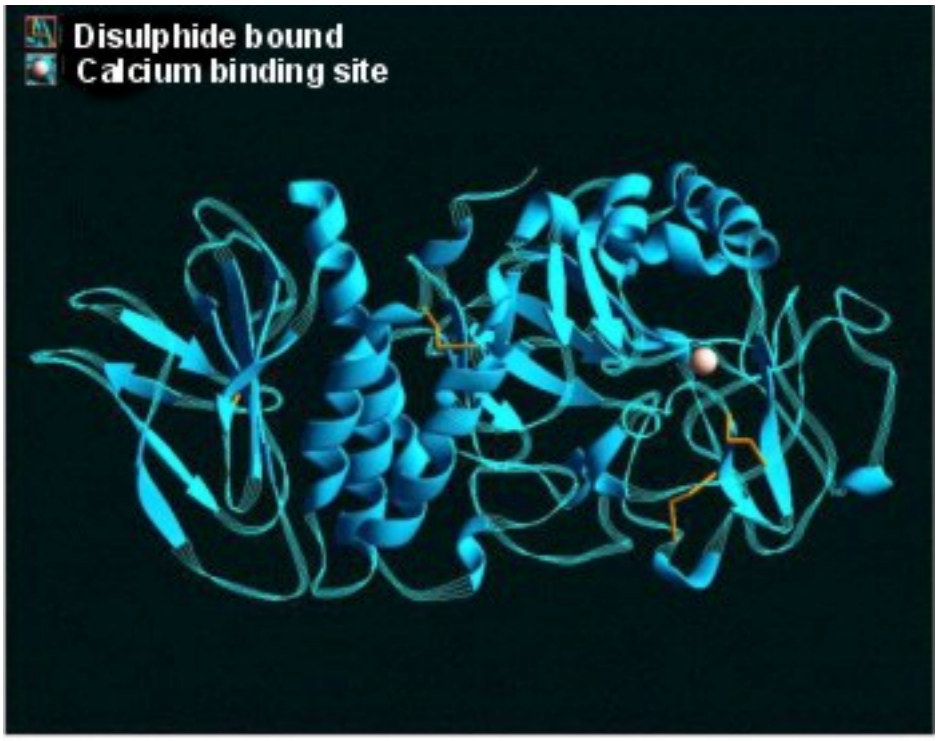


Figure 4Three-dimensional of Taka-amylase A with Ca^{++} binding site
(ftp://pdb.pdb.bnl.gov/Images/JPEG/2TAA_taka-amylase_1.jpg)

2.3.3 Catalytic mechanism

Carbohydrolases may be divided into two classes depending on whether they retain or invert the anomeric configuration of the products (Sinnott, 1990). Based on this classification, α-amylase belongs to class one, which releases the products retaining its anomeric configuration. α-Amylase acts on α(1→4)-glucosidic linkages and produce a new reducing end with α-configuration at C-1 (Fischer & Stein, 1960). Koshland (1953) proposed a double displacement mechanism for retaining enzymes (Figure 5). An enzyme nucleophile (Asp or Glu) attacks the anomeric carbon to generate a covalent intermediate. The departure of the aglycon is

facilitated by protonation of the glycosidic oxygen by a general acid/base catalyst (Asp, Glu or Tyr) (Uitdehaag *et al.*, 1999b) that causes the cleaving of C_1-O linkage. In the second stage of the reaction the covalent intermediate is hydrolysed by a water molecule which is activated by the base form of the general acid/base catalyst. This is able to release the products with α-configuration and reprotonate to original acid group. Transglycolysation can occur if the attacking group in the second displacement of the reaction is free hydroxyl of a sugar residue rather than water (Uitdehaag *et al.*, 1999a; b). Alternatively, retaining enzymes may work by a S_n1 mechanism as proposed first for hen egg white lysozyme (HEWL) by Vernon (1967). Following the departure of the aglycon an oxocarbonium ion intermediate stabilised by a carboxylate group in the enzyme develops. The oxocarbonium ion reacts rapidly with water to generate the final product. Furthermore, a strong electrostatic field that is across the active site cleft (see above) is believed to be important for protonation of the glycosidic oxygen in lysozymes (Dao-Pin *et al.*, 1989).

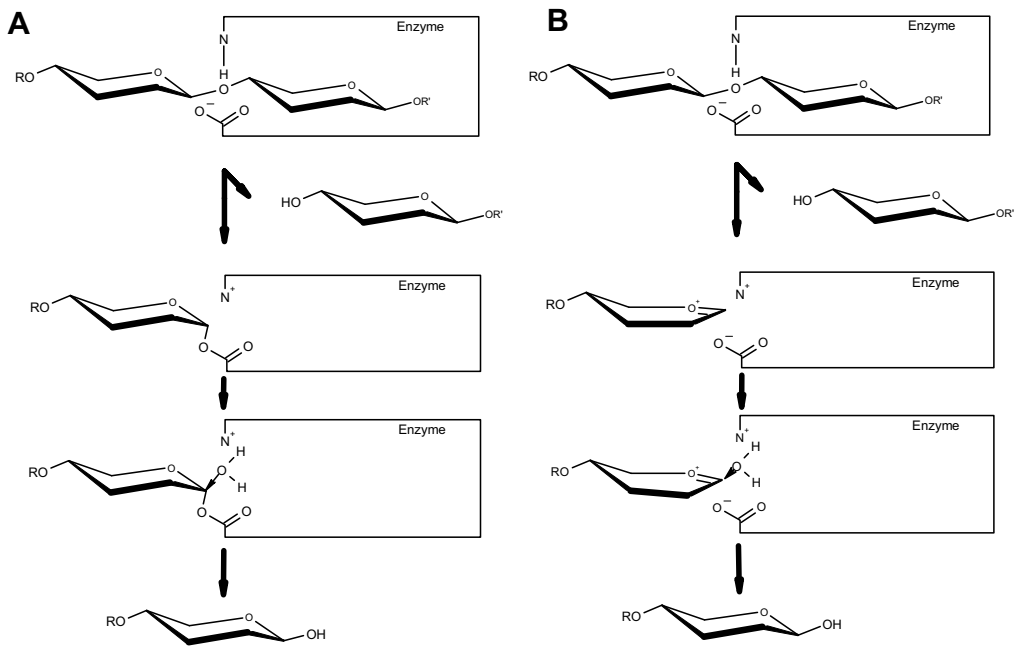


Figure 5 Catalytic pathways for retaining carbohydrases
A: via a covalent intermediate; B: via an oxocarbonium ion intermediate
(Sogaard, 1992)

Based on the crystal structure analysis of TAA (Matsuura *et al.*, 1984), porcine pancreatic α-amylase (Buisson *et al.*, 1987), *Aspergillus* acid α-amylase (Boel *et al.* 1990), and bacterial (*Bacillus subtilis*, *B. licheniformis* and *B. stearothermophilus*) α-amylases (Suzuki *et al.*, 1990; Lee *et al.*, 1991), three highly conserved amino acid residues Glu230, Asp206 and Asp297 were found at the centre of the active site (Figure 6). The role of these three carboxyl and two histidine residues (His122 and His296) has been investigated in a number of α-amylases and

related enzymes using site-directed mutagenesis. Subsequent mutational studies showed that these residues are essential for catalysis (Svensson, 1994; Jenecek, 1997), since mutation of either residue in *B. stearothermophilus* (Holm *et al.*, 1990; Vihinen *et al.*, 1990), *B. subtilis* (Takase *et al.*, 1992) and TAA (Nagashima *et al.*, 1992) cause inactivation of enzyme. Only the D331E (TAA Asp 297) mutant of *B. stearothermophilus* α-amylase has been reported to have enzyme activity, albeit extremely low (Vihinen *et al.*, 1990), which may indicate that this particular residue and its counterparts in related enzymes are somewhat less critical in catalysis than the other two invariant residues. The glutamic acid residue is now believed to be the proton donor, while the first of the two conserved aspartic acids appearing in the amino acid sequence is thought to act as the nucleophile. The role of the second aspartic acid is less certain, but it has been suggested to be involved in stabilising the oxocarbonium ion-like transition state and also in maintaining the glutamic acid in the correct state of protonation for activity (Uitdehaag *et al.*, 1999a). In the active site of TAA two amino acid residues also were postulated as being important, are His122 and His296. This has been confirmed by investigations on mutated proteins and the histidine residues are not invariant throughout the α-amylase family (Janecek, 1997; MacGregor *et al.*, 1996; Svensson, 1994). These five important residues occur near the ends of strands 3, 4, 5 and 7 of the β/α-barrel and are found in four short sequences, long-recognised as being conserved. Oyama and co-workers (1996) with site mutagenesis have substituted the Glu230 with Gln and they reported that the mutational enzyme have a better affinity to amylose than the original TAA.

MacGregor and co-workers (2001) suggested that the active site of enzyme belonging to α-amylase family is considered to be made up of a number of subsites (Figure 6; Figure 7), each subsite being capable of interaction with one glucose residue of the substrate. The subsite nomenclature has been defined by Davies and co-workers (1997). In the α-amylase there are two or three subsites present on the reducing chain-end of the scissile bond, whereas the number of subsites on the non-reducing side of scissile bond varies between two and seven (MacGregor, 1988; Brzozowski *et al.*, 2000). The subsites themselves are composed of site chains of amino acid residues situated on loops in the enzyme structure to connect the *C*-terminal ends of the adjacent helices of the $(\beta/\alpha)_8$-barrel of the catalytic domain. Because the architecture of the β-α loops varies from enzyme to enzyme, the number and nature of subsites at the active site also varies, and it is characteristic for the particular enzyme. Furthermore the number of subsites can be specific properties of the individual enzyme belonging to α-amylase family.

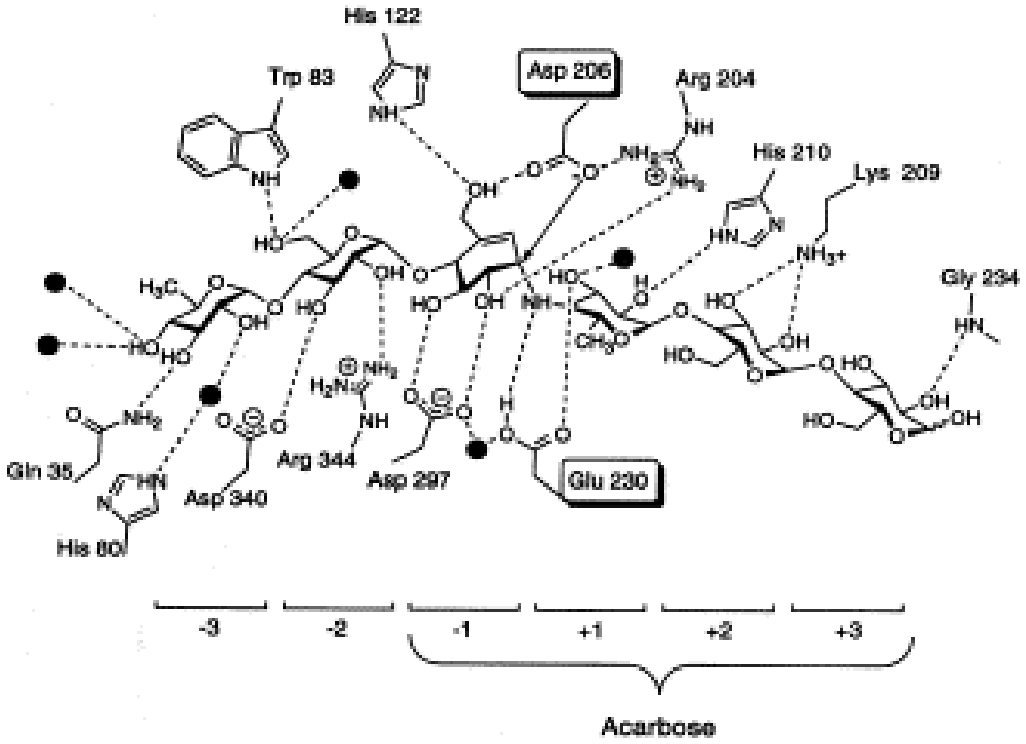


Figure 6 Taka-amylase A and substrate complex model with subsites from -3 throught +3. (MacGregor *et al.*, 2001)

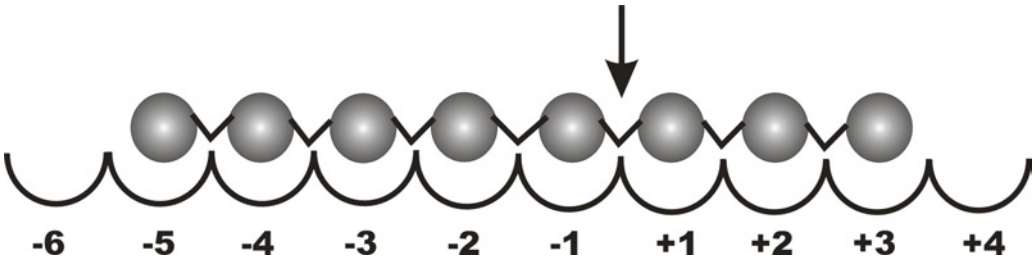


Figure 7 Schema of subsite arrangement with oligosaccharide occupying subsites -5 through +3. Cleavage occurs between subsites -1 and +1 as indicated by the arrow. The reducing end group is bound at subsite +3 (MacGregor *et al.*, 2001)

2.3.4 Aspects of protein engineering and possible biotechnological exploitation

The well known structures and genetic background provide more and more possibilities to make new tools in biotechnology and applied microbiology. The conditions prevailing in the industrial applications in which enzymes used are rather extreme, especially with respect to temperature and pH (van der Maarel *et al.*, 2002). Therefore, there is a continuing demand to improve the stability of enzyme and thus meet the requirements set by specific applications. One approach would be to screen for novel microbial strains from extreme environments such

as hydrothermal vents, salt and soda lakes and brine pools (Sunna *et al.*, 1997; Niehaus *et al.*, 1999; Vieille & Zeikus, 2001). This is being used successfully by various groups. For example, Antranikian and co-workers (1990) has patented the production of hyper thermostable α-amylase by *Pyrococcus woesei*. A second approach that is used with more success is to engineer commercially available enzyme preparations. Early mutational analyses investigated structure/function relationships (Svensson, 1994) and protein engineering, moreover addressed important industrial goals such as improvement of thermostability or changing the pH activity dependence (Declerck *et al.*, 1997; Nielsen *et al.*, 1999; Declerck *et al.*, 2000). Several approaches of protein engineering have been published and applied. First one is finding out what specific regions are important for a given property and hybrid can be made of two or more homologous enzymes. Suzuki and co-workers (1989) applied this technique and successfully made the hybrid of two α-amylases from *B. licheniformis* and *B. amyloliquefaciens* and two regions that are important for thermostability were identified. Conrad and co-workers (1995) as well as Borchert and co-workers (1999) also used this approach for finding regions contributing importance to the functioning of α-amylase from *B. licheniformis* at different temperatures. The second approach of protein engineering is replacing some amino acid in protein structure to make it more stable. Matthews and co-workers (1987) described that the introduction of prolines in loop regions of α-amylase can have a stabilising effects on enzyme. This hypothesis was also proved by Frandzen and co-workers (1996) when they replaced the arginine residue at position 124 by proline. The change resulted a more stable enzyme. Disulfide bonds in the enzyme can also lead to improved stability (Day, 1999). Stability is a prerequisite for activity at extreme pH values, but it alone is not sufficient, as the active site residues must be in a catalytically competent protonation state in order for the enzyme to be active. Thus, the proton donor (Glu230 in case of TAA or Glu261 in case of bacteria α-amylases) is required to be protonated, while the nucleophile (Asp206 in case of TAA or Asp231 in case of bacteria α-amylases) must be negatively charged. Mutagenesis experiments that insert or remove residues near or in the active site in hope to make α-amylase more stable at extreme pH values, were carried out (Sogaard *et al.*, 1993; Nielsen *et al.*, 1999; Wind *et al.*, 1998), but unfortunately no predictable way of changing the pH-activity profile has been found. A currently fashionable approach for engineering protein is random mutagenesis coupled to high throughput screening (Chen, 2001). In this approach, point mutations generate an error in the polimerisation chain reaction leading to such a change in the triplet code that a new amino acid is built into the protein. Shaw and co-workers (1999) used this method and reported that a newly isolated α-amylase from *B. licheniformis* is 23 times more stable than the wild-type one.
Other aspect is modification of product specificity that has major industrial relevance. So far, very few data are available about glycosyltransferase activity of α-amylases. In fact, some α-amylases do not just hydrolyse polysaccharides, but they are also able to transfer the glucose residues to build shorter oligosaccharides. In the last decade of the twentieth century several projects were funded to investigate the glycosyltransferase activity of α-amylases (Kuriki *et al.*,

1996; Uidehaag *et al.*, 1999b; van de Veen *et al.*, 2000; Ishii *et al.*, 2000). Matsui (1991; 1992) with point-mutation have enhanced the glycosyltransferase activity of α-amylase from *Saccharomycopsis* probably by slow product release. The new mutated α-amylase allowed attack of a new substrate donor molecule. Cyclodextrin glucanotransferase specificity was rationally engineered in α-amylase from *B. stearothermophilus* which was closely related in sequence to a cyclodextrin glycosyltrasferase of the same microorganism (Beier *et al.*, 2000). The mutation of glutamine that was found normally as transition state stabilising histidine at subsite, to histidine in glucansucrase from *Streptococcus downei* altered the acceptor reaction to produce new type of oligosaccharides (Monchois *et al.*, 2000). Interestingly the reserve mutation of histidine to glutamine in the related amylosucrase from *Neisseria polysaccharea* was the superior of a series of replacements and gave an enzyme with enhanced sensitivity to glycogen activation (Sarcabal *et al.*, 2000). Random mutation at localised substrate binding region of polypeptide chain such as in β→α loops 4 (Matsui & Svensson, 1997) and 7 (Svensson *et al.*, 1999) in barley α-amylase shifted the position of the preferred productive binding mode of the oligosaccharide substrates. In addition mutated enzymes were obtained with altered substrate specificity, including increased specific activity towards insoluble starch (Svensson *et al.*, 1999). New mutant strains open the possibilities for industry to make large amount of high specificity products and thermostable of enzymes that can be applied in the wide range in the food and feed industry.

2.4 Glucoamylase

2.4.1 Occurrence and general properties

Glucoamylase (EC 3.2.1.3) has one of the largest sales volumes in the world enzyme market. Its main application is in the starch processing industry that uses it in the production of glucose- and isosyrup. These syrups are widely applied in the soft drink industry, in bakeries and in the fermentation industries. The official name of glucoamylase is α(1→4)-D-glucan-glucohydrolase, but it is also known as amyloglucosidase or γ-amylase. This enzyme catalyses the hydrolysis of glucose based polymers such as starch to release glucose and α-dextrin with less polymerisation degree. Glucoamylase acts the polymers from the non-reducing chain-ends and release β-D-glucose residues, thus it is classified as exo-enzyme. Although activity (k_{cat}/K_m) towards the α(1→6) and α(1→3) linkages is only 1 % and 0.2 % of that towards the α(1→4) linkage (Fogarty, 1983; Sierks & Svensson, 1994). Glucoamylases occur almost exclusively in fungi and the enzymes used commercially originate from strains of either *Aspergillus niger* (Pazur *et al.*, 1980) or *Rhizopus* spp. (Adachi *et al.*, 1977). Also, *A. awamori* (Bon & Webb, 1989) and *A. oryzae* (Gomi *et al.*, 1987) still belong to the most intensively studied fungus in the last century.

Molecular mass of glucoamylase varies depending on the sources. Generally it is in the range from 30 kDa to 120 kDa (Table 4), but there are some exceptions such as glucoamylase from

Lipomyces kononenkoae, which has a molecular mass of 811 kDa (Spencer-Martins & van Uden, 1979). Molecular weight of glucoamylase from *A. awamori* is in the range from 83 kDa to 88 kDa (Yamasaki *et al.*, 1977b), but Flor & Hayashida (1983) reported glucoamylase from a mutant *Aspergillus awamori* var. *kawachi* that has a molecular weight of about 250 kDa. The smallest one was from *Cephalosporium eichhorniae* reported by Day (1978) that has a molecular mass of 26.5 kDa. Glucoamylase from *A. oryzae* occurs in different forms. The GAIII and GAII have the equal molecular mass of about 38 kDa while GAI has a molecular weigh of about 76 kDa (Miah & Ueda, 1977b).

Table 4 Physicochemical properties of glucoamylases

Source	Molecular mass [kDa]	Optimum pH	Opt.imum temperature [^{o}C]	References
Acremonium sp. YT-78	74	5.0	50	Mase *et al.,* 1996
Aspergillus awamori	83.7	4.5	60	Yamasaki *et al.,* 1977
A. awamori	110, 86	-	-	Silva *et al.,* 1997
A. awamori var. kawachi	57- 90	3.8-4.5	-	Yoshino & Hayashida, 1978
A. niger	74 96	4.2, 4.5	60, 65	Amirul *et al.,* 1996
A. niger	61-112	4.4	60	Queiroz *et al.,* 1997
A. oryzae	38 76	4.5	50-60	Miah & Ueda, 1977a; b
A. saitri	90	4.5	-	Takahashi *et al.,* 1981
A. terreus	70	5.0	60	Ghosh *et al.,* 1991
Chalara paradoxa	68	5.0	45	Monma *et al.,* 1989
Corticium rolfsii	69,70, 78, 79	-	-	Nagasaka *et al*. 1998
Clostridium	77	4.5	65	Ohnishi *et al.,* 1991
C. thermosaccharolyticum	75	5.0	70	Specka *et al.,* 1991
Lactobacillus amylovorus	47	6.0	45	James *et al.,* 1997
Neurospora crassa	82	5.4	60	Spinelli *et al.,* 1996
Paecilomyces varioti	69	5.0	-	Takeda *et al.,* 1985
Piricularia oryzae	94	4.5	50-55	Yuhki *et al.,* 1977
Rhizopus sp.	58.6- 74	4.5-5.0	-	Takahashi *et al.,* 1985
R. niveus	-	4.5-6.0	-	Saha &Ueda, 1983
Schizophyllum commune	66	5.0	40	Shimazaki *et al.,* 1984
Thermomyces lanuginosa	37	-	70	Basaweswara Rao *et al.,* 1981
(Humicola lanuginose)	-	4.9, 6.6	65-70	Taylor *et al.,* 1978

The pH optimum of glucoamylases is generally in the range from 4.5-5.0 (Table 4) and the enzymes are usually stable at acidic pH (Abe *et al.*, 1988; Hyun & Zeikus, 1985). Yuhki and co-workers (1977) reported that glucoamylase from *Piricularia oryzae* has optimal pH of 6.5. *Humicola lanuginosa* secreted two forms of glucoamylase with pH optima 4.9 and 6.6 (Taylor *et al.*, 1978). The GAII from *H. lanuginosa* stable up to pH 11.0. The enzymes from *Corticium rosfsii* and *Coniophora cerebella* are stable up to pH 9.0 (Kaji *et al.*, 1976).

The temperature optimum of most glucoamylases is in the range from 40 to 60 ^{o}C (Takahashi & Yamanobe, 1981) but some exceptions are detected. Glucoamylases from *Aspergillus niger* (Queiroz *et al.*, 1997) and *Thermomyces lanuginosus* (Basaveswara Rao *et al.*, 1981) have optimum at 60 ^{o}C and 70 ^{o}C, respectively. In general, glucoamylase from mesophile fungi is stable up to 50 ^{o}C (Miah & Ueda, 1977b). Glucoamylases from thermophilic fungi are more stable than those from mesophilic. They lost about 50 % of their activity at 70 ^{o}C after 5-6 hour

incubation (Taylor *et al.*, 1978; Basaveswara Rao *et al.*, 1981). They are more stable in the presence of substrates or substrate homologues.

Unlike α-amylase, glucoamylase do not need any metal ions to form active conformation. The presence of Ca^{++} and/or Ba^{++} ions decreases the enzyme activity. Certainly, in this case the heavy metal ions such as copper, zinc, mercury and lead also inhibit the enzyme activity (Li *et al.*, 1998; Odibo & Ulbrich-Hofmann, 2001).

Most glucoamylases are glycoproteins (Selvakumar *et al.*, 1996; Mishra & Maheshwari, 1996; Spinelli *et al.*, 1996). The carbohydrate contents vary form 2% up to 20% and generally contain glucose, glucosamine, mannose and galactose. The glucoamylase from *S. diastaticus* was reported to have very high carbohydrate content of about 80 % (Kleinman *et al.*, 1988b). Pazur and co-workers (1980) investigated the glycoprotein properties of glucoamylase from *A. niger* and reported that in this enzyme the carbohydrate compounds are present as 20 individual D-mannose units, 11 disaccharides with the structure D-mannopyranosyl-D-mannose, 8 trisaccharides and 5 tetrasaccharides composed in various combination of D-glucose, D-mannose and D-galactose joined by (1→3) and (1→6) glycosidic bonds. They also found that the carbohydrates are linked glycosidically to amino acid residues at *L*-threonine and *L*-serine. The carbohydrate moieties play an important role in the stability as the removal of carbohydrate moiety reduces the stability and activity of the enzyme (Pazur *et al.*, 1970). Such an arrangement of carbohydrate chains in glycoprotein is unusual and may account for some of the unique properties of glucoamylase (Pazur *et al.*, 1987). Also Pazur and co-workers (1987) showed that the carbohydrate side chains are distributed randomly along the polypeptide chain which might be responsible for its high resistance to proteolysis, remarkable stability against heat inactivation and during storage at cold temperature.

2.4.2 Multiplicity

The multiplicity of glucoamylase was reported first time by Ueda (1957). Since then many studies have been reported dealing with the multiplicity of glucoamylase (Table 5). The glucoamylase from *Aspergillus niger* that is widely applied in industries has generally two forms so called GA1 and GA2 (Svensson *et al.*, 1982). Four forms of glucoamylase are produced by *Aspergillus oryzae* (Morita *et al.*, 1968).

These forms have identical enzymatic and physicochemical properties except differences in their electrophoretic mobilities, sedimentation coefficients and pH stabilities. Miah and Ueda (1977b) reported that glucoamylase from *A. oryzae* have three forms having significant differences in enzymatic properties. Hayashida (1975) reported that medium composition had a great influence on formation of different forms of glucoamylase secreted by *A. awamori* var. *kawachi*. Saha and co-workers (1979) found that culture conditions have effects on the relative amount and characteristics of three forms of glucoamylase secreted by *A. oryzae*.

Multiple forms of glucoamylases may be formed by stepwise degradation of native glucoamylase with protease and glucosidases in the source (Hayashida *et al.*, 1976; Hayashida

& Yoshino, 1978). For examples GAII could be formed from GAI by acid protease and glucosidases in the case of *A. awamori* var. *kawachi* (Figure 8) (Yoshino & Hayashida, 1978). Protease might play a major role in modifying the enzyme protein molecule of *Rhizopus* sp. into three multiple forms. This formation mechanism was proved by Hayashida & Flor (1981) that only one form of glucoamylase was obtained from a protease-negative glycosidase-negative mutant of *Aspergillus awamori* var. *kawachi*.

Table 5 Multiplicity of glucoamylases

Sources	Number of forms	References
Aspergillus niger	2	Svensson *et al.*, 1982
	4	Selvakumar *et al.*, 1996
Aspergillus oryzae	3	Miah & Ueda, 1977b
Rhizopus sp.	3	Takahashi *et al.*, 1985
Rhizopus niveus	5	Saha & Ueda, 1983
Penicillium oxalicum	2	Yamasaki *et al.*, 1977a
Aspergillus candidus	2	Manjunath & Raghavendra Rao, 1979
Aspergillus cinnamoreus	2	Kurushima *et al.*, 1974
Aspergillus foetidus	2	Lineback & Baumann, 1970
Aspergillus saitoi	2	Takahashi *et al.*, 1981
Candida pellicusola	2	Kawamura & Sawai, 1968
Cladosporum resinae	2	McCleary & Anderson, 1980
Filobasidium capsuligenum	2	De Mot & Veraachtert, 1985
Thermomyces lanuginosa	1	Basaweswara Rao *et al.*, 1981
(Humicola lanuginose)	2	Taylor *et al.*, 1978
Monaseus kaoliang	2	Iizuka & Mineki, 1977
Piericularia oryzae	2	Yurki *et al.*, 1977
Saccharomyces diastaticus	2	Erratt & Stewart, 1980
Aspergillus awamori	1	Yamasaki *et al.*, 1977b

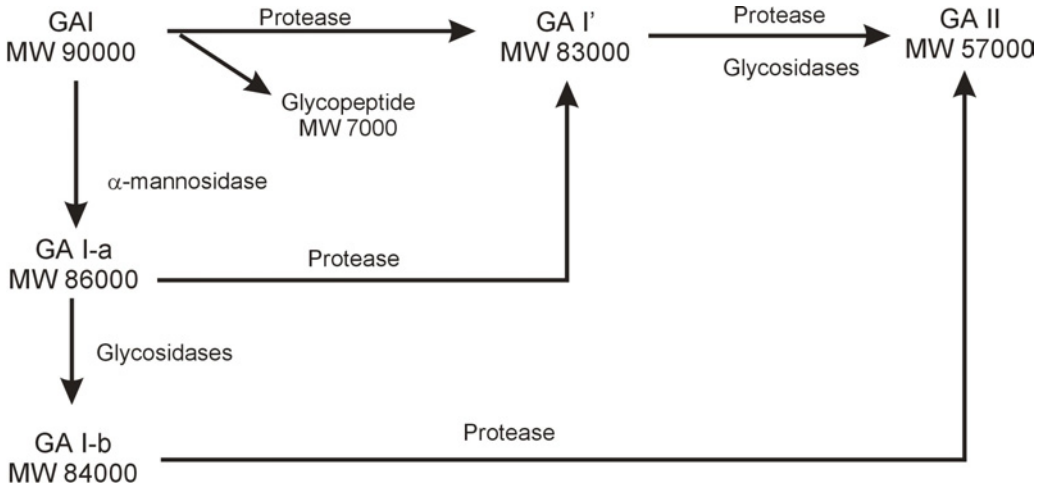


Figure 8 A proposed scheme for the formation of multiple forms of glucoamylase
(Yoshino & Hayashida, 1978)

Different forms of glucoamylases were also resulted from spliced mRNA (Boel *et al.*, 1984). Erratt and Stewart (1981) reported that *Saccharomyces diastaticus* has three unlinked genes STA1, STA2 and STA3 encoding extracellular glucoamylase isoenzymes GAI, GAII and GAIII, respectively. Pretorius and co-workers (1986; 1991) studied on identification and characterisation of yeast glucoamylase structural genes and they confirmed the presence of these three genes. They also identified the restriction endonyclease maps of STA1, STA2 and STA3.

2.4.3 Structure

Most glucoamylase enzymes contain about 600-700 carboxylic residues and can be divided into three important regions. First region containing the first 440-470 amino acid residues from N-terminal is the catalytic domain. The catalytic site includes the general acid/base catalysts Glu179 and Glu400 situated at the bottom of a pocket (Sierks *et al.*, 1990; Svenson *et al.*, 1990). Based on the structure of glucoamylase from *A. awamori* var X100 reported by Coutinho and Reilly (1997) as well as Aleshin and co-workers (1992), the catalytic domain contains 13 α-helices of which 12 form an $(\alpha/\alpha)_6$-barrel. In this fold, six outer and six inner α-helices surround the funnel-shaped active site, constituted by the six highly conserved α→α segment that connect the *N*-termini of the inner with the *C*-termini of the outer helices (Aleshin *et al.*, 1992). The catalytic domain of glucoamylase from *A. niger* completely agree with that one from *A. awamori*.

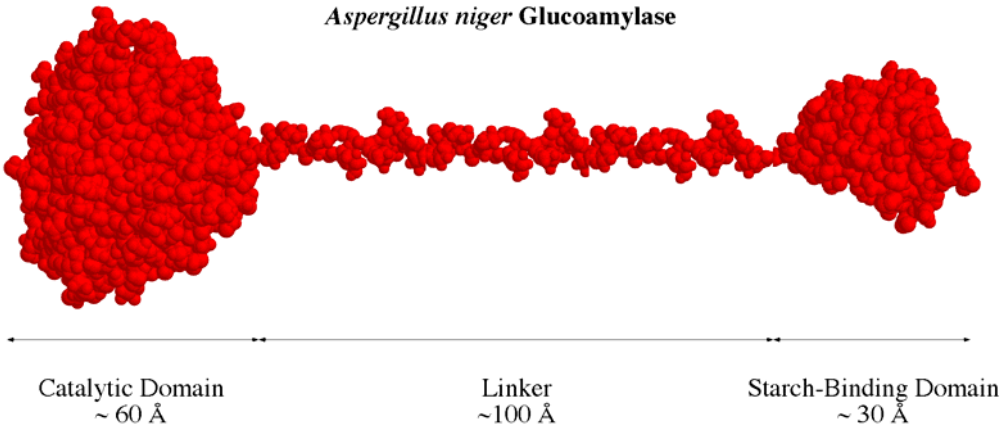


Figure 9 Structure of glucoamylase from *Aspergillus niger*
(http://www.public.iastate.edu/~pedro/glase/glase.html)

The second region (aa 440-508) is linker region (Figure 9) and plays important roles in stability, secretion and digestion of raw-starch (Goto *et al.*, 1999; Christesen & Nielsen, 1999). This region contains a very highly *O*-glycosylated C-terminal segment rich in serine and threonine

amino acid residues (Coutinho & Reilly, 1997; Svensson *et al.*, 1983). The first part of linker region from sequence 440 to 471 carries about 10 exposed single mannosyl residues (Aleshin *et al.*, 1992) which together with the two *N*-glycosidically linked units at Asp171 and Asp395 form a belt of carbohydrate around the globular catalytic domain (Aleshin *et al.*, 1992). The highly *O*-glycosylated part of linker (aa 472-508) has been speculated that this part surrounds the catalytic domain to place the starch-binding domain (SBD) with one of the two binding sites near the active site (Sauer *et al.*, 2000). And the third region is called starch-binding domain that consists of 8 β-strands organised in two β-sheets forming a twisted β-barrel structure (Sorimari *et al.*, 1996; Jacks *et al.*, 1995). Two starch-binding sites (Figure 10) are located on opposite sides of the top of the domain (Sorimari *et al.*, 1996; 1997). Earlier the starch-binding domain was shown to be required for degradation of raw starch by glucoamylase, the natural GAII form (1-512) without starch-binding domain having very low activity on raw starch (Svensson *et al.*, 1982). Now isolated the starch-binding domain acting on starch granules together with GAII showed synergistic effects on the degradation of the insoluble substrate, suggesting that starch-binding domain binds onto starch as an individual entity and disrupts the compact structure of starch granule facilitating the hydrolysis by the catalytic domain (Southall *et al.*, 1999).

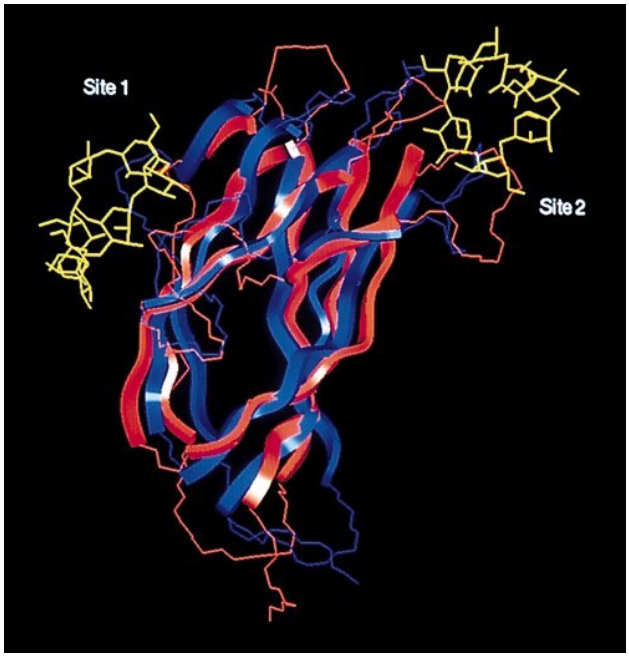


Figure 10 Substrate binding domain of glucoamylase from *Aspergillus niger* in complex state with β-cyclodextrin indicated by yellow ones (Sorimari *et al.*, 1997)

2.4.4 Catalytic mechanism

The widely accepted mechanism of hydrolysis of glucoamylase involves proton transfer to the glycosidic oxygen of the scissile bond from a general acid catalyst; formation of an oxocarbonium ion; and a nucleophilic attack of water assisted by a general base catalyst (Sinnot, 1990; Tanaka *et al.*, 1994). Also based on the classification proposed by Sinnott (1990) mentioned above, glucoamylase belongs to the class two, which releases the products with invert anomeric configuration.

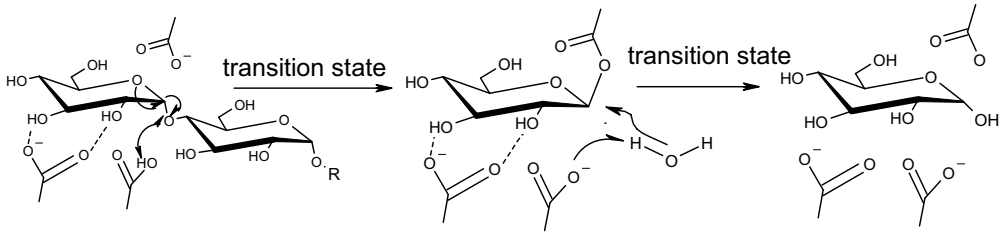


Figure 11 Double displacement mechanism and the formation of a covalent intermediate by which inverting glycosylhydrolase act

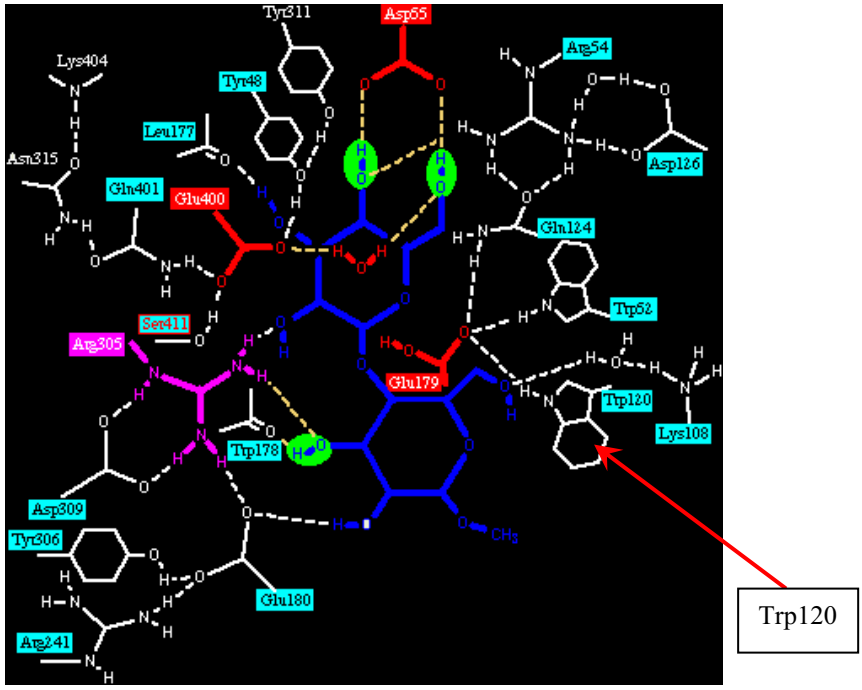


Figure 12 Glucoamylase and substrate complex model.
blue ones: substrate molecules, red ones: three catalytic residues
(http://www.punlic.iastate.edu/~reilly/glase/glase.html)

The glucoamylase acts on α(1→4)- and α(1→6)-glycosidic linkages and release β-glucose residues from non-reducing chain-ends. An enzyme nucleophile (Asp or Glu) attacks the anomeric carbon to generate a covalent intermediate (Figure 11). Glu179 and Glu400 (Figure 12 red ones) in case of glucoamylase from *A. niger* have been identified as the general acid and the general base catalyst, respectively, and pH-dependences of steady-state kinetic parameters are in accordance with a rate determining hydrolysis step involving these two catalytic residues (Sierks *et al.*, 1990). Sierks and co-workers (1990) investigated the catalytic mechanism of glucoamylase from *A. awamori* and they found that Asp176 and Glu179 were identified as base and acid, respectively, meanwhile Glu180 residue plays a role in the formation of covalent intermediate complex of substrate and protein. Investigating the substrate binding mechanism, Clarke & Svensson (1984) reported that the conserved Trp120 amino acid residue has an important role (Figure 12). This residue plays the same role like the Trp83 in Taka-amylase A mentioned above.

2.4.5 Aspects of protein engineering and biotechnological exploitation

Glucoamylase is an industrially extremely important enzyme, used in the enzymatic conversion of starch into high glucose and fructose corn syrups (Saha & Zeikus, 1989; Holló & Hoschke, 1993). Recently, the disadvantages of the technology applied in industry are that the saccharification takes too long time (48-72 h) and this is batch technology. Glucoamylases from most sources are unstable at temperatures higher than 60°C that is the temperature applied in industrial saccharification processes. Also, most glucoamylases are inhibited by Ca^{++} ion needed in liquefaction by α-amylase. Development of thermostable and/or Ca^{++} tolerant glucoamylase, capable of performing industrial saccharification at elevated temperatures, would thus be significant importance to the starch processing industry. Small achievements towards thermostable glucoamylase were fulfilled through protein engineering of the enzymes from *A. niger* and *A. awamori* (Ford, 1999). Several approaches, such as replacement of glycines in α-helices (Chen *et al.*, 1996a), elimination of fragile Asp-X bonds (Chen *et al.*, 1995) and substitution of asparagines in Asn-Gly sequences (Chen *et al.*, 1994) have been attempted using site-directed mutagenesis. The most successful strategy applied seems to be engineering of additional disulphide bonds into the enzyme molecule. With the application of this technique the operation temperature of glucoamylase can increase by about 4°C per bond (Fierobe *et al.*, 1996; Allen *et al.*, 1998).

Alternately, screening of thermophilic bacteria and subsequent cloning and expression of the target gene could result a thermostable glucoamylase. Some fungi have been reported to be able to produce thermostable glucoamylase. They are *Humicola grisea* var. *thermoidea* (Tosi *et al.*, 1993), *Aspergillus fumigatus* (da Silva & Peralta, 1998) and *Thermomyces lanuginosus* (Basaveswara Rao *et al.*, 1981; Jensen & Olsen, 1992; Mishra & Maheshwari, 1996). A common molecular feature for the enzymes from thermophilic fungi seems to be the lack of

helices 9, 10 and 11 of the $(\alpha/\alpha)_6$-barrel of catalytic domain (Ducki *et al.*, 1998). Information on these and forthcoming sequences of thermostable glucoamylases may guide future rational protein engineering towards a glucoamylase that exhibits activity and stability at elevated temperatures and/or Ca^{++} and other ions tolerance.

The replacement of soluble glucoamylase by immobilised enzyme may allow a more efficient process in which the enzyme can be readily re-used (Solomon, 1978). On other hand the glucose repression or/and reserve reaction of glucoamylase may be decreased by immobilisation. This opens the future to reach a high degree of conversion. In the last few decades numerous studies were published dealing with the immobilisation of glucoamylase by covalent binding or absorption. The first attempt to obtain immobilised glucoamylase was carried out by Wilson and Lilly in 1969 by binding enzyme to DEAE-cellulose using 2-amino-4,6-dichloro-S-triazine functional agent. Other groups (Maeda & Suzuki, 1970) also tried the immobilisation with inert carriers such as cellulose, nylon and glass and carboxymethyl-cellulose-glucoamylase complex. Gel entrapment methods to immobilised glucoamylase were published by several authors (Gruesback & Rase, 1974; Maeda *et al.*, 1974). The properties of the immobilised enzyme preparation have been described and their possible application in the laboratory and on industry scale, but unfortunately, no large scale commercial use has been reported. So far none of the immobilised glucoamylase preparation published can work at higher than industrially required temperature (60^oC). The thermostable glucoamylase enzyme which after immobilisation is capable to meet this demand of dextrose manufacturers. Further research on the fundamental aspects of the reaction mechanism catalysed by immobilised glucoamylase may generate new conceptions that will finally provide the basic information required for the production of crystalline dextrose. It should be mentioned that in the last decade, the intensity of research dealing with immobilised glucoamylase decreased, because of the dropping price of soluble glucoamylase in the world enzyme market.

2.5 Transfructosylases

2.5.1 Nomenclature

In the case of plants, many authors (van den Ende & van Laere, 1996; Luscher *et al.*, 1996; Simmen *et al.*, 1993) clearly reported that different enzymes are responsible for hydrolytic and transfructosylating activities. However, the nomenclature of fructooligosaccharides-producing enzymes from micro-organisms remains still in dispute. Some authors use the term of fructosyltransferases (Lee *et al.*, 1992; L'Hocine *et al.*, 2000; Yun *et al.*, 1997), whereas others designated it as β-fructofuranosidase (invertase) (Chiang *et al.*, 1997; Chen & Liu, 1996; Chang *et al.*, 1994). Andersen (1967) applied yeast invertase to synthesise fructooligosaccharides from fructose and the formation of difructoside was detected. Thus, it can be concluded that yeast invertase is able to synthesise fructooligosaccharides. So far, only L'Hocine and co-workers (2000) reported that they successfully separated two enzymes, a fructosyltransferase and β-

fructofuranosidase, from *A. niger* AS0023. The fructosyltransferase catalysed exclusively fructosyltransfer reaction, while β-fructofuranosidase did not show any transfer activity. Generally, the accepted opinion is that invertase has very low transfructosylating activity, even at high sucrose concentration, while the fructosyltransferase have much higher transfructosylating activity. Additionally, the affinity of fructosyltransferase to sucrose or shorter fructooligosaccharides as acceptors of fructosyl residues is much higher than water (Park & Almeida, 1991; Duan *et al.*, 1993; Chen & Liu, 1996; Chiang *et al.*, 1997). Thus, it is difficult to differentiate them consequently, but it cannot be excluded that one enzyme may possess both activities. In this study it is suggested by the author that the enzymes that are able to catalyse the transfer of fructosyl residue to synthesise fructopyranoside are to be classified in the class of transfructosylases. According to this classification both fructosyltransferase (EC 2.4.1.9) and β-fructofuranosidase (EC 3.2.1.26) belong to the transfructosylase class.

2.5.2 Occurrence and some properties

Transfructosylases (as classified above) fructosyltransferase and β-fructofuranosidase known as invertase occur in many higher plant such as asparagus (Shiomi 1982), chicory (van den Ende & van Laere, 1996), onion (Shiomi *et al.*, 1997), Jerusalem artichoke (Koops & Jonker, 1994; Luscher *et al.*, 1996) and grasses (St. John *et al.*, 1997a). These enzymes are also secreted by micro-organisms (Table 6).

Table 6 Physicochemical properties of microbial transfructosylases

Sources	Molecular mass [kDa]	Optimum pH	Optimum temperature [^{o}C]	References
Arthrobacter sp	-	6.0	40	Fujita *et al.*, 1990
Aspergillus japonicus	236	5.4	60	Duan *et al.*, 1993
Aspergillus foetidus	-	-	-	Rehm *et al.*, 1998
Aspergillus nidulans	98-185	-	-	Chen *et al.*, 1996b
Aspergillus niger	300 8-168	4.4	55	Hirayama *et al.*, 1989 L'Hocine *et al.*, 2000
Aspergillus oryzae	85-90	4.5	-	Kida *et al.*, 1988
Aspergillus phoenicis	-	8.0	55	van Balken *et al.*, 1991
Aspergillus sydowi	50	5.2	55	Muramatsu & Nakakuki, 1995
Aureobasidium pullulans	300-346	5.2	55	Yun *et al.*, 1990
Aureobasidium sp.	190	-		Hayashi *et al.*, 1990
Bacillus macerans	66	5.0	50	Park *et al.*, 2001
Bacillus stearothermophilus	600	6.5	65	Belamri *et al.*, 1994
Bacillus subtilis	-	-	-	Euzenat *et al.*, 1997
Bifidobacterium adolescentis	74	6.1	-	Muramatsu *et al.*, 1993
Fusarium oxysporum	-	4.0-5.5	55-60	Patel *et al.*, 1994; Kaur et al., 1992
Pycnoporus sanguineus	84	-	-	Quiroga *et al.*, 1995
Scopulariopsis brevicaulis	110	6-9	40	Hatakeyama *et al.*, 1996
Saccharromyces cerevisiae	-			Straathof *et al.*, 1986

The molecular masses of β-fructofuranosidase and fructosyltransferase from micro-organisms are generally bigger than those from plants. Plant transfructosylases have weight about

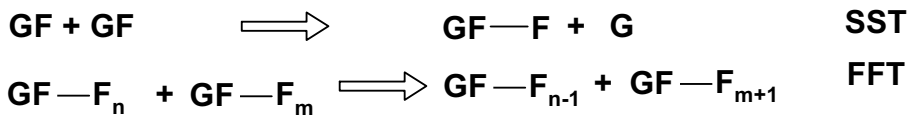
60-100 kDa with two subunits. The bigger subunit has molecular mass about 50-60kDa and smaller one about 20 -30 kDa (Koops & Jonker, 1994; St. John *et al.*, 1997a; b; Luscher *et al.*, 2000). The enzymes originating from microorganisms are homopolymers with at least two monomers. The molecular mass of one monomer is in the range from 40 to 200 kDa (Duan *et al.*, 1993; Rubio & Maldonado, 1995). In the native form, the molecular mass of them vary from 90 up to 600 kDa (Table 6).

Various reports have placed the optimum of pH and temperature for activity of tranfructosylases (β-fructofuranosidase and fructosyltransferase) between 5-6.5 and 50-60 ^{o}C, respectively (Park *et al*., 2001). These enzymes accept Ca^{++}, Mg^{++}, Co^{++} and Li^{+} ions as co-factors (L'Hocine *et al.*, 2000). Glucose well known as by-product competitively inhibits the enzyme transfer reaction (Yun *et al*., 1994). This causes the maximum yield of industrial production of fructooligosaccharides by *A. niger* to reach only about 60 % (w/w) based on total sugars. To overcome the glucose repression of transfer reaction and improve the yield of FOS production a mixed enzyme system of fructosyltransferase and glucose oxidase was proposed (Jung *et al.*, 1993; Yun *et al.*, 1994). Metal ions such as Hg^{++}, Pb^{++}, Cu^{++}, Ag^{+}, Zn^{++} and Fe^{++} are known to act on the transfrutosylases as competitive inhibitors (Lee *et al.*, 1992; Muramatsu & Nakakuki*,* 1995; Hatakeyama *et al.,* 1996). Sulfhydryl reagents such as *N*-bromosuccinimide and p-chloromercuribenzoate do not only cause the inhibition of microbial transfructosylases, but these cause the inactivation of enzymes too (Hayashi *et al.*, 1991a; Hayashi *et al.,* 1994; Rubio & Maldonado, 1995). These results indicate that some thiol groups are probably located at or near the active site. So far, in protein data bank, some sequences of invertase from micro-organisms are published, but no three dimensional structure of transfructosylase is known yet.

The transfructosylases from both micro-organisms and plants are glycoproteins with *N*-glycolysation. The carbohydrate content varies from 5 % up to 45 % (Patel *et al.*, 1997). The glycosyl part of enzyme is formed mainly by mannose, glucose galactose and *N*-acetylglucosamine (Wallis *et al.*, 1997). Deglycolysation of enzyme decreases the stability, but has little effects on the enzyme activity and specificity (Hayashi *et al.*, 1994).

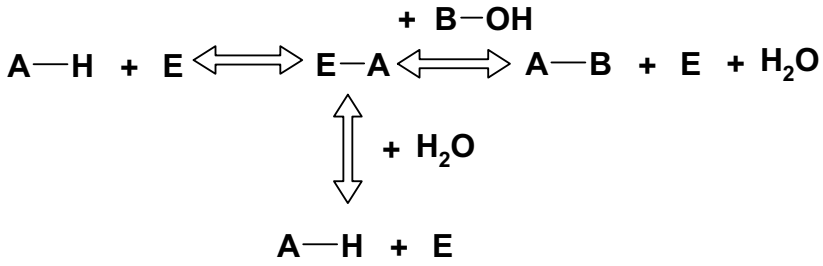
2.5.3 Catalytic mechanism

The reaction mechanism of transfructosylases depends on the source of the enzyme. In plants and some micro-organisms, a series of enzyme act together where as a single enzyme works in most other micro-organisms (Yun, 1996). For example, fructosan metabolism in Jerusalem artichoke (*H. tuberous*) is established by two enzyme: sucrose:sucrose 1-fructosyltransferase (SST) and β(2→1)fructan: β(2→1)fructan 1-fructosyltransferase (FFT). SST converts sucrose into glucose and an oligofructoside but is unable to promote polymerisation above the trisaccharide level; further higher polymers are consecutively synthesised by FFT. Arnold (1965) suggested the description of this mechanism as following:



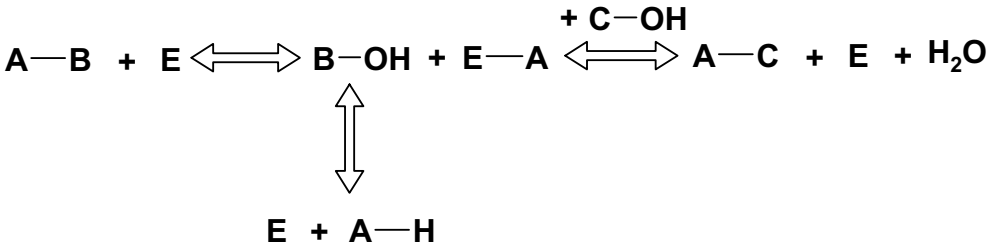
where GF is a sucrose (glucose-fructose) and n, m are the number of extra fructose residues.

In the case of single enzyme the catalytic mechanism varies depending on enzyme. β-fructofuranosidase (invertase, EC 3.2.1.26) does not only catalyse the hydrolysis of sucrose to glucose and fructose, but depending on its origin and reaction conditions, this enzyme is able to exhibit significant transfructosylating activity. Two possible paths could describe the catalytic mechanism of this type of enzyme: reverse hydrolysis and transfructosylation (Antosova & Polakovic, 2001).
The reverse hydrolysis is an equilibrium process in which the reaction equilibrium is shifted from the hydrolysis towards the synthesis of oligosaccharides according to the following scheme:



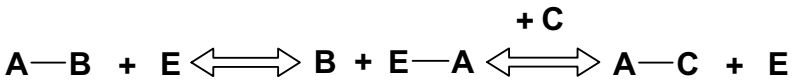
where A is the fructosyl group of donor, B is the glycosyl group of acceptor (sucrose, and fructooligosaccharides), E is the β-fructofuranosidase enzyme, A-B is the new product (fructooligosaccharides with one fructose residue larger than the acceptor molecule).

In the first stage of the reaction, the complex of enzyme-donor is formed and activated. The acceptors are water (hydrolysis), sucrose or oligosaccharide (reverse hydrolysis). The complex reacts with the hydroxyl group of the acceptor to form products (fructooligosaccharides/fructose and glucose). Perhaps, the amount of oligosaccharide depends on the equilibrium constants of the two transformation reactions of the E-A complex to free enzyme and fructose, or oligosaccharides. In this case of increased concentration of substrate (sucrose) lead to increased yields of oligosaccharides.
In the case of transglycosylation, a fructosyl group is transferred from an activated donor to the acceptor. Like the mechanism mentioned above, the enzyme-donor complex is formed in the first stage of the reaction.



where A-B is the donor of fructosyl group, B-OH is glucose, C-OH the acceptor, E is enzyme, A-C is the product.

Transglycosylation is usually controlled process and the product A-C is also a potential acceptor (substrate) for the next reaction. Therefore, the yield of FOS depends on the rate of synthesis and hydrolysis reactions. Hirayama and co-workers (1989) observed that the amount of total FOS rose at the beginning of conversion process and then dropped.
In the case of fructosyltransferase (EC 2.4.1.9), the catalytic mechanism is more simple, because this enzyme is unable or have very little activity to hydrolyse fructose residue from fructooligosaccharide. The catalytic mechanism is described as following:



where A-B is the donor, E is the enzyme, C the acceptor and A-C is the product.
The enzyme catalyses the transfer of fructosyl group from donor molecule to acceptor (sucrose or fructooligosaccharide) to synthesise fructooligosaccharides with higher fructose residues.

2.5.4 Biotechnological exploitation

Transfructosylases as classified above are usually accumulated intracellularly. This is one of the drawbacks that cause the increase of production cost when exploitation of these types of enzymes on industrial scale. To reduce the production cost, some approaches can be applied.
The first approach could be the immobilisation technique, because immobilised enzymes have long self-life. Moreover, the substrates and products of transfructosylases catalysed reactions are small molecules (sucrose, 1-kestose, nystose etc.) which are not subject to great diffusion limitations when the enzyme is in immobilised form. Applying immobilised biocatalyst continuous technologies can be elaborated. Some studies are published dealing with immobilisation of β-fructofuranosidase (Kida *et al.*, 1988; Cantarella *et al.*, 1993; Hayashi *et al.*, 1991a; b; 1994). Cantarella and co-workers (1993) reported that the β-fructofuranosidase can be immobilised using poly-HEMA hydrogels, meanwhile Hayashi and co-workers (1993) tried it with porous silica. In both cases the immobilised enzyme was more stable and active than the free enzyme. In 1994, also Hayashi and co-workers successfully immobilised enzyme using

alginate gel with very high efficiency (92 %). The immobilised β-fructofuranosidase had extremely long half-life (275 days) and worked at high sucrose concentration (500 mg/ml). This technology was applied in commercial fructooligosaccharides production (Hayashi *et al.*, 1994). The second approach should be immobilisation of cells. The first experiment dealing with usage intact mycelia of *Aspergillus phoenicis* to produce 1-kestose was published by van Balken and co-workers in 1991. The immobilisation of mycelia of *Aspergillus japonicus* was carried out by Chen and co-workers (1996c). In this study they reported that after immobilisation of mycelia with calcium alginates, the mycelium-bound β-fructofuranosidase lost only 17 % of its activity after 35 days. They also reported that the enzyme was active in a wider pH range, and had improved thermostability, but did not increase the total amount of fructooligosaccharides produced by immobilised bioreactors.

It seems that there is now little scope for advances in the basic concepts involved in immobilisation of transfructosylases, but there is possibility for improvements in the performance of immobilised transfructosylase preparations, even immobilised cell preparations. Manufacturing of fructooligosaccharides (FOS) require simple, cheap, effective and durable plant which will be used as crude a substrate as possible. The reactors containing immobilised enzyme/cells may be smaller and therefore cheaper to buy and to run as well as easily to control. They usually provide the required amount of products. Presumably purified preparations of the enzyme would give extremely high activities or operate at specific environments when immobilised.

One approach that is worth considering is the use of a suitable inducer. Gupta and co-workers (1994) reported that inulin was a better inducer for production of β-fructofuranosidase than sucrose. In their study, they also mentioned that when using inulin as inducer some *A. niger* and *Kluyveromyces fragilis* secreted acid invertase extracellularly. Inulin is a fructan with about 15 to 70 residues of fructose and big enough to to be transferred through cell membranes. To utilise these type of substrates micro-organisms have to secrete the extracellular enzymes. More study is needed to clarify the physicochemical and catalytic properties of the new type of enzyme.

2.6 The *Aspergillus* genus

Aspergillus niger is generally regarded as non-pathogenic fungus that is widely distributed in nature and became an industrially exploited organism since 1919 (Schuter *et al.*, 2002). Humans are exposed to its spores every day without disease becoming apparent. Taxonomically the genus *Aspergillus* can be divided into two groups based on the colour of the conidiospores (Raper & Fennel, 1965). *Aspergilli* with brown to black shaded spores constitute the *A. niger* group. Although the members of this group vary considerably, only few differ clearly enough from the majority that they can be classified as separate species (e.g. *A. carbonarius*, *A. japonicus*, *A. ellipticus*, *A. heteromorphus* and *A. aculeatus*). Most of the brown to black *Aspergilli* belong to the other group, within which the species are difficult to distinguish such as

A. ficuum, *A. phoenicis*, *A. niger* and *A. awamori*. In practice, this group of species is often called *A. niger* van Tieghem (Shuster *et al.*, 2002). The most recent supraspecific scheme for the genus *Aspergillus* was suggested by Gams and co-workers (1985). They placed all species with dark-brown to black-shaded conidia into the section *Nigri* of a proposed subgenus *Circumdati*. Their proposal has been accepted by the International Commission on *Penicillium* and *Aspergillus* (Samson, 1992).

Table 7 Industrial enzymes and organic acids produced by filamentous fungi

Product	Organism
α-Amylase	*A. niger, A. oryzae*
Cellulase	*Humicola insolens, Penicillium funicolosum, Trichoderma viride*
Glucoamylase	*A. niger, A. awamori, A. phoenicis, Rhizopus delemar, R. niveus*
Glucose oxidase	*A. niger*
Invertase	*A. niger, A. oryzae*
Laccase	*Coriolus versicolor*
Pectinase	*A. niger, A. oryzae, Humicola insolens*
Proteinase	*A. niger, A. melleus, R. delemar*
Rennin (microbial)	*Mucor miehei, M. pusillus*
Citric acid	*A. niger*
Itaconic acid	*A. terreus*

The first food product that was produced by fermentation with *A. niger* was citric acid that is originally isolated from citrus fruits. The development of a commercial process by Pfizer, in Brooklyn, New York, USA (Bennett, 1998) soon made citric acid widespread in various industries including the food and beverage industries, and greatly exceeds other metabolites such as gluconic acid (Rouskas, 2000). Additionally, the common uses of citric acid include tablets, cosmetics, detergents, antifoaming agentsand textile treatment as well as a preservative in blood preparations. Recently, citric acid is produced almost exclusively by fermentation applying *A. niger* and *A. wentii*, because the yields of these organisms are economic and formation of undesired side products is minimal (Shuster *et al.*, 2002). The Food and Drug Administration (FDA) has listed *A. niger* as a source of citric acid (21 Code of Federal Regulations §173.280). Now, as we know, *A. niger* not only have industrial importance in production of citric acid, but these organism is a rich source of enzymes, too. It is able to produce enzymes in considerable quantity (in the order of ten gram per litre under appropriate conditions). The enzymes involved in the degradation of plant cell wall materials (cellulases, hemicellulases, pectinases, glycosidases, phytases), starch (α-amylase, glucoamylase), lipids (lipases) and proteins (proteases, pectinases) as well as the oxidation of phenolic compounds (laccases), are produced commercially by *Aspergillus* species (Table 7). The FDA has accepted these enzymes for use in foodstuff by issuing the recognising opinion letters in the early 1960's. They declared that these enzymes can be "generally recognized as safe" (GRAS) under the condition when non-pathogenic and non-toxigenic strains of *A. niger* and current good manufacturing practices (GMP) are used in their production. Godfrey and Reichelt (1983)

claimed GRAS status for β-galactosidase and protease from *A. niger*. Carbohydrolases and cellulases from *A. niger* are also approved as a secondary food additive by the FDA (21 Code of Federal Regulations §173.120). As mentioned above, the functional food has come to the front during the last decade in human nutrition. Some non-digestible oligosaccharides (fructooligosaccharides and galactooligosaccharides) are well known as prebiotics. Industrial production of these oligosaccharides has been carried out also by using transfructosylation and transgalactosylation with enzymes from *A. niger* strains. FOS and GOS are allowed to use as foodstuffs in Japan. In the United States of America FOS produced by β-fructofuranosidase from *A. niger* was accepted given GRAS status. European countries, now, also use them as food additives in dairy products (Yun, 1996).

2.7 Thermophilic fungi

Whether temperature is the most important variable in the environment of living things somebody may debate, but no one will doubt its cardinal role that temperature plays in the vital processes of all living things. In all warm-blooded animals (homoiotherms) a highly complicated mechanism is involved for the control of body temperature that operates within amazingly narrow limits. In the great assemblage of poikilothermic or heterothermic organism, relatively few can be found thriving at temperatures below 10°C or above 40°C. Those that grow and multiply vigorously outside this range may be called psychrophiles if they occur at low temperatures or thermophiles if they thrive at high temperatures.

Thermophily – literally, a heat lover – is a characteristic that appears among widely different groups of organisms. Crustacea, molluscs and insects are reported at temperatures up to 50°C and nematodes too may occur at temperatures as high as 50°C or even higher. Thermophilic bacteria such as *Bacillus* and *Thermus* species have also been the subjects of intensive research because of their great importance in many natural microbial processes. They are found growing where temperatures range up to 85°C or possibly slightly higher. These organisms are easy to maintain and grow on both small and large scale (Kristjansson, 1989). The enzymes from such bacteria are generally stable and active up to at least 80 ^{o}C and often up to 100 ^{o}C (Wart & Moo-Young, 1988). These enzymes also have the advantage over conventional proteins that refrigeration is often not needed. Besides algae, bacteria and *Actinomycetes* there is another group of quite different micro-organisms that are thermophile though to a less spectacular degree. They belong to the true fungi – *Eumycetes* or *Eumycota* – with temperature maximum between 50°C and 60°C. Based on classification suggested by Brock (1978), and Weigel and Ljungdahl (1986) this group can be classified as thermophilic fungi. Of course the term of "thermophilic" organisms is not very precise and its meaning depends on the group of organisms that are being considered. Here I will only use the term of "thermophilic" dealing with fungi. The thermophilic fungi are now defined as those that have a growth temperature minimum at or above 20°C and a growth temperature maximum at or below 50°C, and the thermotolerant forms have temperature ranges of growth from below 20°C to approximately

55°C. Thermophily in fungi is not as extreme as in eubacteria or archaea, some species of which are able to grow near or above 100°C in thermal springs, solfatara fields, or hydrothermal vents (Blöchl *et al.*, 1997; Brock, 1995).

Thermophilic fungi are to be found in nature where organic materials were decomposing at elevated temperatures that usually resulted from microbial thermogenesis, but sometimes depended upon isolation or the presence of warm-blooded animals. Accordingly, damp hay, leaf mould and other composting materials, straw and dung of several herbivorous animals were the first materials collected for study. Soil samples from several widely separated localities, birds nets and wood-rats nests were also investigated. The true thermophilic fungus was described firstly by Tsiklinskaya (1899), but until now very few species (about 30 species of 50000 recorded fungal species) are listed as thermophilic or thermotolenrant fungi (Table 8). Perhaps because of their moderate degree of thermophily and because their habitats are not exotic, thermophilic fungi have not received much publicity and attention (Maheshwari *et al.*, 2000).

The thermophilic fungus, *Thermomyces lanuginosus* has been reported to produce high levels of extracellular thermostable, cellulase free xylanase with broad pH optimum, when grown on cheap carbon source such as corn cob (Gomes *et al.*, 1993). This fungus is also reported to produce extracellular thermostable amylolytic enzymes (α-amylase and glucoamylase) when cultured on starch-based medium (Baraweswara Rao *et al.*, 1981; Mishra & Maheshwari, 1996).

Historically, the first known isolate of *Humicola lanuginosa* was made in 1899 by Tsiklinskaya, who observed the fungus on a potato which had been inoculated with garden soil. Tsiklinskaya grew the organism on white bread kept at 52-53°C and it was she who first described the fungus, to which she gave the name *Thermomyces lanuginosus*. A few years later Miehe (1907) reported the species to be common on leaves obtained from warm compost piles, which were placed in covered dishes and held at about 50°C. Miehe, who included a more comprehensive description of the organism and assumed his isolate was identical to the species isolated by Tsiklinskaya. Griffon and Maublanc (after Bhat, 1995) later isolated this fungus from moist oats incubated at 50°C. Although they recognised the similarity of their isolate to that of Miehe, they placed their organism in the genus *Sepedonium* as the species *S. lanuginosum*, since *Thermomyces lanuginosus* according to them, was incompletely described earlier by Tsiklinskaya. Velich (after Bhat, 1995) succeeded in isolating two thermophilic molds to which he applied the names *Sepedonium thermaphilum cyclosporum* and *S. thermaphilum ovosporum*. The former organism according to Velich is identical with the thermophilic mold *Thermomyces lanuginosus*, which was isolated by Tsiklinskaya and which was also found by Miehe. At a later date, Rege (after Bhat, 1995) isolated a species of *Acremoniella* from decomposing wheat straw (at 50°C) treated with assimilable compounds of nitrogen. Rege`s description leaves little doubt that his isolate was the same organism studied by Tsiklinskaya, Miehe and others. Shortly thereafter, Curzi (after Bhat, 1995) obtained from moist grain a thermophilic organism which he named *Acremoniella thermophila*. This species has subsequently been regarded as the same organism studied by the previously named investigators. Other, more recent workers have also

reported the presence of *Humicola lanuginosa*, particularly in composting material. These later authors uniformly use the original name *Thermomyces lanuginosus* as proposed by Tsiklinskaya, for their isolates. Reese (after Bhat, 1995) reported the organism is his study of the decomposition of cellulosic material, and La Touche (after Bhat., 1995) noted the species on incubating straw at temperatures of 40-50°C. More recently Henssen (after Bhat, 1995) observed and accurately described the organism from stable manure. And Crisan (after Bhat, 1995) succeeded in isolating it from composting plant material. From the 1990s most studies used *Thermomyces lanuginosus* name instead of *Humicola lanuginosa* synonym.

Thermomyces lanuginosus species was easily established and developed rapidly on most standard media, and little or no cultural variation was observed when colonies on different media were compared. The typical morphology of *T. lanuginosus* was demonstrated in Figure 13 when pure culture material growing on YpSs agar (Cooney & Emerson, 1964) at 45°C.

Thermophilic fungi are chief components of the microflora that develops in heaped masses of plant material, piles of agricultural and forestry products, and other accumulations of organic matter wherein the warm, humid, and aerobic environment provides the basic conditions for their development (Miehe, 1907; Allen & Emerson, 1949). Moreover, this group of fungi provides scientists with valuable experimental material for investigations of the mechanisms, which although allowing their growth at moderately high temperatures, limit it beyond 60 to 62 ^{o}C.

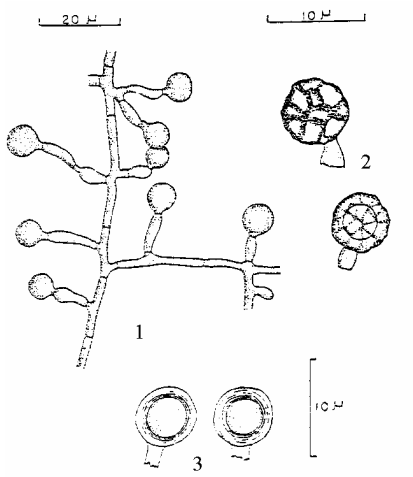


Figure 13 Morphology of *Thermomyces lanuginosus* (Bhat, 1995)
1 Hyphae bearing typical lateral aleuriophores and aleuriospores; **2** Mature aleuriospores, showing attached pedicel and reticulate sculpting; **3** Mature aleuriospores in optical sestion

Table 8 Taxonomic status and cardinal temperatures of thermophilic and thermotolenrant fungi (Maheshwari *et al.*, 2000)

Fungus (present nomenclature)	**Other name(s)**	**T_{opt} (^oC)**	**T_{max} (^oC)**
Canariomyces thermophila Guarro & Samson		45	
Chaetomium mesopotamicum Abdullah & Zora		45	52
Chaetomium thermophile La Touche	*C. thermophilum*, *C. thermophilium*	45-55	58-61
Coonemeria aegyptiaca (Ueda & Udagawa) Mouchacca	*Themoascus aegyptiacus*, *Paecilomyces aegyptica*	40	55
Coonemeria crustacean (Apinis & Chesters) Mouchacca	*Thermoascus crustaceus, Dactylomyces crutaceus, Paecilomyces crustaceus*	40	<60
Coonomeria verrucosa (Yaguchi, Someya et Udagawa) Mouchacca	*Theromoascus crustaceus*	30-40	55
Corynascus thermophilus (Fergus & Sinden) van Klopotek	*Thielavia thermophila, Myceliophthora fergusii, Chrysosporium fergusii*	50	60
Dactylomyces thermophilus Soop	*Thermoascus thermophilus, Thermoascus aurantiacus* (misapplied name)	40-45	
Malbranchea cinnamomea (Libert) van Oorschot & de Hoog	*Trichothecium cinnamomeum, Thermoidium sulfureum, Malbrenchea pulchella* var. *sulfurea*	45	57
Melanocarpus albomyces (Cooney & Emerson) von Arx	*Myriococcum albomyces, Thielavia albomyces*	45	57
Melanocarpus thermophilus (Abdullah & Al-Bader) Guarro, Abdullah& Al-Bader	*Thielavia minuta* var. *thermophila*	35	50
Myceliophthora hinnulea Awao & Udagawa		40-45	>50
Myceliophthora thermophila (Apinis) van Oorschot	*Sporotrichum thermophilum/thermophile, Chrysosporium thermophilum, Myceliophthora indica, Corynascus heterothallicus*	45-50	55
Myciococcum thermophilum (Fergus) van der Aa		45	53
Paecilomyces varioti Bainier		50	55
Rhizomucor miehei (Cooney & Emerson) Schipper	*Mucor miehei*	35-45	57
Rhizomucor pusillus (Lindt) Schipper	*Mucor pusillus*	35-45	55
Scytalidium thermophilum (Cooney & Emerson) Austwick	*Torula thermophila, Humicola grisea* var. *thermoidea, Humicola insolens*	40	58
Stilbella thermophila Fergus		35-50	55
Talaromyces byssochlamydioides Stolk & Samson	*Paecilomyces byssochlamydioides*	40-45	>50
Talaromyces emersonii	*Geosmithia emersonii; Talaromyces duponti; Penicillium duponti* (misapplied names)	40-45	55
Talaromyces thermophilus	*Penicillium duponti*	45-50	60
Thermoascus aurantiacus	*Thermoascus aurantiacus* sensu Cooney & Emerson (misapplied name)	49-52	61
Thermomyces ibadanensis Alpinis & Eggins		42-47	61
***Thermomyces lanuginosus* Tsiklinskaya**	***Humicola lanuginosa***	**45-50**	**60**
Thermomyces stellatus (Bunce) Apinis	*Humicola stellata*	40	50
Thielavia australienses Tansey & Jack		35-40	50
Thielavia pingtungia Chen KY & Chen ZC		40	>50
Thielavia terrestris (Apinis) Malloch & Cain	*Allescheria terrestris; Acremonium alabamensis*	40-45	52

T_{opt}: optimal temperature; T_{max}: maximum temperature

2.8 Filamentous fungi as cell factories

Humans in very early time have recognised and used micro-organisms for their own benefit. Egyptians and Chinese have used yeast for beer and wine making for several centuries. Since then the humans in the world have developed technology to apply micro-organisms in bread and cheese making as well as manufacture of alcoholic beverages. But people started to speak about biotechnology at the beginning of the twentieth century with respect to industrial processes involving living organisms (Punt *et al.*, 2002) such as yeasts, fungi, bacilli and lactic acid bacteria. Major goals are enhanced production of ingredients and improved properties of starter cultures, increased flavour formation and proteolytic characteristics or better autolytic properties (Kuipers, 1999). The aims of food biotechnology are not only directed towards improving food production, but are also defined by consumer demands for safe, natural, fresh, tasteful and convenient products (Richardson, 1996). Since then many research groups and programmes are officially funding to exploit the potential and possibilities of application of micro-organisms in human nutrition and animal feeding. Further research revealed that fungi, including both true filamentous fungi and yeasts, have a very important role in many processes. Now, the "mycotechnology" took all-worthy place in biotechnology. Thirty five enzymes of 51 listed by the Association of Manufacturers of Fermentation Enzyme Products (AMFEP, http://www.amfep.org) are produced by filamentous fungi.

Filamentous fungi are widely exploited as cell factories in the food and beverage industries worldwide. Numerous foodstuffs including beer, bread, various dairy-origin products such as cheese, yoghurt, saké, jiu, soy sauce, tempé and miso (Sakaguchi *et al.*, 1992) have been produced by using enzyme activities. In the case of fermentation-derived foods the source of these activities is microbial culture metabolism (MacCabe *et al.*, 2002). The production of enzymatic activities by fungal cultures was commenced in the latter part of the 19th century. After patenting the first enzymatic catalyst namely Taka Diastase in 1894, Takamine (1914) reported production of amylolytic enzymes by *Aspergillus oryzae*. Since then numerous studies and patents have been listed about productions of commercial enzymes by filamentous fungi (Table 7) such as *Aspergillus* spp. that became important micro-organisms.

From the beginning, surface cultures were applied to produce pectinase, protease and glucoamylase using *Aspergillus niger*. This technology evolved slowly, because the specific volume of bioreactor needed was very high. But companies used the surface culture processes up until the mid 1960's (Barbesgaard *et al.*, 1992). The milestone of production of enzymes by submerged culture was put as early as 1932 by Kluyver's group (Kluyver & Perquin, 1932) and from 1950 the production technology for fungal products gradually changed from surface culture to stirred-tank processes (Barbesgaard *et al.*, 1992). The majority of microbial enzymes produced today on a commercial scale are extracellular (Lambert, 1983) using the submerged fermentation technology. These tend to be hydrolases which are secreted by the micro-organism in order to make substrates available to the cell by hydrolysing high molecular weigh

compounds such as carbohydrates and proteins. Because these enzymes must work outside the cell, they usually have good stability characteristics to chemical and physical changes in the medium. In contrast, intracellular enzymes remain associated with the cell, are not normally secreted into the surrounding medium, and are generally stable as long as the integrity of the cell is maintained. Since they have properties similar to other constituent proteins and are contained in an environment often protected by tough cell walls, their isolation and purification is generally more complex than that of the extracellular enzymes. Good recovery efficiencies can be achieved with most cytoplasmic enzymes.

Table 9 Advantages and disadvantages of industrial application of thermostable enzymes (Kristjansson, 1989; Holló & Hoschke, 1993)

Properties	Advantages	Disadvantages
Thermostability	Tolerates high temperature, last longer	Some chemicals, raw materials and cofactors are damaged by heating. Difficult to inactive the enzyme
High optimum temperature	Little activity at low temperature, long shelf life	May be too high for some applications
Resistance to denaturing agents	Tolerates organic solvents, high and low pH	
General robustness	Tolerates harsh purification; gives better yield	
Solubility	Higher concentration of poorly soluble compounds are possible	Generally decreased for gases such as oxygen
Viscosity	Decreases; mixing and pumping can be accelerated; mass transfer rate increases	
Microbial contamination	Growth of all pathogens and most environmental mesophiles prevented	
Biological activity in raw materials	Heating kills most interfering enzyme or microbial activities	
Genes can be cloned in *E. coli*	A heating step makes purification easier	
Chemical reaction rates	Diffusion and other chemical processes are accelerated	
Material stress		More stress on equipment and contains the materials that can be used

The enzyme industry as we know it today is the result of a rapid development seen primarily over the past four-five decade thanks to the evolution of modern biotechnology. The majority of currently used industrial enzymes are hydrolytic in action, being used for degradation of various natural substances. Proteases remain the dominant enzyme type, because of their extensive use in the detergent and dairy industries (Kirck *et al.*, 2002). Various carbohydrolases, primarily amylases and cellulases — used in industries such as the starch, fuel alcohol, textile, detergent and baking industries — represent the second largest group (Godfrey & West, 1996). This

group slices about 30-33 % of the cake of $1.5 billion worldwide enzyme market estimated in 2000 (McCoy, 2000). The growth, however, has stagnated in some of the major technical industries such as the detergent industry causes the percentage of carbohydrolases estimated to be relatively grown in next years to the detriment of proteases. The advantages of using these enzymes in industrial applications such as starch and fuel alcohol are well recognised in recent years. However, most of the commercially available amylases and technologies have several disadvantages that cause the less suitable and uneconomical uses of them in industrial scale. For examples, most of the thermostable α-amylases that used now in starch conversion industry are from bacteria and these enzymes need Ca^{++} ion to operate at high temperature and pH in the range from 6.0 to 7.0. The pH of 35 — 40 % of starch slurry is about 4.5. The industrial used glucoamylases and pullulanases are from fungi and operate at low temperature (55-60 ^{o}C) and pH in range from 4.2 to 4.6, respectively. The saccharification process usually takes a long time from 12-96 hours as compared with the time of the liquefaction (1.5 — 2 h). Other considerable aspects are contamination and reaction time. Thus, on the industrial scale, the reactions above 60 ^{o}C minimises the contamination and decrease the reaction time. Firstly, for every 10 ^{o}C increase in temperature the reaction rates approximately double. Thus, for each 10 ^{o}C increase in operating temperature, the amount of enzyme required for a given conversion can theoretically be halved. Alternatively, holding time can be shortened. And secondly, temperatures of 60 ^{o}C or greater are inhibitory to microbial growth (Wasserman, 1984). The advantages and disadvantages of application of thermostable enzymes are summarised in Table 9. Therefore, to meet the current demand and improve the economics, there is a need for novel enzymes, which can catalyse desired reactions under industrially suitable conditions, in most economical manner (Bhat *et al.*, 1998).

Mesophiles and moderate thermophiles have thus been received the greatest attention as thermostable enzyme source. Few mesophilic organisms are reported to produce relatively thermostable amylases (Krishnan & Chandra, 1983; Morgan & Priest, 1981). Generally the enzymes from thermophilic organisms are more stable than those from mesophiles. Polysaccharases from the thermophilic micro-organisms appear to possess properties ideal for industrial sector. In recent years, research efforts have been directed towards isolating and characterising polysaccharides from these micro-organisms. It is hoped that thermostable polysaccharides with desirable properties will commercially be available in the near future.

Because thermophilic fungi occur in terrestrial habitats which are heterogeneous in terms of temperature and the types and concentrations of nutrients, chemicals, gases, water activity, competing species, and other variables, they may be able to adapt to several factors besides just high temperatures. From this perspective, research should extend to their nutrient uptake system, their ability to utilize mixed substrates, the nature and concentrations of their intracellular ions and osmolytes, and their effects on enzyme function.

Enzymes of thermophilic fungi have been studied primarily to explore their suitability in bioprocesses and to a lesser extent, to probe similarities and differences in physicochemical

properties between enzymes from mesophilic and thermophilic fungi. Since culture filtrates can be obtained in substantial quantities, the enzymes that are secreted in the growth media have been studied more frequently than cell-associated enzymes, although such investigations have focused mainly on the identification of suitable thermophilic fungal sources for desired enzymes, the development of protocols for the purification of these enzymes, and the study of their general properties (Maheshwari *et al.*, 2000).

Filamentous fungi are capable of producing large amounts of specific proteins. The production level of any protein of interest in naturally occurring strains is usually too low for commercial exploitation. However, impressive improvements in protein yield have been obtained with two approaches: optimisation of medium compositions and traditional strain development based on various mutagenesis approaches. For commercial processes yields of higher than 30 g/l of specific protein are not uncommon (Punt *et al.*, 2002).

Within the frame of optimisation of medium composition, on the one hand experiments for searching of appreciable raw material for growth and production of specific protein using filamentous fungi should be carried out. On the other hand from economical points of view, the optimum concentration of component also plays important roles in optimal fermentation technology. The one-variable-at-a-time approach (OVAT) is used very frequently to study the effects of culture environmental factors. This method is time consuming and does not guarantee the real optima under cultivation conditions. Moreover, it ignores the interactive effects among different variables. To overcome these disadvantages, numerous experimental design techniques (two-level factorial designs, two-level screening designs, three-level designs, mixed two- and three-level designs, central composite designs, Latin squares, Taguchi robust design, mixture designs) were developed based on mathematical-statistical analysis (Montgonery, 1991). These methods - consist of factorial design and regression analysis - give proper solution in the technological optimisation (Logothetis & Wynn, 1989) and have been successfully applied for optimising of media composition and operating conditions in many bioprocess.

Until transformation techniques for filamentous fungi became available in the mid to late 1980s, improvement of fungi production strains was largely restricted to laborious programmes of mutagenesis, screening and subsequent selection (Crueger & Crueger, 1984). Recently, two new gene cloning approaches have been used relatively successfully. One approach is "expression cloning", which combines simple enzyme assays with the use of *Saccharomyces cerevisiae* expression system for fungal cDNA (Dalboge, 1997; Dalboge & Lange, 1998). The limited metabolic scope of laboratory yeast strains allows transformants that express a wide variety of fungal hydrolases to be identified by sensitive enzyme screening. The resulting cDNA clones can easily be characterised and subsequently expressed in more appropriate high level expression host such as *Aspergillus* spp. (Dalboge, 1997; Dalboge & Lange, 1998). Similar strategies are currently being developed for filamentous fungi (Emalfarb, 2001). Another opinion is the combined use of database mining and molecular screening. Besides that of *S. cerevisiae*, at least two fungal genome sequences are publicly available: *Neurospora*

crassa (http://www.genome.wi.mit.edu/annotation/fungi/neurospora/) and *Aspergillus fumigatus* (http://www.tigr.org/tdb/e2k1/afu1/index.shtml). Publication of various other fungal genomes is expected shortly. This will help us to make comparison and identify fungal genes encoding proteins. These approaches will allow new fungal enzymes to be developed. Where specific activities have been described for fungal species, database mining in combination with polimerisation chain reaction based molecular screening, will allow genes from fungal species with preferred enzyme activities to be cloned and then expressed in suitable host. The choice of expression host strain can not be made solely on the basis of production yields, but other aspects such as regulatory issues, have also a very important roles. The potential preferred host strains are chosen from among those for which GRAS or GRASP (Generally Recognised as Safe Petition submitted) status at FDA have been filed. Moreover, patents and intellectual property rights have necessitated searching for expression hosts other than the species traditionally used.

3. Materials and Methods

3.1 Materials

3.1.1 Microorganisms

Aspergillus niger IMI 303386 was purchased from International Mycological Institute, Surrey, UK and maintained on Potato-Dextrose-Agar (PDA) slopes. *Thermomyces lanuginosus* strains were obtained from different culture collection and maintained on different media (Table 10).

Table 10 *Thermomyces lanuginosus* strains and maintained conditions

No.	Strain number	Maintained media	Temperature (^oC)	Notes
1	ATCC 28083	ATCC 336	45	
2	ATCC 34626	ATCC 663	45	
3	ATCC 36350	ATCC 336	45	
4	ATCC 38905	ATCC 350	37	
5	ATCC 44008	ATCC 350	50	RM - B
6	ATCC 46882	ATCC 338	37	
7	ATCC 16455	ATCC 350	45	
8	CBS 218.34	PDA, MA_2, NUT	40 - 45	
9	CBS 224.63	PDA, MA_2, NUT	40 - 45	
10	CBS 288.54	PDA, MA_2, NUT	40 - 45	
11	CBS 395.62	PDA, MA_2, NUT	40 - 45	
12	DSM 5826	YPSS	45	
13	IMI 084400	PDA, OA	37	ATCC 22070
14	IMI 096218	OA	40	
15	IMI 110803	PDA, OA	37	
16	IMI 158749	YPSS, OA	37	
17	IMI 131010	PDA	45	
18	IMI 140524	PDA	37	

ATCC : American Type Culture Collections, Rockwille, Maryland, U.S.A.
CBS : Centraalbureau voor Schimmelculture, Baarn, The Netherlands.
DSMZ : Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.
IMI : International Mycological Institute, Surrey, UK.

3.1.2 Chemicals

DEAE-Sepharose Fast Flow, Q-Sepharose Fast Flow, Sepharose CL-6B and Superose 12 resins were purchased from Pharamacia Biotech (Uppsala, Sweden) as well as pI calibration kits. The Ultrogel AcA44 was from Biosep (CA, USA). The BioGel P-2 resin, $3NH_2$-Spherisorb column and molecular mass markers were from Bio-Rad Laboratories (Hercules, CA, USA). All used chemicals were analytical grade and purchased either from Sigma, Merck, Reanal or BDH or other companies.

3.2 Methods

3.2.1 Cultivation of *A. niger* and production of β-fructofuranosidase

The spore suspension was prepared by adding 5 mL sterile distilled water into an *A. niger* PDA slope. This was used to inoculate 250 mL conical flask containing 100 mL pre-culture medium with composition as follows (w/v): glucose, 1 %; yeast extract, 1 %; $NaNO_3$, 2 %; $MgSO_4.7H_2O$, 0.05 %; K_2HPO_4, 0.5 %. The pH was adjusted to 7.4 using 2 % K_2HPO_4 before autoclaving. The pre-culture was grown for 24 h at 28 ^oC and in orbital shaker (200 rpm) and 15 mL mycelial suspension was used to inoculate 300 mL of medium in 1 L flask which contained (w/v) sucrose 2 % or inulin, 1 %;L-asparagine, 0.4 %; K_2HPO_4, 0.1 %; $MgSO_4.7H_2O$, 0.05 %; $CaCl_2$, 0.01 % and 0.1 % of trace element solution with the following composition: 0.5 g $FeSO_4.7H_2O$, 0.16 g $MnSO_4.4H_2O$, 0.14 g $ZnSO_4.7H_2O$, 0.02 g $CoCl_2.6H_2O$ in 100 mL distilled water. The fungus was cultured for 48 h under the same conditions as pre-culture.

3.2.2 Cultivation and production of amylolytic enzymes by *T. lanuginosus*

A three-stage cultivation technique was used. In the first stage, the fungus was grown on YPSS or PDA slant agar for 8 to 10 days at 50 °C in a humidified thermostat. In the second stage a suspension of spores was prepared using 0.1 % of Triton X-100 solution. A volume of 5mL of the suspension was added to 100mL glucose-asparagine medium (pH=6.0) to initiate the cultivation at 50 °C and 220 rpm in an orbital shaker for 1 to 2 days to obtain a homogeneous mycelium growth. In the third stage, 10mL of the mycelial suspension was used as inoculum for initiating the production of amylolytic enzymes in 150mL starch-asparagine medium (soluble starch: 40g, L-asparagine: 4g, KH_2PO_4: 3g, K_2HPO_4: 2g, $MgSO_4.7H_2O$: 0.5g, Vogel's (1956) trace elements solution: 1mL was dissolved in 1L distilled water). Samples (10mL) were taken under a laminar box from duplicate flasks at varying times. The samples were filtered and the α-amylase and glucoamylase activities were assayed in the filtrate.

3.2.3 Enzyme extraction

After 48 h culturing, the mycelia was collected by filtration using Whatman No. 1 filter paper and washed several times with 0.1 M phosphate buffer, pH 6.5. The wet mycelia were ground with approximately 2 times of sand and minimal amount of buffer. The homogenised mycelia were centrifuged at 15000 x g for 20 min and supernatant was used as a crude enzyme.

3.2.4 Enzyme activity assays

β–Fructofuranosidase

Hydrolytic and transfructosylating activities

A 3 mL reaction mixture containing 0.75 mL of 0.2 M phosphate buffer pH 6.5, 1.5 mL of 5 % (w/v) sucrose solution and 0.65 mL of distilled water was pre-incubated at 50 ^oC for 10 min. A

0.1 mL of appropriately diluted enzyme solution was added, and incubation continued for further 15 min. The reaction was terminated by placing the tubes in polyethylene glycol 400 boiling bath for 10 min. After cooling, a two and one mL of reaction mixture were used for determining the reducing sugar and glucose by Somogyi/Nelson (Nelson, 1944; Somogyi, 1952) and glucose oxidase-peroxidase (GOD/POD) (Wood & Bhat, 1988) methods, respectively. The colour developed in both cases was read at 520 nm and converted into reducing sugar and glucose using glucose standard curve prepared under identical conditions. The hydrolytic activity was determined by measuring the reducing sugar (R) released from sucrose as described above. The transfructosylating activity was determined by measuring both reducing sugar and the glucose (G) released from sucrose. The concentrations of free fructose (F) and transferred fructose (F') in the reaction mixture (Chen & Liu, 1996) were calculated as follows:

$F = R - G$
$F' = G - F = 2G - R.$

One unit of hydrolytic activity was defined as the amount of enzyme required to release 1 µmol reducing sugar per min. One unit of transfructosylating activity was expressed as µmol of fructose transferred per minute from donor molecular to acceptor molecular.

α-Amylase

A reaction mixture containing 1 ml of 0.1 mol sodium-acetate buffer (pH 5.0) and 1 ml of 0.5% (w/v) soluble starch solution was mixed and pre-incubated at 50 ^{o}C for 10 min before adding 1 ml of appropriately diluted culture filtrate as an enzyme source. After 5 min the reaction was stopped by adding 1 ml of 0.5 mol HCl. The unhydrolysed starch in this aliquot was estimated by the iodine method described below. One unit of α-amylase activity was defined as the amount of enzyme that hydrolyses 1mg of soluble starch in 1 min.

Glucoamylase

One ml of reaction mixture containing 0.25 ml of 0.1mol phosphate buffer (pH 4.6) and 0.25 ml of 1% (w/v) soluble starch solution was pre-incubated at 50 ^{o}C for 10 min. Half a ml of appropriately diluted culture filtrate as an enzyme source was added, and the incubation was continued for further 15 min. The reaction was stopped by placing the tubes in a boiling bath for 30 min. After cooling the released glucose concentration was estimated by the glucose-oxidase/peroxidase method (McComb & Yushok, 1957; Wood & Bhat, 1988). One unit of glucoamylase activity was defined as the amount of the enzyme that releases 1 µmol of glucose in 1 min.

3.2.5 The iodine method for starch estimation

An aliquot of the sample (starch hydrolysate) was mixed with 1 ml of iodine reagent and this mixture was adjusted up to 2 ml by distilled water. The iodine reagent contained 0.02% (w/v) of iodine and 0.2% (w/v) of KI in 0.5 N HCl. Five ml of distilled water was added to this mixture and the colour developed was read at 590 nm against blank. The amount of starch was

estimated using a standard curve prepared with potato soluble starch (Merck) under the same conditions.

3.2.6 Determination of sugar concentration

The analyses of sugars were carried out using chromatographical (HPAELC and HPLC) and other analytical methods such as BCA, Somogyi/Nelson and GOD/POD. For more details see Appendix 1, 3 and 4.

3.2.7 Determination of protein concentration

The protein concentration was estimated by measurement of 280 nm absorbance and/or by Bicinchoninic acid (BCA) microtiter plate method using Protein Assay Kit from Pierce (USA) following the instruction given by distributor. The Lowry and modified Lowry methods for protein estimation were also applied using Protein Assay Kits from Sigma (USA).

3.2.8 Electrophoretic analysis

The protocols are described by Laemmli (1970). The proteins were stained with 0.25 % Coomassie brilliant blue G-250. In case of the molecular mass estimation the following markers (SDS-PAGE molecular weight standard, high range, Bio-Rad) were used: myosin (200 kDa), β-galactosidase (115.25 kDa), phosphorylase B (97 kDa), serum albumin (66.2 kDa) and ovalbumin (45 kDa). 1 % Agarose gel was used for estimation of pI of β−fructofuranosidase by isoelectric focusing at 4 ^{o}C in the pH range 2.5 - 10. The pI calibration kit from Pharmacia (human carbonic anhydrase B pI 6.55, bovine carbonic anhydrase B pI 5.85, β-lactoglobulin A pI 5.2, soybean trypsin inhibitor pI 4.55 and glucose oxidase pI 4.15, amyloglucosidase pI 3.5) and ampholine carried ampholytes from Bio-Rad (BioRad, USA) were used. The proteins were stained with 0.1 % Serva blau G.

3.2.9 Effects of pH and temperature on enzyme stability

The one-variable-at-a-time approach (OVAT) is used very frequently to study the effects of pH and temperature on enzyme stability. This method does not guarantee the real optima under operating conditions. Moreover it ignores the interactive effects among different variables. To overcome these disadvantages, the response surface method (RSM), which includes the factorial design and regression analysis, gives proper solution in the technological optimisation (Logothesis & Wynn, 1989; Montgonery, 1991). In our case the pH and temperature were selected as independent factors (variables). The various pH levels were adjusted by application of different buffer systems: 50 mM sodium acetate buffer (pH 3.5 – 5.6), 50 mM phosphate-citrate buffer (pH 4.0 - 8.0) and 50 mM Tris-HCl buffer (pH 7.5 - 9.0). Appropriate amounts of enzymes were incubated in 50 mM buffers pH from 3.5 to 9.0 with 0.5 stepwise and temperatures from 50 ^{o}C to 85 ^{o}C with 5 ^{o}C stepwise. Samples were taken in different times and

assayed for activities at standard conditions described above. The matrix of half-life time with two dimensions (pH and temperature) was built and the half-life time values in matrix were maximised conventionally as 1440 minutes (one day). The RSM was applied to evaluated matrix data using SPSS 10.0 for MS Windows statistical software package (Copyright © 1994-2000 by SPSS Inc.).

3.2.10 Determination of carbohydrate content of enzymes

Enzyme samples (280 ml containing 50 µg purified protein) were hydrolysed by adding 120 µl of 100 % trifluoracetic acid (TFA) and incubation at 100 °C for 4 h. After cooling to room temperature the mixture was concentrated in vacuo. To remove the excess of TFA three times 1 ml distilled water was added and evaporated before carbohydrate analysis. The sugars were quantified by determination of reducing sugar and by high pressure anion exchange liquid chromatography (HPAELC) using CarboPack 10 column (DIONEX) connected to a DIONEX system and a PAD detector (DIONEX). Elution was done with 0.1 M NaOH.

3.2.11 Determination of kinetic parameters of amylolytic enzymes

The kinetic parameters of α-amylase for soluble starch were investigated in 50 mM sodium acetate buffer system pH 5.6 containing 7.5 mM Ca^{2+}. The reducing sugar concentration was determined by method described above. Based on the results of preliminary experiments, 10 U (3.384 µg protein) of enzyme was applied in 1 mL of total volume of reaction mixture.
In the case of glucoamylase, different substrates (maltose, maltotriose, maltotetraose, maltopentaose and soluble starch) were used. These were dissolved in 50 mM sodium acetate buffer system pH 5.0 and 0.5 U (8.33 µg protein) of glucoamylase was applied. The released glucose concentration was determined by GOD/POD method. The reactions were carried out at 65 °C and the Hanes-Woolf method (Cornish-Bowden, 1995) was applied to evaluate experimental data. All experiments were done in triplicates and the average data were used to calculate the K_m and V_{max}.

3.2.12 *N*-terminal amino-acid sequence

The purified α-amylase enzyme was applied onto Applied BioSystem 476A pulsed liquid sequenator (Foster City, Ca, USA) to determine the primary sequence of the peptide chain. The primary sequence was analysed using the BLAST database (http://pbil.univ-lyonl.fr/BLAST/).

4. Results and Discussion

4.1 β-Fructofuranosidase from *Aspergillus niger*

4.1.1 Effects of sucrose and inulin on the production of β-fructofuranosidase by *A. niger*

The effects of sucrose and inulin on the production of β-fructofuranosidase by *A. niger* was studied. In the case of sucrose the fungus produced mainly intracellular β-fructofuranosidase, in the case of inulin the hydrolysis activity can be detected in both fractions. These values were 3 and 4.5 fold higher than the activity of intracellular β-fructofuranosidase from sucrose medium. These results agree with results published by Gupta and co-workers (1994). They investigated the effects of inulin and sucrose on production of acid invertase by 6 *Aspergillus* species and one *Kluyveromyces fragilis* strain and found that inulin is better inducer than sucrose. In the case of *A. niger* IMI 303386 both intra- and extracellular invertase from inulin-based medium only showed the hydrolysis activity. More detailed results were demonstrated in Appendix 1.

4.1.2 Purification of β-fructofuranosidase from *A. niger*

The results of purification procedure are summarised in table 1. A 40 mL of enzyme extract containing 93 mg protein and 96.5 units of transfructosylase activity was precipitated over ice-bath by solid ammonium sulphate at 80 % saturation. The mixture was kept in fridge for overnight to complete the precipitation. The precipitated proteins were collected by centrifugation at 15000 x g, 4 ^{o}C for 20 min. After that it was dissolved in minimal amount of 0.02 M phosphate buffer (pH 6.5). About 90 % of total β-fructofuranosidase activity with 2 fold of purification was recovered after this step.

Total amount of sample from above step was applied to a DEAE Sepharose Fast Flow column (25 cm x 2.5 cm) connected to FPLC system equilibrated with 50 mM sodium acetate buffer pH 5.5 to carry out ion exchange chromatography. The bound protein was eluted with sodium acetate buffer pH 5.5 containing 0.2 % sodium azide using linear gradient of sodium chloride from 0 to 0.5 M in 500 mL of total volume of buffer. The profile of this step is demonstrated in Figure 14. According to Figure 14, numbers of contaminating proteins were removed and about 75 % of hydrolytic activity of β-fructofuranosidase was recovered with 5.7 fold of purification. The active fractions (Figure 14, activity peak) were pooled and concentrated by ultrafiltration using 10 kDa cut-off membranes as well as dialysed with 0.02 M phosphate buffer pH 6.5.

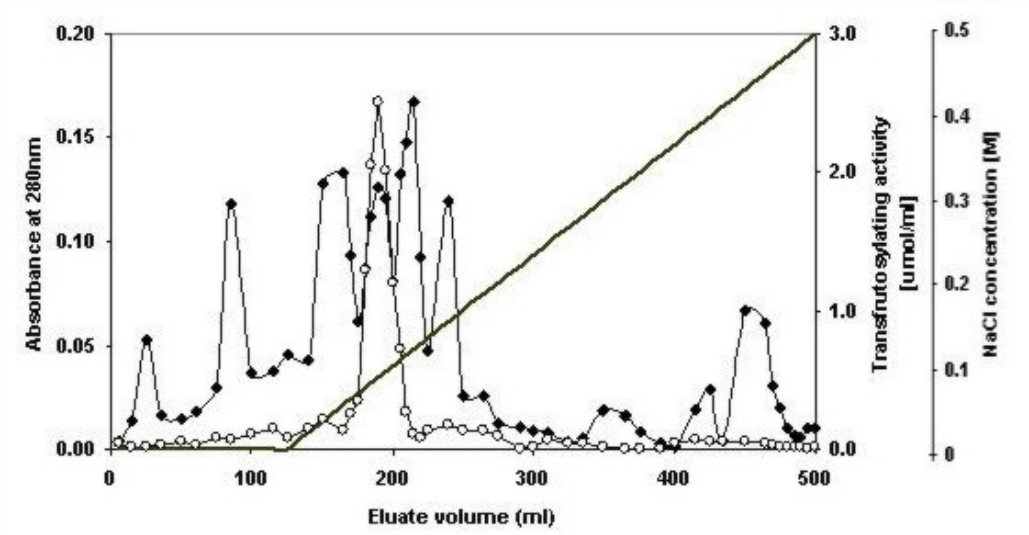


Figure 14 Profile of ion-exchange chromatography with DEAE-Sepharose fast flow resin.
protein enzyme activity NaCl gradient

The concentrated sample from previous step was loaded onto Ultogel AcA44 column (90 cm x 1.6 cm) equilibrated with 50 mM phosphate buffer pH 6.5 containing 0.2 % NaN_3. The proteins were eluted by the same buffer at flow rate 16 ml/h.

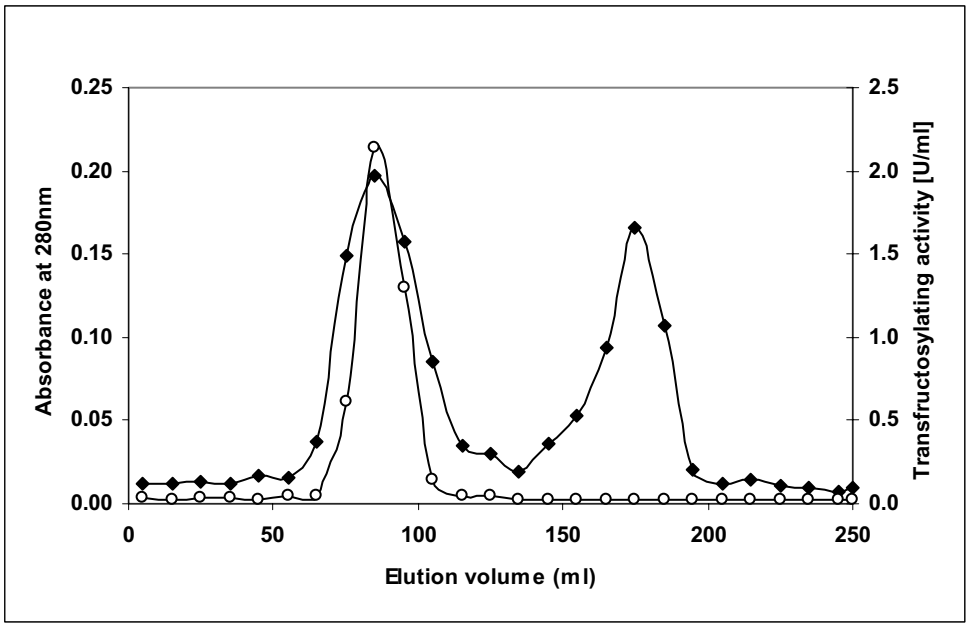


Figure 15 Elution profile of Ultrogel AcA44 chromatography of β–fructofuranosidase from *A. niger* IMI 303386 protein enzyme activity

Two protein components were detected by measuring absorbance at 280 nm and the enzyme activity was associated with (Figure 15). The active fractions were pooled and concentrated using membrane filter cell (Amicon UV 10). About 50 fold of purification and 42 % recovery of enzyme activity were gained after this step.

Table 11 Summary of purification of β-fructofuranosidase enzyme from *A. niger*

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	93.00	96.50	1.04	1	100.0
Precipitation	43.60	86.40	1.98	1.9	89.5
DEAE-Sepharose	1.76	73.04	41.50	40.0	75.7
Ultrogel AcA44	0.78	40.30	51.67	49.8	41.8

The results of purification procedure were summarized in Table 11. According to this protocol, β-fructofuranosidase form *A. niger* IMI 303386 can be purified by three steps (ammonium sulphate precipitation, ion-exchange chromatography and gelfiltration) with 50 purification fold and 42 % recovery.

4.1.3 Characterisation of β-fructofuranosidase

Physicochemical properties of β-fructofuranosidase from A. niger

Showing single protein species on SDS-PAGE staining with 0.25 % Coomassie brilliant blue G-250, the enzyme was purified to apparent homogeneity. From electrophoretic results the molecular mass was estimated to be in the range from 115 to 135 kDa. This result is agreed with the values reported by L'Hocine and co-workers (2000). In this report, they mentioned that *A. niger* AS0023 produced two forms: fructosyltransferase have molecular weight from 81 to 168 kDa according to SDS-PAGE and invertase have a molecular weight in range from 71 to 111 kDa. In the native form the fructosyltransferase seems to undergo association-dissociation; its subunits tend to aggregate to form oligomers (hexamer, tetramer and dimer). The native invertase is dimer with an approximate average subunit size of 91 kDa. Hirayama and co-workers (1989) also characterised β-fructofuranosidase from *A. niger* ATCC 20611 and reported that its molecular mass was 100 kDa on SDS-PAGE and 340 kDa by gelfiltration. In 1995, Rubio and Maldonado reported that invertase from *A. niger* isolated from mouldy lemons have molecular mass 47 kDa on SDS-PAGE and 95 kDa according to gelfiltration with Sephadex G-150. The β-fructofuranosidase from *A. japonicus* MU-2 gave three bands with 95 kDa, 65 kDa and 37 kDa of molecular mass, respectively (Hayashi *et al.*, 1992b). It can be concluded that β-fructofuranosidase from *A. niger* have at least two equal molecular mass subunits. The molecular mass of one subunit varies from 40 kDa and 200 kDa. Based on the result of isoelectric focusing, β-fructofuranosidase from *A. niger* showed one major band at pH 5.4 and one minor band at pH 4.4. Interestingly, very few data are available relating to pI values of invertase from *A. niger*. These results are close to results reported by Chen and

co-workers (1996) who studied the secretion, purification and characterisation of two forms (S-and F-form) of invertase secreted by *A. nidulans*. The pI of S (slow) and F (fast) forms were in range of 4.9-5.2 and 3-4.2, respectively.

Effects of pH on β-fructofuranosidase enzyme activity and stability

The β-fructofuranosidase from *A. niger* IMI 303386 is most active in the range from 5.0 to 6.5, and this enzyme have pH optimum at 5.5 (Figure 3). This result agreed with that from *A. niger* reported by Park & Almeida (1991) as well as from *A. japonicus* MU-2 and TIT-KJ1 reported by Hayashi and co-workers (1992b) and Duan and co-workers (1993), respectively. Interestingly, this value is close to the pH optimum of fructosyltransferase (pH 5.8), but higher than invertase (pH 4.4) published by L'Hocine and co-workers (2000).

Based on the results demonstrated in figure 3, the β-fructofuranosidase from *A. niger* IMI 303386 is completely stable in pH range from 4.0 to 8.0. Incubating the enzyme solution at temperature 40 ^{o}C in these pH values, at least 90 % of enzyme activity was retained after 5 h incubation. The enzyme lost its activity rapidly at pH below 4.0 or above 8.0. These results partially agree with that reported by L'Hocine and co-workers (2000). In their study the effects of pH and temperature on stability of fructotransferase and invertase from *A. niger* AS0023 were investigated. Both enzymes were stable between pH 4.0 - 11.0 for 2 h incubation at 25 ^{o}C and more than 80 % residual activity was retained.

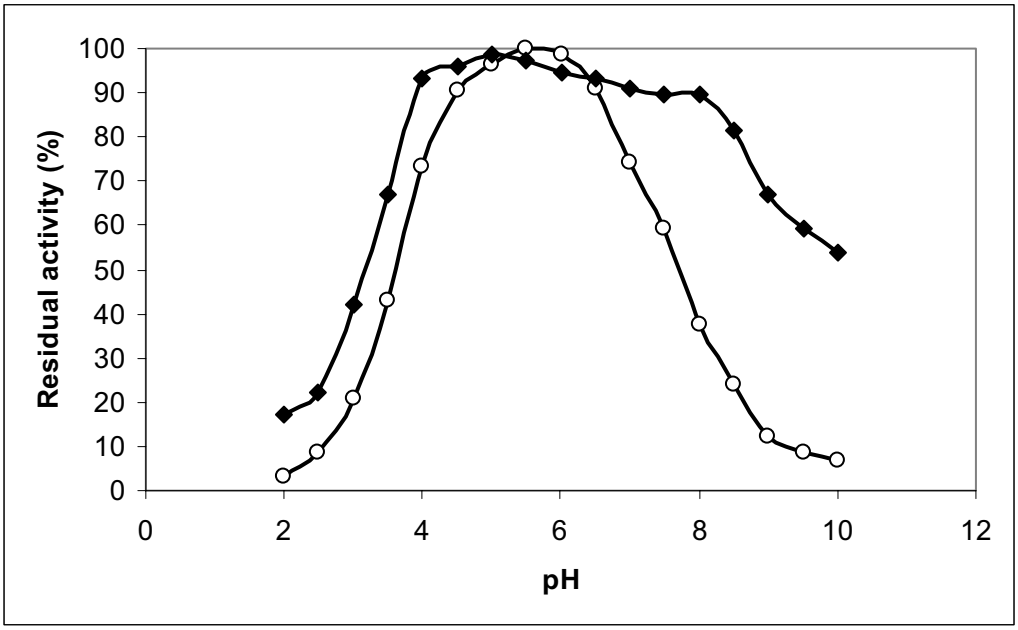


Figure 16 Effects of pH on activity (—○—) and stability (—◆—) of β-fructofuranosidase

Effects of temperature on β-fructofuranosidase enzyme activity and stability

Based on figure 4, the enzyme shows maximum activity at 50 ^{o}C and lost its activity rapidly when measured at temperature higher than 60 ^{o}C. The optimum temperature (60-65 ^{o}C) of *A. japonicus* (Hayashi *et al.*, 1992c) was higher than it. The β-fructofuranosidase from other *A. niger* strains (L'Hocine *et al.*, 2000, Park & Almeida, 1991) also showed optimum temperature at 50 ^{o}C.

After 5 h incubation at pH 5.5, β-fructofuranosidase was found to be stable at temperature lower than 60 ^{o}C. More than 90 % of residual activity was retained (Figure 4). The activity decreased rapidly, when the enzyme was incubated at temperature higher than 60 ^{o}C. This result suggests that the long-term bioconversion should be carried out at 50 – 55 ^{o}C.

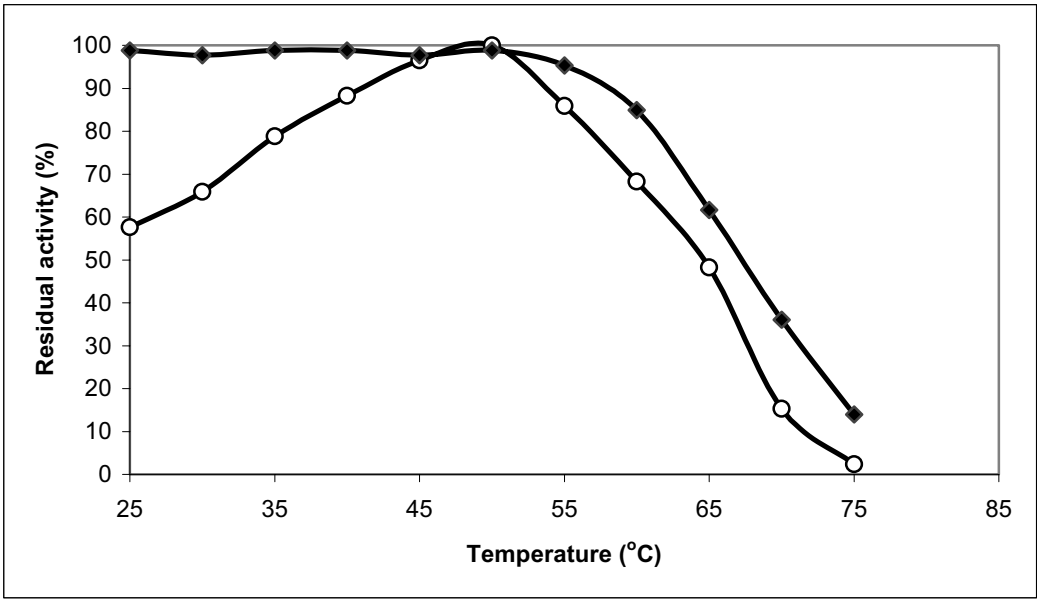


Figure 17 Effect of temperature on activity (—○—) and stability (—◆—) of β-fructofuranosidase

Effect of some compounds on β-fructofuranosidase activity

The results summarised in table 2 show that with 1 mM of Ba^{++}, Mg^{++}, Ca^{++} and 10 mM sodium-EDTA the enzyme more active than without them (Table 2). The best results were gained by adding 1 mM of $MgSO_4$ (15 % increase) and 10 mM of sodium-EDTA (17 % increase). The Hg^{++}, Zn^{++}, Ag^{+} and Ni^{++} ions are very strong inhibitors. Adding 1 mM of them β-fructofuranosidse from *A. niger* lost most activity. Adding 1 mM of Co^{++} and Cu^{++} ions, the enzyme lost about 15 – 20 % of its activity.

Table 12 Effects of various metal ions and compounds on the activity of β-fructofuranosidase from *A. niger*

Compound (1 mM)	Relative activity (%)
Control	100
$HgCl_2$	2
$ZnSO_4$	0.5
$CuSO_4$	83
$AgNO_3$	0
$FeSO_4$	92
$CoCl_2$	78
$MnSO_4$	91
$BaCl_2$	108
$MgSO_4$	115
$CaCl_2$	112
EDTA (10 mM)	117
NaN_3	98
$NiSO_4$	15
Urea (10mM)	103

Carbohydrate content of β-fructofuranosidase from A. niger

β-fructofuranosidase from *A. niger* IMI 303386 was found to be a glycoprotein with the carbohydrate content of 17 % according to phenol-H_2SO_4 method. This result fits to the general hypothesis that the enzymes from fungi are glycoproteins and the carbohydrate content of them is in the range of 2 – 20 %. Further studies are needed to determine the components of oligosacchrides and mode of glycosylation.

4.2 Fructooligosaccharides

4.2.1 Action of β-fructofuranosidase from *A. niger* on sucrose

The action of 1U and 2U β-fructofuranosidase on different concentration (1 %, 2 %, 5 %, 10 %, 15 %, 20 %, 25 %, 30%, 35 %, 40 %, 45 % and 50 %) of sucrose was investigated in 5 ml of total volume of reaction mixture. In the initial stage of incubation, sucrose was converted to glucose, fructose and FOS. The maximum amounts of fructooligosaccharides were gained at 25 % of sucrose as substrate after 72 h of incubation time. For more detailed of the results see Appendix 1.

4.2.2 Production and purification of fructooligosaccharides

The production of FOS was carried out using scale up technique in laboratory volume using 20 units of β-fructofuranosidase from *A. niger*. The reaction mixture containing 25 mL of 50 %

(w/v) sucrose, 12.5 mL of 0.2 M phosphate buffer pH 6.5, 0.01 g sodium azide and 12.5 mL of distilled water, was incubated at 39C for 72 hours.

For separation and purification of FOS, the samples were loaded onto Bio-Gel P-2 column and eluted with distilled water at flow rate of 15 mL h^{-1}. Fractions were collected and analysed for total sugar by phenol sulphuric acid method. Chromatogram was demonstrated in Figure 18.

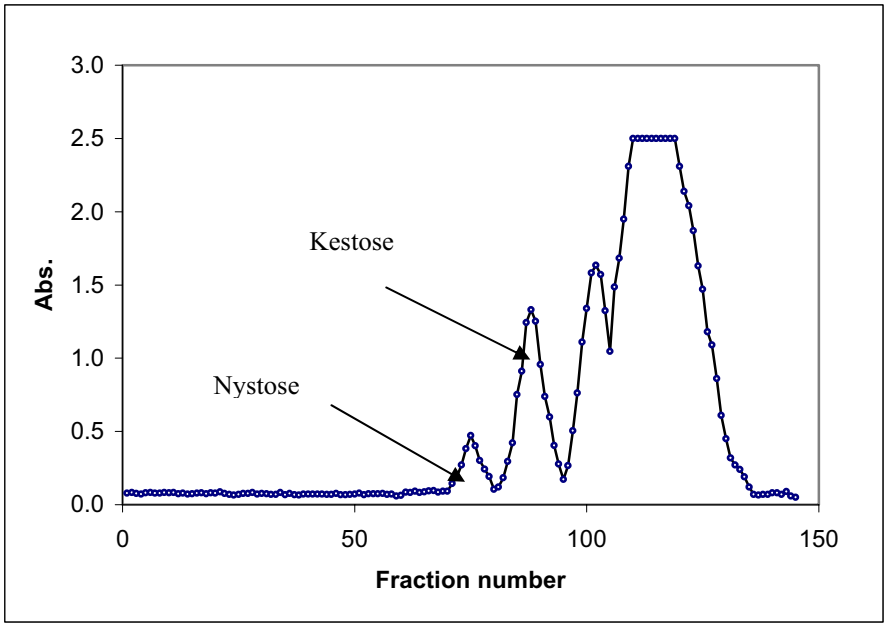


Figure 18 Profile of purification of fructooligosaccharides

The fractions belonging to individual sugar peak were pooled freeze-dried and identified by HPLC. The results were demonstrated in Appendix 1.

4.3 Amylolytic enzymes from *Thermomyces lanuginosus*

4.3.1 Morphological characterisation of *Thermomyces lanuginosus*

The morphology of the individual strains showed significant deviations in the frame of the species. Growth of this organism on Potato-Dextrose Agar (PDA) was rapid when it was incubated at 45 ^{o}C. After three days of propagation at least 60-80 mm in diameter colonies were observed (Figure 17). At first, the colonies appear white and fealty but soon turn grey or greenish grey from the centre of the colony. Gradually the colony turns purplish brown, and at this time the agar stains a deep pink or wine colour, due to diffusible substances secreted by the colony but the strains showed considerable variety in this respect (Figure 17, 18, 19, 20).

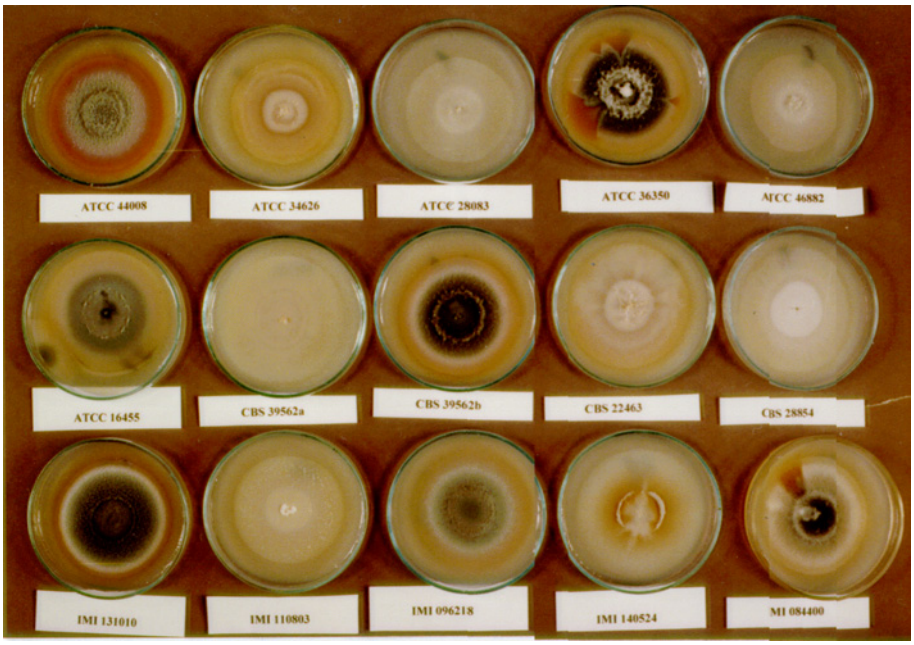


Figure 19 Colonies of *Thermomyces lanuginosus* strains[1]

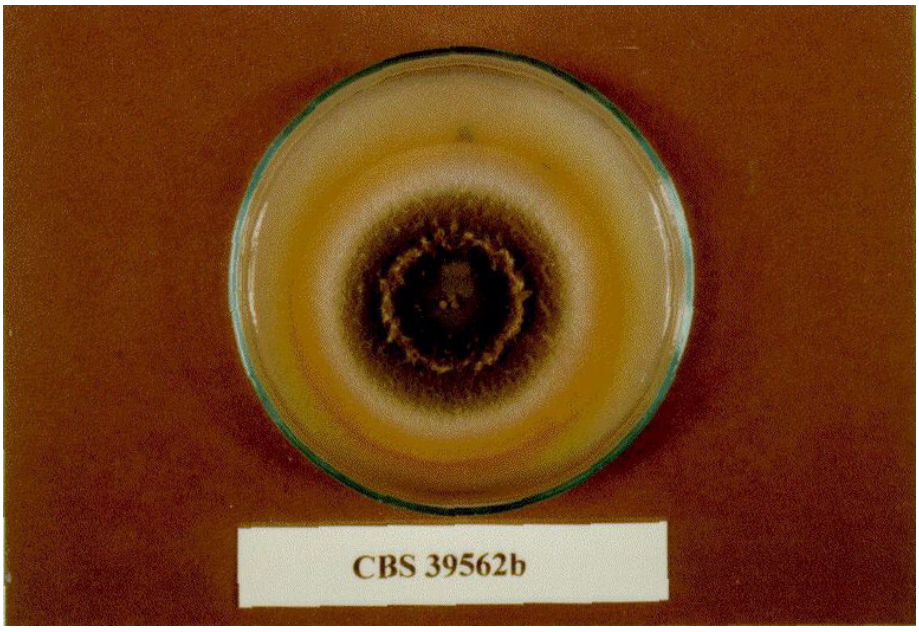


Figure 20 Strongly sporulated, dark colony of CBS 395626b strain of *Thermomyces lanuginosus* (PDA, 3 days, 50°C)

[1] All photos were taken by Dr. Csaba DOBOLYI

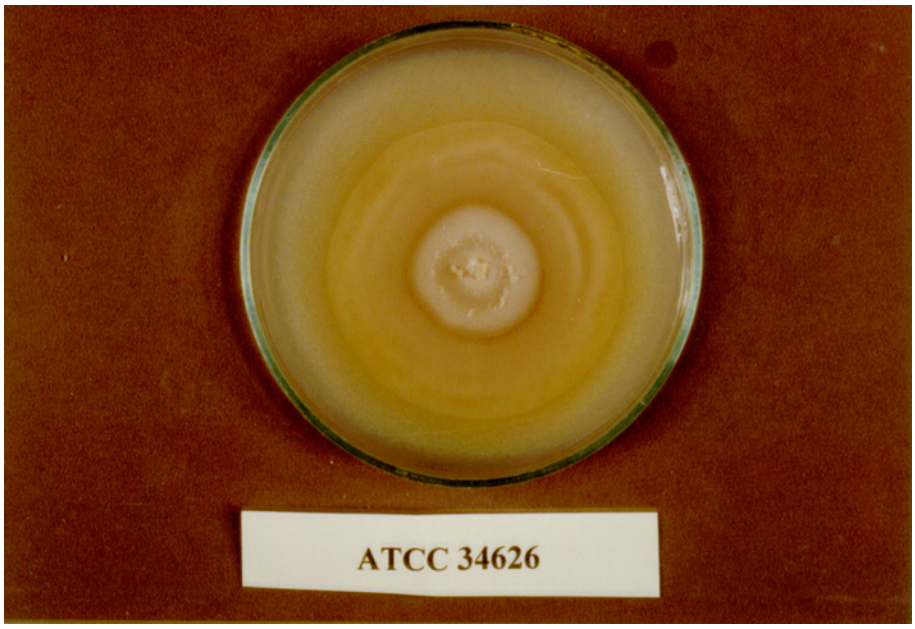


Figure 21 Weakly sporulated colony of ATCC 34626 strain of *Thermomyces lanuginosus* (PDA, 3 days, 50°C)

The CBS 28854, IMI 110803 and DSM 5826 strains showed light-brown in culture of PDA agar, while in colony of IMI 96218, ATCC 44008 strains the pink-dark brown can be observed. In the central of colony of CBS 39562, IMI 131010, IMI 84400 and ATCC 36350, the arenate filled with black pigments is found (Figure 18, 21). Deviations of colonial morphology could be observed from both among the various strains and during the growth of some strains because of the segregation of their properties of fructification (Figure 21). Mature colonies appear dull dark brown to black (Figure 17, 18, 19)**.**

In respect of the microscopic morphological properties the tested strains showed only slight differences. The mycelia of the tested strains consist of branched partitioned *hyphae* with diameter of 1.5-4.0 µm in the nutrient agar. At the tip of 8-15 µm air *hyphae* from right angle with the *hyphae* that is running on the surface of the medium, there are some 6-8 µm diameter, dark-brown spores (Figure 22). They are unicellular spherical asexual spores.

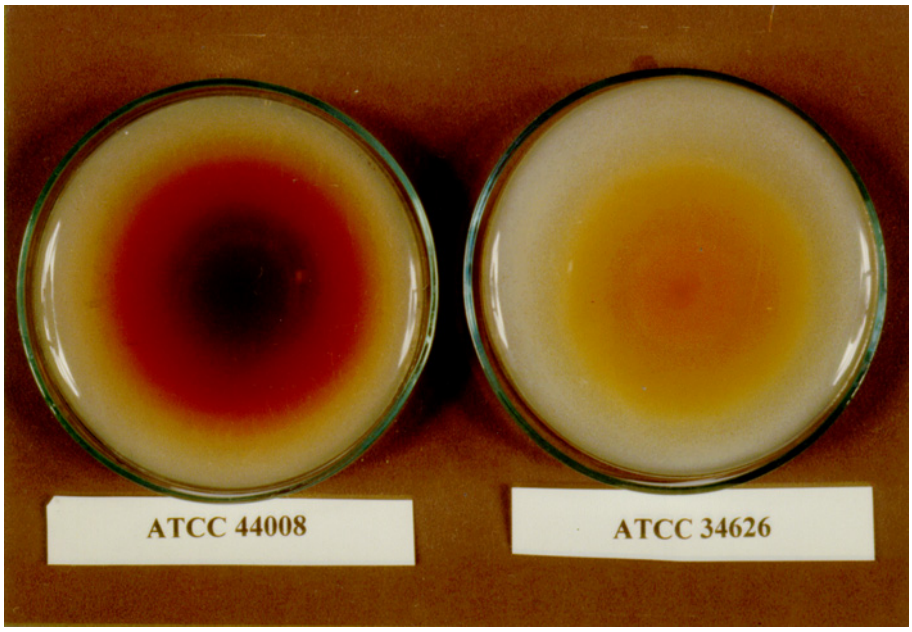


Figure 22 The backside of the colonies of *Thermomyces lanuginosus* strains (PDA, 3 days, 50°C)

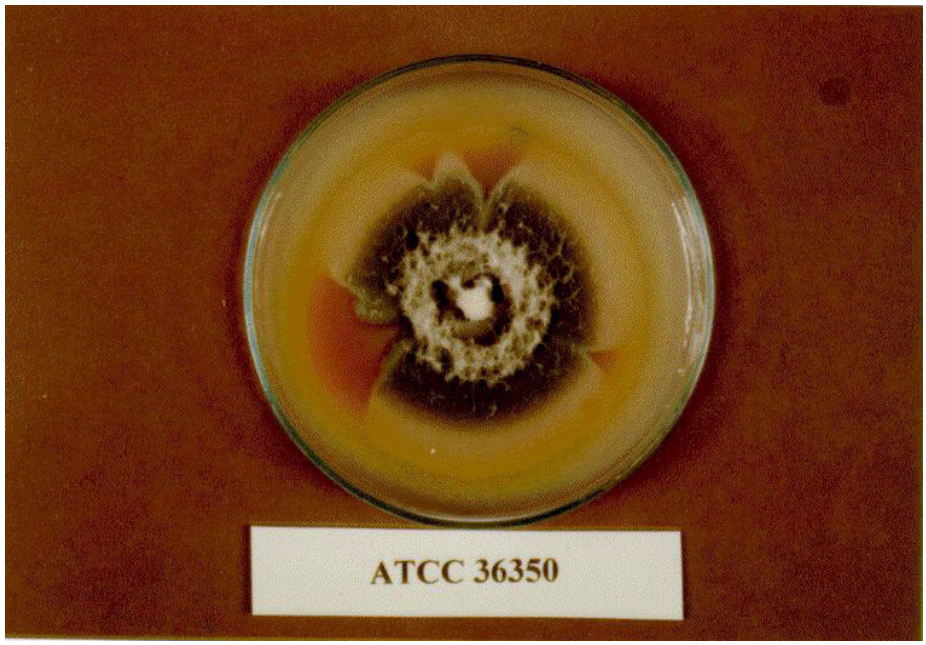


Figure 23 Segregation of the fructification capability of *Thermomyces lanuginosus* strain ATCC 36350 (PDA, 3 days, 50°C)

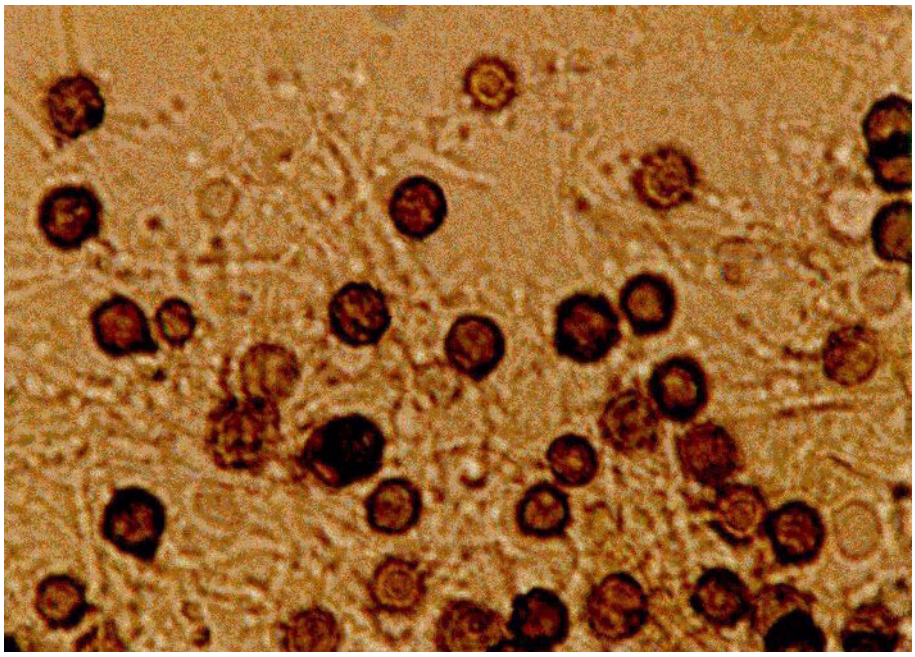


Figure 24 Aleuriospores on the fine colourless hyphae (*Thermomyces lanuginosus* ATCC 34626, native preparation, 1200x)

When young colonies were examined microscopically, masses of developing aleuriospores were evident on the fine, colourless hyphae. The rather short aleuriophores, which measure from 10 to 15 µ in length, arise at right angles to the hyphae (Figure 22, 23, 24). They generally are un-branched but occasionally they may branch once or twice near the base and thus appear as a cluster. Saptations commonly occur in the aleuriophores but they are difficult to observe. The aleuriospores are borne singly at the tips of the aleuriophores. Immature spores are colourless and smooth-walled, but as maturation proceeds they turn dark brown and the thick exo-spore becomes characteristically wrinkled. Mature spores are spherical, irregularly sculptured, and range from 6 to 10 µ in diameter (Figure 22, 23). Both immature and mature spores separate easily from the aleuriophore. The aleuriophore usually ruptures slightly below the point of attachment of the spore in which case free, immature or mature spores may be found with the upper portion of the aleuriophore still attached.

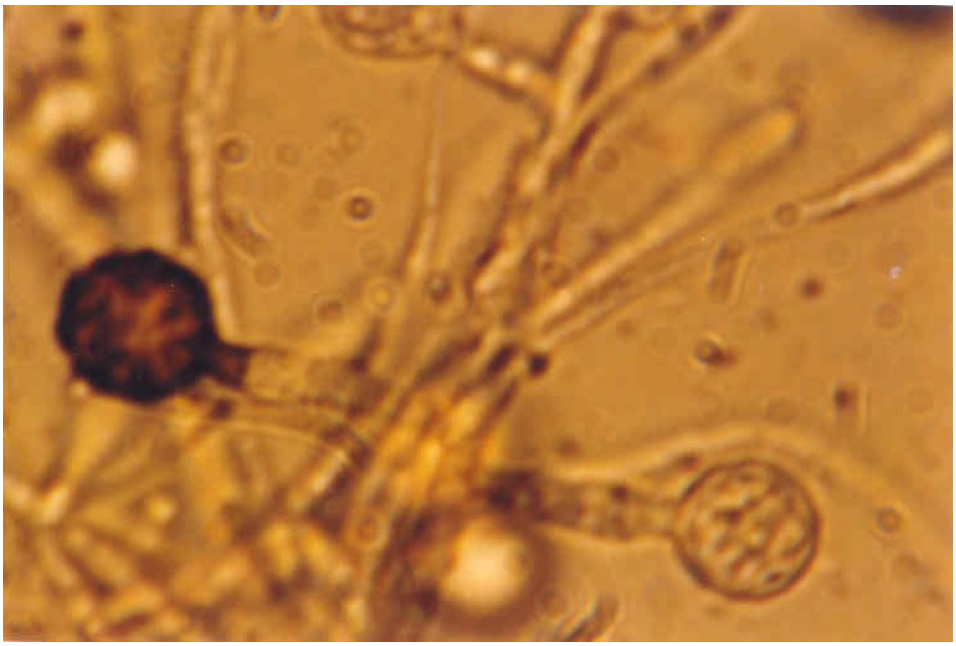


Figure 25 Aleuriospores on the right angles hyphae
(*Thermomyces lanuginosus* ATCC 34626, native preparation, 2800x)

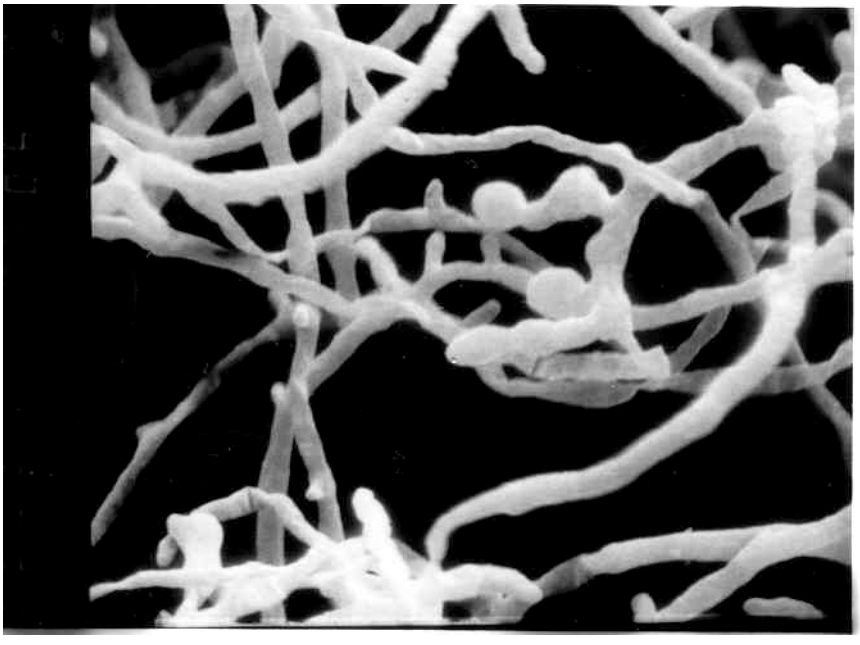


Figure 26 Hyphae of ATCC 34626 strain (Electronmicroscop picture, 2000x)

4.3.2 Screening of *T. lanuginosus* strains for production of amylolytic enzymes

Eighteen strains of thermophilic fungus *Thermomyces lanuginosus* have been screened for amylolytic activities. The *T. lanuginosus* strains were obtained either from different international culture collection such as ATCC, IMI, DSM or CBS. The new rapid screening method has been developed based on ratio of cleaning zone for amylolytic activities. The results of rapid screening method showed that all of *T. lanuginosus* strains produced extracellular amylolytic enzymes. These results also were investigated applying submerged fermentation using 500-ml shaken flasks. Three strains (ATCC 34626, ATCC 44008 and ATCC 28083) have been selected for detailed studies on the production of glucoamylase and α-amylase. The optimum conditions of the amylolytic activities have been selected. The optimum parameters were the following: 70^{o}C and pH 4.6, 70^{o}C and pH 5.0 to glucoamylase and to α-amylase, respectively. The crude α-amylase from *T. lanuginosus* showed about 1.5 times higher in presence of Ca^{++} than absence of this ion. The detailed results were given in Appendix 2.

4.3.3 Optimisation of composition of media for the production of amylolytic enzymes by *Thermomyces lanuginosus* ATCC 34626

The composition of medium for the production of amylolytic enzymes by *Thermomyces lanuginosus* was optimised in different ways. Effects of various carbon and nitrogen sources were investigated. *Thermomyces lanuginosus* grown on starch, maltodextrin, dextrin, maltose, amylopectin, glucose and dextran substrates showed good α-amylase (92-125 U/mL) and glucoamylase (6-13 U/mL) activities. The maltodextrin seem to be best carbon source for α-amylase production. Applying this substrate in submerged fermentation in shaken flask cultivation the α-amylase activity reach about 125 U/ml after 96 h. In the case of glucoamylase, dextrin was proved to be best carbon source. The maximum production (12.6 U/ml) occurs at 96 h fermentation. Among the tested nitrogen sources L-asparagine was the best one for both amylolytic enzymes. The initial pH of fermentation medium has significantly effects on amylolytic enzymes production by *T. lanuginosus* ATCC 34626. The optimum pH of fermentation medium was found and fixed at 4.9 by using 100 mM citrate buffer for the production of amylolytic enzymes. Response Surface Method (RSM), one of modern optimisation techniques, was applied for searching the optimum concentration of components of media for amylolytic enzyme production. To evaluate data collected from experimental design the second-order polynomial model was applied. This polynomial model was fitted at significance level 95 % ($P<0.05$) for both α-amylase and glucoamylase according to statistical software (SPSS 9.0 for MS Windows). The developed composition of media was checked experimentally. The concentration of L-asparagine was adjusted to 0.75 %. The concentration of starch was set at 6.5 % for α-amylase and 2 % for glucoamylase. The effects of concentration of K_2HPO_4 and KH_2PO_4 were also investigated. Using the newly developed

media with optimised concentration of components, *T. lanuginosus* ATCC 34626 produce about 260 U/ml and 23 U/ml activities in the case of α-amylase and glucoamylase, respectively. This was about 5 times higher than when using standard conditions. The detailed results are summarised on Appendix 3.

4.3.4 Purification and characterisation of amylolytic enzymes from thermophilic fungus *Thermomyces lanuginosus* strain ATCC 34626

The purification and characterisation of amylolytic enzymes (α-amylase and glucoamylase) secreted by *T. lanuginosus* were evaluated. Amylolytic enzymes from *Thermomyces lanuginosus* ATCC 34626 were purified to electrophoretic homogeneity using precipitation with ammonium sulphate, ion-exchange chromatography with DEAE-Sepharose fast flow and Q Sepharose fast flow and gelfiltration with Sepharose CL-6B. This purification protocol gave 27 % and 23 % of recovery with purification fold of 16.7 and 8.6 in the case of α-amyalse and glucoamylase, respectively. The molecular mass of purified α-amylase and glucoamylase were estimated to be 61 kDa and 75 kDa, respectively, using 1-D-gelanalysis software v2.4b (Signum/Biotech Fisher GMBH, Germany). Their pI values were calculated to be 3.5 - 3.6 and 4.1 - 4.3. The amylolytic enzymes from *T. lanuginosus* exhibit pH optima in the range 4.6 – 6.6 in the case of α-amylase and 4.4 - 5.6 in the case of glucoamylase. Both purified enzymes have temperature optima at 70 ^{o}C. Effects of numerous metal ions and compounds on amylolytic enzyme activities were investigated. Zn^{++} ions strongly inhibit both enzyme activities. Mn^{++} and Fe^{++} ions are activators in the case of glucoamylase; Ca^{++} and Ba^{++} are activators in the case of α-amylase. With half-life times longer than one day at 60 °C both enzymes prove to be thermostable in the pH range 4.5 - 8.5. The amylolytic enzymes from *T. lanuginosus* loose activities rapidly when incubated at temperature higher 80 ^{o}C or at pH lower than 4.0. In the presence of 10 mM Ca++ ion the half-life time of α-amylase was 5 times longer than absence of this one. In the case of glucoamylase the half-life time was 3 times longer in presence of 10 mM α-cyclodextrin. Both enzymes are found to be glycosylated; 8.5 % carbohydrate in the case of α-amylase and 3.3 % in the case of glucoamylase (Appendix 4).

The K_m and V_{max} of α-amylase on soluble starch were 0.68 mg/ml and 45.19 U/mg, respectively. The K_m values of glucoamylase on maltose, maltotriose, maltotetraose, maltopentaose and soluble starch were 6.5 mM, 3.5 mM, 2.1 mM, 1.1 mM and 0.8 mg/ml, respectively.

The first 37 residues of *N*-terminal of the purified α-amylase of *T. lanuginosus* ATCC 34626 were sequenced. Almost complete homology with the α-amylase from *Aspergillus oryzae* and *Emericella nidulans* was observed. The sequence alignment was done using protein database available on internet (http://pbil.univ-lyonl.fr/BLAST/). The results of sequence analysis suggested that α-amylase from *T. lanuginosus* ATCC 34626 should belong to hydrolases class 13 (Appendix 4).

4.4 New scientific results

β-Fructofuranosidase from Aspergillus niger

- A method was developed for extraction and purification to homogeneity of β-fructofuranosidase from *Aspergillus niger*. The physicochemical properties of purified β-fructofuranosidase were also determined. The enzyme consists of at least two monomers. Molecular mass of each was 115-135 kDa according to SDS-PAGE and the major and minor monomers have isoelectric point 5.4 and 4.4, respectively.
- The bioconversion time (72 hours) was determined and fixed to reach maximum amount of fructooligosaccharides during transfructosylation applying β-fructofuranosidase from *Aspergillus niger*.
- A method for purification of fructooligosaccharides to homogeneity was developed. For gelfiltration two BioGel P-2 columns connected in series were applied using distilled water as eluent.

Thermostable amylolytic enzymes from Thermomyces lanuginosus

- New fermentation media were developed by applying experimental design methods to production of α-amylase and glucoamylase enzymes by *Thermomyces lanuginosus* ATCC 34626 strain. Applying these media in the case of α-amylase five-times and in the case of glucoamylase ten-times higher activities were detected than when the original medium was used.
- Methods were developed for the purification of α-amylase and glucoamylase enzymes from *Thermomyces lanuginosus*. The molecular mass and isoelectric point of α-amylase was calculated to be 61 kDa and 3.5-3.6, respectively, while molecular mass of glucoamylase was 75 kDa and its isoelectric point was in the range between 4.1 and 4.3.
- New concept for the evaluation of a thermostability of thermostable enzyme was introduced. Investigating the effects of pH and temperature as well as their interaction and selecting the half-life time of the enzyme as dependent factor, model can be constructed using Response Surface Method. Application of this methodology makes possible the objective characterisation and comparison of enzyme from different sources.
- The kinetic parameters of α-amylase and glucoamylase from *Thermomyces lanuginosus* on various substrates (soluble starch, maltose, maltotriose, maltotetraose and maltopentaose) were determined.
- The first 37 residues from *N*-terminal of α-amylase from *Thermomyces lanuginosus* ATCC 34626 were determined. Using database search (http://pbil.univ-lyonl.fr/BLAST/) the segment shows strong homology with sequences of α-amylase isolated from *Emericella nidulans* and *Aspergillus species.* Based on these results the α-amylase from *T. lanuginosus* can be classified into carbohydrolase class 13.

5. Summary

Enzymes play important roles in production of various products that can be applied in food and beverage industry as well as in production of fine chemicals and pharmaceuticals. Enzymatic methods have already replaced some conventional chemical processes. Nowadays, most of them are produced by fermentation using microorganisms. The enzyme market is grown dynamically at the level of about 10 % annually. At present, the most commonly used enzymes in biotechnology are hydrolytic enzymes, which catalyse the breakdown of large biopolymers into smaller units. Belonging to them, carbohydrolases are now widely used in food and beverage industry especially in production of bioalcohol, high glucose, maltose or fructose syrups as well as thiosugars. In the last few decades, the interest in transferase activities of carbohydrolases increased because they are able to catalyse the transfer reaction to produce oligosaccharides such as fructooligosaccharides, galactooligosaccharides, thiosugars and cyclodextrins. Because of promising biological properties of these oligosaccharides, in the past years they have been gaining substantial attention in human nutrition, animal feeding and on medical fields as well as in analytical chemistry. New developments, especially in the synthesis and medical chemistry of thio-sugars have become important for carbohydrate drug design. In the present thesis on the one hand *Aspergillus niger* was selected to investigate the properties of β-fructofuranosidase as well as the production and purification of fructooligosaccharides synthesised by this enzyme. On the other hand thermophilic fungus *Thermomyces lanuginosus* was selected for research topic dealing with thermostable amylolytic enzymes. Moreover, the understanding of these enzymes will help us in the future to develop new types of fungal α-amylase or more thermostable glucoamylase as well as complex enzyme preparations containing both amylolytic enzymes that work under the same conditions with synergistic effect. Using these enzyme preparations, the "one step" technology of starch biodegradation can be realised.

β-Fructofuranosidase from Aspergillus niger

- β-fructofuranosidase activity of some *Aspergillus niger* strains was screened and the IMI 303386 strain was selected for future studies. This strain was able to produce higher levels of intra- and extracellular β-fructofuranosidase and inulinase on inulin than on sucrose. Inulinase hydrolysed inulin in an exo-fashion and released mainly fructose.
- These enzymes are stable in frozen forms for at least 2 weeks, but after precipitation with ammonium sulphate the stability of the enzymes decreased for 4-5 days.
- The intracellular β-fructofuranosidase from *A. niger* IMI 303386 was purified to homogeneity according to SDS-PAGE by various steps by $(NH_4)_2SO_4$ precipitation, DEAE Sepharose Fast Flow ion-exchange chromatography and Ultrogel AcA44 gelfiltration. This protocol gave fifty-fold purification and 42 % recovery of enzyme activity.

- The molecular mass of β-fructofuranosidase monomer from *A. niger* was estimated to be in the range between 115 and 135 kDa. This enzyme shows two different monomers on isoelectric focusing with agarose gel. The pI value of the major and the minor monomers was calculated to be 5.4 and 4.4, respectively.
- The β-fructofuranosidase showed maximal transferase activity when it was incubated at 55 ^{o}C and pH 5.5. Among to the investigated metal ions and other compounds Ba^{++}, Mg^{++}, Ca^{++} and EDTA are activators, while Hg^{++}, Ag^{+} and Ni^{++} are strong inhibitors of β-fructofuranosidase activity.
- The enzyme is completely stable in the pH range from 4 to 8 and up to 55 ^{o}C. More than 90 % of residual activity was recovered after 5-hour incubation. The enzyme lost its activity drastically when it was incubated at temperature higher than 60 ^{o}C.
- β-Fructofuranosidase from *A. niger* IMI 303386 is a glycoprotein and its carbohydrate content was calculated to be approximately 17 %.
- Intracellular β-fructofuranosidase from sucrose-based medium catalysed the best transfructosylating reaction. The concentration of fructooligosaccharides reached a maximum using 25 % (w/v) sucrose as substrate at 72 h.
- The purification procedure was carried out applying gelfiltration using BioGel P-2 columns. The main fructooligosaccharide components were kestose and nystose. These components were purified to homogeneity according to HPLC analysis.

Thermostable amylolytic enzymes from Thermomyces lanuginosus

- *Thermomyces lanuginosus* strains were cultured on different media. On potato-dextrose agar their colony have reached diameter of 6-8 cm at 45°C after three-day incubation. The structure and the colours of their surface were different. Deviations of colony morphology are observed among the various strains. During the growth some strains showed segregation of their fructification properties.
- A new rapid method was developed to screen *Thermomyces lanuginosus* strains for production of amylolytic enzymes. The screening was carried out applying this method on solified medium containing soluble starch. The utilisation of starch was detected by the iodine vapour method. The ratios of clear zone to colony were calculated and according to these values the strains were ranked. These results were confirmed in fermentation trials assaying amylolytic activities of the individual strains. The ATCC 34626 strain, which secretes appreciable amount of both amylolytic enzymes (α-amylase and glucoamylase), was selected for further studies.
- The pH of ferment broth has significant influences on amylolytic enzyme activities during fermentation. The maximal enzyme activities were measured when applying fermentation media prepared with 100 mM citrate buffer pH 4.9.
- *Thermomyces lanuginosus* grew very well on all tested carbon sources such as soluble starch, native starch, glucose, maltodextrin, dextrin, maltose, dextran and amylopectin. In

the case of α-amylase the maltodextrin, in the case of glucoamylase dextrin substrates proved to be good for enzyme production by *T. lanuginosus*, but the fungus also secreted high levels of amylolytic enzymes on native and soluble starch.

- Among the tested inorganic and organic nitrogen sources, L-asparagine was the best. Cultivating the *T. lanuginosus* ATCC 34626 strain on yeast extract the gained enzyme activities were half of that, which were detected on L-asparagine.
- To determine the optimal concentration of carbon and nitrogen sources, the Response Surface Method (RSM) was applied. The optimal concentration of soluble starch and L-asparagine for α-amylase were 6.5 % and 0.75 %, for glucoamylase were 2.0 % and 0.75 %, respectively. Calculated values were confirmed experimentally.
- The concentration of K_2HPO_4 and KH_2PO_4 in fermentation media were also optimised and confirmed.
- The following media were suggested for the production of amylolytic enzymes (α-amylase and glucoamylase) using *Thermomyces lanuginosus* ATCC 34626 strain:
  - α-amylase: soluble starch 6.5%, L-asparagine 0.75%, KH_2PO_4 0.15%, K_2HPO_4 0.1 %, $MgSO_4.7H_2O$ 0.05%, Vogel's trace element solution 0.1 mL
  - glucoamylase: soluble starch 2.0 %, L-asparagine 0.75%, KH_2PO_4 0.15%, K_2HPO_4 0.1 %, $MgSO_4.7H_2O$ 0.05%, Vogel's trace element solution 0.1 mL

  Both media should be prepared with 100 mM citrate buffer pH 4.9.
- The extracellular amylolytic enzymes from *T. lanuginosus* were purified to homogeneity according to SDS-PAGE applying multi-steps procedures. Different techniques were applied such as precipitation with ammonium sulphate, ion-exchange chromatography with DEAE Sepharose Fast Flow, gelfiltration with Sepharose CL-6B or Superose 12, ion-exchange chromatography with Q Sepharose Fast Flow. Applying these protocols approximately 17-fold purification and 27 % of recovery in the case of α-amylase, and 8.6-fold purification and 23 % of recovery in the case of glucoamylase were achieved.
- The molecular mass of purified α-amylase and glucoamylase were estimated to be 61 kDa and 75 kDa, respectively, according to SDS-PAGE using 1-D-gelanalysis software v2.4b (Signum/Biotech Fisher GMBH, Germany). Their pI values were calculated to be 3.5 - 3.6 and 4.1 - 4.3, respectively.
- The amylolytic enzymes from *T. lanuginosus* exhibit pH optima in the range between 4.6 and 6.6 in the case of α-amylase and 4.4 - 5.6 in the case of glucoamylase. Both purified enzymes have temperature optima at 70 ^{o}C. Effects of numerous metal ions and compounds on amylolytic enzyme activities were investigated. Zn^{++} ions strongly inhibit both enzyme activities. Mn^{++} and Fe^{++} ions are activators in the case of glucoamylase; Ca^{++} and Ba^{++} are activators of α-amylase.
- The accepted hypothesis of the work was that enzymes with half-life times longer than 12 hours at 60 °C are regarded as thermostable. Based on this hypothesis both amylolytic

enzymes are thermostable in the pH range between 4.5 and 8.5. The amylolytic enzymes from *T. lanuginosus* loose activities rapidly when incubated at temperature higher 80 ^{o}C or at pH lower than 4.0. In the presence of 10 mM Ca^{++} ion the half-life time of α-amylase was five-times longer than in the absence of it. The half-life time of glucoamylase was three-times longer in the presence of 10 mM α-cyclodextrin.

- ❖ Both enzymes are found to be glycosylated; α-amylase contains 8.5 % carbohydrate and glucoamylase 3.3 %. The major monosaccharides were mannose, glucosamine and galactosamine.
- ❖ The K_m and V_{max} of α-amylase on soluble starch were 0.68 mg/ml and 45.19 U/mg, respectively. The K_m values of glucoamylase on maltose, maltotriose, maltotetraose, maltopentaose and soluble starch were 6.5 mM, 3.5 mM, 2.1 mM, 1.1 mM and 0.8 mg/ml, respectively.
- ❖ The first 37 residues of *N*-terminal of the purified α-amylase of *T. lanuginosus* ATCC 34626 were determined. Almost complete homology (95 %) with the α-amylase from *Aspergillus oryzae* and *Emericella nidulans* was observed. The sequence alignment was done using BLAST protein database (http://pbil.univ-lyonl.fr/BLAST/). The results of sequence analysis suggested that α-amylase from *Thermomyces lanuginosus* ATCC 34626 should belong to glycohydrolase class 13.

Aspects of biotechnological exploitation of results and future works

Aspergillus niger IMI 303386 strain is able to synthesize adequate amount of intracellular β-fructofuranosidase enzyme on medium containing sucrose. This enzyme catalyses the fructosyltransfer to build up fructooligosaccharides that can be used in the production of functional foods for human nutrition. Future works should go to different ways:

- Studies on structure of β-fructofuranosidase enzyme.
- Development of a technology for the immobilisation of β-fructofuranosidase enzyme, even immobilisation of fungus cell.
- Scaling up experiments and design of bioreactor for the production of large amount fructooligosaccharides.

The results of optimisation of media composition can be exploited in the industrial scale production of amylolytic enzymes. Applying these techniques in combination with strain development, a new technology can be developed for the production of thermostable amylolytic enzymes complex using *Thermomyces lanuginosus*.

The new concept for evaluation of thermostable enzymes gives possibilities to compare thermostable enzymes from different sources.

The *N*-terminal sequence of α-amylase from *Thermomyces lanuginosus* gives preliminary information to further works on studies of the structure of this enzyme.

6. Összefoglaló

Az enzimek fontos szerepet játszanak az élelmiszeripari termékek, a finomvegyszerek és gyógyszerek gyártásában. Jelenleg az élelmiszeripari biokonverziók jelentős részét fermentációs úton előállított enzimek segítségével valósítják meg. Az hetvenes évektől kezdve az enzimek éves forgalma világ viszonylatban dinamikus, mintegy 10 %-os növekedést mutatott. A legnagyobb mennyiségben előállított és forgalmazott enzimkészítmények a hidrolitikus enzimcsoportba tartoznak, amelyek a természetben található polimerek lebontását katalizálják. A karbohidrolázok a legáltalánosabban alkalmazott enzimek az élelmiszeriparban, pl. a bioalkohol, a glükóz, a maltóz és a fruktóz szörpök előállításában valamint a tiocukrok gyártásában. Az utóbbi évtizedben egyre nagyobb érdeklődés mutatkozik a karbohidrolázok transzferáz aktivitása iránt, mert transzfer reakciók segítségével oligoszacharidok szintetizálhatók, pl. fruktooligoszacharidok, galaktooligoszacharidok, tiocukrok és ciklodextrinek, amelyek bizonyítottan pozitív élettani hatással rendelkeznek. A PhD kutatómunkámban az *Aspergillus niger* fonalas gombát választottam a β-fruktofuranozidáz enzim termeltetésére és tanulmányozására. Az amilolitikus enzimekkel kapcsolatos kutatásokban, pedig *Thermomyces lanuginosus* fonalas gombát alkalmaztam. Az elért eredményeim az alábbiak szerint összegezhetők:

β-Fruktofuranozidáz

- Néhány *Aspergillus niger* törzs β-fruktofuranozidáz enzimtermelési képességét feltérképezve, az IMI 303386 jelzésű törzset választottam vizsgálataim tárgyául.
- Az *Aspergillus niger* IMI 303386 képes szintetizálni mind az intra- mind az extracelluláris β-fruktofuranozidáz és inulináz enzimeket, szacharózt és inulint tartalmazó tápközegeken.
- A nyers enzim stabilitása szempontjából megállapítható, hogy az ammónium-szulfáttal történő kicsapás után az enzim stabilitása csökkent (14 napról 4-5 napra).
- Az intracelluláris β-fruktofuranozidáz enzimet különböző műveletekkel (ammónium-szulfátos kicsapás, ioncserélő kromatográfia, gélszűrés) homogenitásig tisztítottam. Ezen eljárásokat alkalmazva 50-szeres tisztulási hányadost és 42 %-os kitermelést értem el.
- Az SDS gélelektroforetikus módszerek alapján a β-fruktofuranozidáz monomereinek molekulatömege 115 és 135 kDa tartományba esett. Agaróz gélen történő izoelektromos fókuszálás során két különböző izoelektromos pontú monomer különböztethető meg. Az egyik monomer pI-je 5,4, a másik pI-je 4,4 volt.
- Az *Aspergillus niger* IMI 303386 eredetű β-fruktofuranozidáz maximális aktivitást pH = 5,5-nél és 50 ^{o}C hőmérsékleten mutatott. Számos fémion és egyéb komponenseknek az enzimre gyakorolt hatását megvizsgálva megállapítottam, hogy a Ba^{++}, Mg^{++}, Ca^{++} és EDTA jelenléte pozitívan hat az enzim aktivitására, míg a Hg^{++}, Ag^{+} és Ni^{++} inaktiválja az enzimet.

- Az enzim 5 órán át tartó 55 °C-nál kisebb hőmérsékleteken és pH = 4-8-ig terjedő tartományban történő inkubálása során aktivitásának több mint 90 százalékát megtartotta. A β-fruktofuranozidáz enzim aktivitását gyorsan elvesztette 60 °C-nál magasabb hőmérsékleten történő inkubálás során.
- Az *Aspergillus niger* eredetű β-fruktofuranozidáz enzim által katalizált transzfruktozilációs reakcióban 72 óra után értem el a maximális fruktooligoszacharid koncentrációt 25 % szacharóz szubsztrátumon. A főbb fruktooligoszacharidok a kesztóz és a nisztóz voltak, amelyet BioGel P-2 oszloppal sikerült elválasztani a kísérő komponenstől.

Termostabilis amilolitikus enzimek

- A *Thermomyces lanuginosus* törzsek előzetes szkrínelését amilolitikus enzimtermelésre keményítő tartalmú, szilárd tápagaron végeztem. A keményítő hasznosításának kimutatása jód gőz alkalmazásával történt. A feltisztulási zóna és a telep átmérő hányadosa alapján rangsoroltam az egyes törzseket, melyet szubmerz fermentáció során hagyományos aktivitásmérési módszereket alkalmazva is megerősítettem.
- Ennek alapján kiválasztottam az ATCC 34626 jelzésű törzset a további vizsgálatokra, mivel ez a törzs mind megfelelő α-amiláz, mind glükoamiláz aktivitással rendelkezett.
- Megállapítottam, hogy a fermentlé pH-ja jelentős hatással van az enzimaktivitások alakulására. A fermentációs táptalaj pufferolásával (pH=4,9) 96 óra után tapasztaltam a maximális enzimaktivitásokat.
- A *T. lanuginosus* fonalas gomba jól szaporodik számos szénforráson pl. vízoldható keményítő, natív keményítő, glükóz, maltodextrin, maltóz, dextrán, amilopektin stb.. A gomba képes az előbb említett szénforrások mindegyikén amilolitikus enzimeket szintetizálni. Az α-amiláz esetén a maltodextrin bizonyult a legjobb szénforrásnak, míg glükoamiláz esetén a dextrin.
- Számos szerves és szervetlen nitrogénforrást megvizsgálva megállapítottam, hogy az L-aszparagin a legjobb nitrogénforrás az enzimtermelés szempontjából. Ezt az élesztőkivonat követte.
- Az optimális szén- illetve nitrogénforrás koncentráció megállapítására hatásfelületi módszert (Response Surface Method, RSM) alkalmaztam. A mért adatok értékelésére SPSS 10.0 for MS Windows statisztikai szoftver csomagot használtam. A prognosztizált adatokat kísérletesen megerősítve megállapítottam, hogy a maximális amilolitikus enzimtermelés α-amiláz esetén 6,5 % keményítő és 0,75 % L-aszparagin, glükoamiláz esetén pedig 2 % keményítő és 0,75 % L-aszparagin tartalmú tápközegen érhető el.
- A K_2HPO_4 és KH_2PO_4 optimális koncentrációinak és arányának meghatározását szintén elvégeztem.

- Az optimalizálási eredmények birtokában az alábbi összetételű tápközegeket ajánlom *T. lanuginosus* ATCC 34626 törzs segítségével történő amilolitikus enzimek termelésére szubmerz technológiával:

  - α-amiláz: vízoldható keményítő 6,5 %, L-aszparagin 0,75 %, KH_2PO_4 0,15 %, K_2HPO_4 0,1 %, $MgSO_4.7H_2O$ 0,05 %, Vogel-féle nyomelem oldat 0,1 mL

  - glükoamiláz: vízoldható keményítő 2,0 %, L-aszparagin 0,75 %, KH_2PO_4 0,15 %, K_2HPO_4 0,1 %, $MgSO_4.7H_2O$ 0,05 %, Vogel-féle nyomelem oldat 0,1 mL

  Mindkét fermentációs táptalaj 100 mM citrát pufferben (pH=4,9) készítendő.

- Az amilolitikus enzimek kinyerésére és tisztítására több lépésből álló tisztítási eljárásokat dolgoztam ki, amelyek segítségével megfelelő enzim mennyiséget állítottam elő enzim jellemzési céljaim megvalósításához. Ezen eljárással 16,7-szeres tisztulást és 27 % kitermelést értem el α-amiláz esetében, míg glükoamiláz esetén 8,6-os tisztulást és 23 % kitermelést.
- Gélelektroforézissel (SDS-PAGE) a *T. lanuginosus* α-amiláz molekulatömege 61 kDa volt. Az izoelektromos pontja pedig 3,5-3,6 adódott agaróz és poliakrilamid gélen. Glükoamiláz esetében a molekulatömegnek 75 kDa adódott és a pI-e 4,1-4,3 volt.
- Mind a két enzimnek meghatároztam az optimális működési paramétereit a maximális aktivitás értékekben. Az α-amiláz esetében a pH optimum tartománya 4,6-6,6 volt, glükoamiláz esetén pedig 4,6-5,6. Mindkét enzim hőmérséklet optimuma 70 °C volt. A Zn^{++} ion erősen gátolja a *T. lanuginosus* amilolitikus enzimeinek aktivitását, a Mn^{++} és Fe^{++} ionok pozitív hatást gyakorolnak a glükoamiláz aktivitására, a Ca^{++} és Ba^{++} pedig az α-amiláz aktivitására.
- Új koncepciót vezettem be az egyes enzimek termostabilitásának tanulmányozására. Az eddigi alkalmazott vizsgálatok szerint általában külön-külön határozzák meg a pH és a hőmérséklet hatását az enzim stabilitására vonatkozóan és önkényesen határozzák meg az inkubálás időtartamát. Ezzel szemben itt a két faktor együttes hatását is vizsgáltam és a felezési időt tekintettem függő változónak. Így matematikai-statisztikai módszerek alkalmazásával, célfüggvények segítségével pontos modell állítható fel az enzimek stabilitására vonatkozóan. Vizsgálataim során azt az enzimet tekintettem termostabilisnak, amely 60 °C-on inkubálva 12 óránál nagyobb felezési idővel rendelkezik. Ezen hipotézis alapján arra a következtetésre jutottam, hogy a *T. lanuginosus* gombának mind az α-amiláz, mind a glükoamiláz enzime stabilisnak tekinthető 60 °C-on és a pH 4,5-től 8,5-ig terjedő tartományban.
- Egyes ionok vagy komponensek hatással lehetnek az enzimek stabilitására. A *T. lanuginosus* α-amiláza esetén Ca^{++} jelenlétében a felezési idő 5-ször nagyobb, mint nélküle.

Glükoamiláz esetén pedig az α-ciklodextrin jelenlétében 3-szor nagyobb felezési időt találtam.

- A *T. lanuginosus* eredetű α-amiláz gyorsan bontja mind a vízoldható, mind a natív keményítő szubsztrátumokat. A vízoldható keményítő esetén a K_m érték 0,68 mg/ml, a V_{max} pedig 45,19 U/mg volt.
- A *T. lanuginosus* eredetű glükoamiláz K_m értéke a maltóz, maltotrióz, maltotetraóz, maltopentaóz és vízoldható keményítő szubsztrátumokon 6,5 mM, 3,5 mM, 2,1 mM, 1,1 mM és 0,8 mg/ml volt. Ezen enzim képes bontani a maltóz szubsztrátumot, de nagyon kicsi sebességgel.
- Meghatároztam a *T. lanuginosus* ATCC 34626 törzs által termelt α-amiláz fehérjének *N*-terminális első 37 aminosav sorrendjét. A szekvencia analízist a BLAST fehérje adatbázis (http://pbil.univ-lyonl.fr/BLAST/) felhasználásával végeztem el és megállapítottam, hogy ez a szekvencia 95 %-os homológiát mutat az *Aspergillus niger* és az *Emericella nidulans* által szintetizált α-amiláz aminosav sorrendjével. Ennek alapján a *T. lanuginosus* eredetű α–amilázt a glikohidrolázok 13. csoportjába soroltam.

Eredmények hasznosítási lehetőségei

- Az *Aspergillus niger* IMI 303386 törzs megfelelő tápközegen képes megfelelő mennyiségű β–fruktofuranozidáz enzimet szintetizálni. Ez az enzim alkalmas fruktooligoszacharidok gyártására, amely prebiotikumként hasznosítható funkcionális élelmiszerek előállításánál.
- Megfelelő enzimmennyiség előállítása után lehetővé válik az enzimszerkezet feltárása és az enzimrögzítési módszerek kidolgozása.
- A laboratóriumi enzimes szintézisek továbbfejlesztésével megvalósítható a léptéknövelés, esetleg rögzített enzimes technológia a fruktooligoszacharidok előállítására.
- A táptalaj összetétel optimalizálás eredményei alapját képezhetik egy ipari léptékű enzimtermelés megvalósításának. Törzsnemesítéssel párosítva – megítélésem szerint – olyan technológiát lehet kifejleszteni, amely alkalmas a termostabilis amilolitikus enzimkomplex előállítására.
- Az enzim stabilitás meghatározásában bevezetett új koncepció a matematikai-statisztikai módszereket használva lehetőséget ad a termostabilis enzimek fogalmának tényszerű és egységes megfogalmazására, valamint a különböző forrásból származó enzimek összehasonlítására a stabilitás szempontjából.
- Az α-amiláz elsődleges szerkezetismerete alapot szolgáltathat a molekuláris szintű mechanizmus tanulmányozásához, amely az enzim térbeli szerkezetét, a konformációját és katalitikus mechanizmusát is magába foglalja. Ez pedig utat nyit az enzimmérnökség felé, ahol tudatos módosításokkal a számunkra kedvező és specifikus tulajdonságú enzimek alakíthatók ki.

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Appendix 1

Production, purification and identification of fructooligosaccharides produced by β-fructofuranosidase from *Aspergillus niger* IMI 303386

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Abstract

Aspergillus niger IMI 303386 produced higher levels of intra- and extracellular β-fructofuranosidase and inulinase on inulin than on sucrose. Intracellular β-fructofuranosidase from sucrose medium catalysed the best transfructosylation reaction. The concentration of fructooligosaccharides (FOS) reached a maximum in 72 h with 25 % (w/v) sucrose. The FOS were purified and the main products were kestose and nystose. Inulinase hydrolysed inulin in an exofashion and released mainly fructose.

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Introduction

Nowadays the production and application of fructooligosaccharides (FOS) have gained tremendous commercial importance because of their favourable functional properties. These include: (a) improving the intestinal microflora, (b) relieving the constipation, (c) decreasing the total cholesterol and lipid in serum, (d) the promotion of animal growth and (e) as low calorie non-cariogenic sweetners (Chen & Liu 1996). Currently, FOS are produced commercially using either microbial fructosyltransferase (EC 2.4.1.9) or β-fructofuranosidase (EC 3.2.1.29) with high transfructosylation activity (Hayashi *et al.* 1992). However, commercial FOS contains high levels of glucose, fructose and sucrose which are released as by-products (Yun *et al.* 1994). Thus, in commercial products, FOS can account for only 55-60 % of the total dry weight (Yun *et al.* 1997). To overcome this problem, suitable methods for the separation and identification of FOS are important. The present paper reports on the production of FOS by crude enzyme from *Aspergillus niger* IMI 303386, as well as their separation and identification. In addition, the effect of sucrose and inulin on the production of intra and extracellular β-fructofuranosidase by *A. niger* was studied.

Materials and methods

Materials

Aspergillus niger IMI 303386 was purchased from the International Mycological Institute, Surrey, UK and maintained on PDA (potato-dextrose-agar) slopes. The Bio-Gel P-2 resin and $3NH_2$-Spherisorb column were from Bio-Rad and HPLC chromatography, respectively. All remaining chemicals used were analytical grade and purchased either from Sigma or BDH.

Cultivation of A. niger and production of β-fructofuranosidase

The spore suspension was prepared by adding 5 mL sterile distilled water into an *A. niger* PDA slope. This was used to inoculate 250 mL conical flask containing 100 mL pre-culture medium with composition as follows (w/v): glucose, 1 %; yeast extract, 1 %; $NaNO_3$, 2 %; $MgSO_4.7H_2O$, 0.05 %; K_2HPO_4, 0.5 %. The pH was adjusted to 7.4 using 2 % K_2HPO_4 before autoclaving. The pre-culture was grown for 24 h at 28 ^oC and in orbital shaker (200 rpm) and 15 mL mycelial suspension was used to inoculate 300 mL of medium in 1 L flask which contained (w/v) sucrose 2 % or inulin, 1 %;L-asparagine, 0.4 %; K_2HPO_4, 0.1 %; $MgSO_4.7H_2O$, 0.05 %; $CaCl_2$, 0.01 % and 0.1 % of trace element solution with the following composition: 0.5 g $FeSO_4.7H_2O$, 0.16 g $MnSO_4.4H_2O$, 0.14 g $ZnSO_4.7H_2O$, 0.02 g $CoCl_2.6H_2O$ in 100 mL distilled water. The fungus was cultured for 48 h under the same conditions as pre-culture.

Enzyme extraction

From sucrose medium, the mycelia was collected by filtration using Whatman No. 1 filter paper and washed three times with 0.1 M phosphate buffer, pH 6.5. The wet mycelia were ground with approx. 2 times of sand and minimal amount of buffer. The homogenised mycelia were

centrifuged at 15000 x g for 20 min and the supernatant was used as an intracellular enzyme. The culture filtrate from inulin medium was used as an extracellular enzyme, whereas the intracellular enzyme was extracted in the same way as described in case of sucrose medium.

Enzyme activity assay

Hydrolytic and transfructosylating activities

A 3 mL reaction mixture containing 0.75 mL of 0.2 M phosphate buffer pH 6.5, 1.5 mL of 5 % (w/v) sucrose solution and 0.65 mL distilled water was pre-incubated at 50 ^{o}C for 10 min. A 0.1 mL of appropriately diluted enzyme was added, and the incubation continued for further 15 min. The reaction was terminated by placing the tubes in polyethylene glycol 400 boiling bath for 10 min. After cooling, a two and one mL of reaction mixture were used for determining the reducing sugar and glucose by Somogyi/Nelson (Nelson 1944, Somogyi 1952) and glucose oxidase-peroxidase (Wood & Bhat, 1988) methods, respectively. The colour developed in both cases was read at 520 nm and converted into reducing sugar and glucose using glucose standard curve prepared under identical conditions. The hydrolytic activity was determined by measuring the reducing sugar (R) released from sucrose as described above. The transfructosylating activity was determined by measuring both reducing sugar and the glucose (G) released from sucrose as described above, and by using the following equations. The concentrations of free fructose (F) and transferred fructose (F') in the reaction mixture (Chen & Liu 1996) were calculated as follows:

$F = R - G$

$F' = G - F = 2G - R.$

One unit of hydrolytic activity was defined as the amount of enzyme required to release 1 μmol reducing sugar per min. One unit of transfructosylating activity was expressed as μmol of fructose transferred per min.

Stability of β-fructofuranosidase

The stability of crude and $(NH_4)_2SO_4$ precipitated β-fructofuranosidase during storage at -20^{o}C was tested for 14 days by measuring the hydrolytic activity.

Action of β-fructofuranosidase on sucrose

A 5mL reaction mixture containing 2.5 mL of 50 % (w/v) sucrose, 1.25 mL of 0.2 m phosphate buffer pH 6.5, two units of β-fructofuranosidase (based on hydrolytic activity) and distilled water was incubated at 39 ^{o}C for 96 h. The aliquots (0.5 ml) were taken at different time intervals and 0.1 ml of each was used to determine the reducing sugar by Somogyi/Nelson method. The remaining sample (0.4 ml each) was deionised using Bio-Rad mixed bed resin (AG 501-X8) and analysed by HPLC using a $3NH_2$-Spherisorb column at a flow rate of 0.75 ml/min with acetonnitrile-water (75:25, v/v) mobile phase and an Evaporative Light-Scattering detector.

Production and purification of FOS

The production of FOS was carried out using 20 units of β-fructofuranosidase in a 50 mL reaction mixture containing 25 ml of 50 % (w/v) sucrose, 12.5 mL of 0.2M phosphate buffer pH 6.5, 0.01 g sodium azide and 12.5 mL distilled water, for 72 h at 39 ^{o}C. The reaction was terminated before deionising the samples as described above, and freeze dried.

For the separation and purification of FOS, a 2 mL reaction sample containing 500 mg of total sugar was applied on to Bio-Gel P-2 column (90 x 1.6 cm) and eluted with distilled water at a flow rate of 15 mL.h^{-1}. Two ml fractions were collected and analysed for total sugar by phenol-sulphuric acid method (Dubois, 1962). Fractions containing sugars were also analysed by HPLC using 3NH_2-Spherisorb column as described above. The fractions containing oligosaccharides with the same retention time were pooled and freeze dried. The purity of pooled samples was rechecked by HPLC as described above.

Result and discussion

Effect of sucrose and inulin on the production of β-fructofuranosidase by A. niger

The effect of sucrose and inulin on the production of intra and extracellular β-fructofuranosidase by *A. niger* was studied. The fungus produced mainly intracellular β-fructofuranosidase on sucrose and both intra and extracellular β-fructofuranosidase on inulin. The level of extracellular β-fructofuranosidase produced on inulin was 3 and 4.5 fold higher than the intracellular β-fructofuranosidase produced on inulin and sucrose, respectively (Figure 1). Furthermore, both extra and intracellular enzyme samples obtained from inulin medium hydrolysed inulin rapidly, while that obtained from sucrose medium showed negligible activity towards inulin. Interestingly the intracellular β-fructofuranosidase obtained from sucrose medium found to catalyse transfructosylation reaction efficiently.

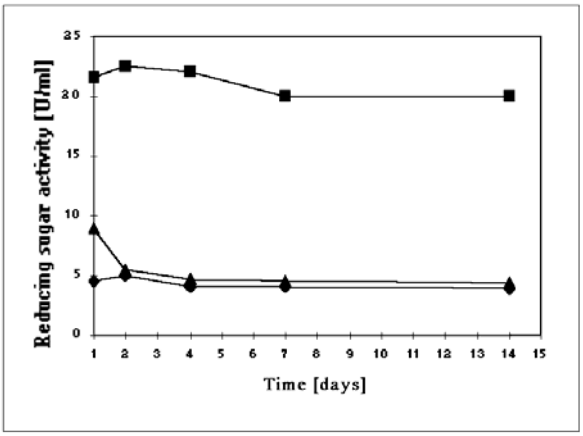

Fig. 1. Stability of β-fructofuranosidase from *A. niger* IMI 303386 during storage at -20 ^{o}C. —◆— intracellular enzyme from sucrose medium, —■— extracellular enzyme from inulin medium and —▲— intracellular enzyme from inulin medium.

Stability of β-fructofuranosidase

The crude enzyme was completely stable at least for two weeks, when stored at -20°C (Figure 1). The enzyme precipitated with $(NH_4)_2SO_4$ and redisolved in phosphate buffer, pH 6.5 and stored under the same conditions, retained only 50 % of its original activity after two weeks. These results suggested that β-fructofuranosidase from *A. niger* is more stable when stored in crude form than after $(NH_4)_2SO_4$ precipitation.

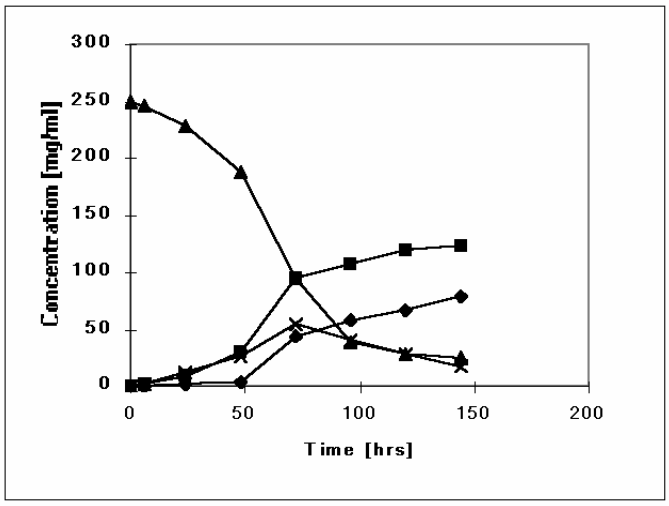

Fig. 2. Profile of formation of fructooligosaccharides produced by β-fructofuranosidase from *Aspergillus niger*.

glucose; fructose; sucrose; fructooligosaccharides

Action of β-fructofuranosidase from A. niger on sucrose

Hydrolysis of sucrose and formation of FOS by intracellular β-fructofuranosidase from *A. niger* grown on sucrose medium is shown in Figure 2.

During the initial 50 h of incubation, the sucrose was slowly converted to glucose, fructose and FOS, thereafter the sucrose conversion was rapid and reached maximum by ~120 h of incubation (Figure 2). The concentration of FOS reached maximum by 72 h and thereafter gradually decreased (Figure 2). Nevertheless, the concentrations of both glucose and fructose increased steadily throughout the incubation, but the concentration of glucose was at least two fold higher than that of fructose throughout the incubation. The concentration of kestose and nystose in the reaction mixture were approximately 62 and 5 mg/ml, respectively. Higher FOS were not present in the reaction mixture.

Action of inulinase from A. niger on inulin.

The action of inulinase from *A. niger* on varying concentration of inulin was examined. HPLC analysis revealed that the inulinase from *A. niger* hydrolysed inulin in an exo-fashion and released mainly fructose.

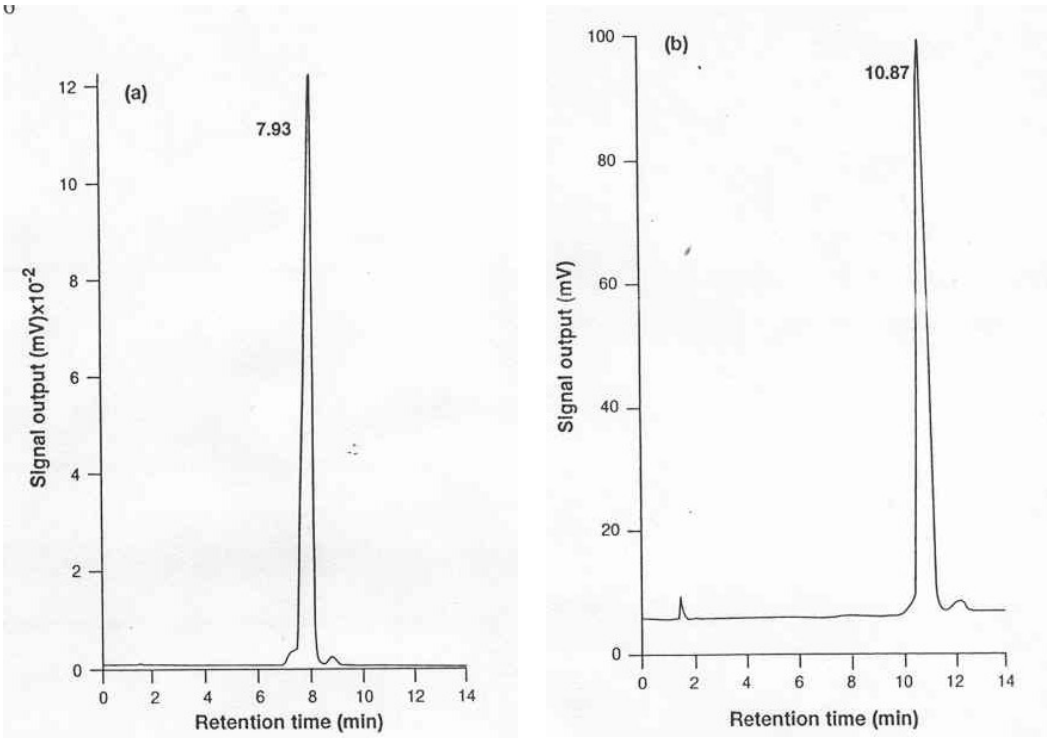

Fig. 3. Demonstration of the purity of individual fructooligosaccharides by HPLC a, kestose; b, nystose

Production and purification of FOS

FOS were produced and purified as described in Methods. It was found that β-fructofuranosidase from *A. niger* catalysed the synthesis of two FOS namely kestose and nystose. This is in agreement with the observation by Park & Almeida (1991), where they reported that a purified extracellular transfructosylase from *A. niger* catalysed the synthesis of mainly 1-kestose and nystose. In the present study, not only the conditions for the production of FOS were optimised but olso an ideal method for the purification of FOS was developed. Using Bio-Gel P-2 column chromatography, reasonably pure kestose and nystose were obtained (Figure 3). This method will provide not only better FOS for commercial purposes, but also for nutrition and clinical studies in the future.

Acknowledgement

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Appendix 2

Screening the strains of thermophilic fungus *Thermomyces lanuginosus* for amylolytic activities

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Abstracts

Eighteen strains of thermophilic fungus *Thermomyces lanuginosus* were screened for amylolytic activities. All of them produced extracellular amylolytic enzymes. Three strains were selected for detailed studies on their production of glucoamylase and α-amylase. The optimum conditions for the assays of the amylolytic activities were selected. The optimum parameters were found to be the following: 70 °C and pH 4.6 for glucoamylase and 70 °C and pH 5.0 for α-amylase.

Keywords: *Thermomyces lanuginosus*, amylolytic activity, glucoamylase, α-amylase, screening, thermophilic fungus

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Over the last few decades, there has been an increasing demand for polysaccharide-degrading enzymes all over the world. At present, most of the commercially available polysaccharidases have many disadvantages. Therefore, to meet the current demand and improve the economics of enzyme production, there is a need for enzymes which can catalyse the desired reactions under industrial conditions.

The use of amylolytic enzymes for processing starch is preferred over acid hydrolysis because of the high specificity of enzymes and the mild reaction conditions, lower energy requirements and the absence of undesirable side reactions associated with their use. However, the efficient hydrolysis of natural starch not only requires amylolytic enzymes, but also requires these enzymes to be stable and active at temperatures around 90-95 °C. α-Amylases from *Bacillus* species are stable and active between 90-95 °C (JANECEK, 1993) and are used widely in food industries and others. Their narrow pH optimum makes them less suitable for industrial processing of starch from an economic viewpoint. Furthermore, the bacteria do not produce appreciable amounts of glucoamylase, so this has to be produced from an additional source. Currently, the glucoamylase from *Aspergillus* is used in industry, but this enzyme is stable and active only up to 55-65 °C and between pH 4.0-6.0 (UNDERKOFLER, 1976; GODFREY, 1983). Therefore, a thermophilic fungus, which produces thermostable α-amylase and glucoamylase appeared to be an ideal source for developing an economically attractive method for the industrial processing of starch.

Thermomyces lanuginosus, formerly known as *Humicola lanuginosa* (DOMSCH *et al.*, 1980), had been suggested as an excellent test organism for the study of extracellular amylase from a thermophilic fungus (BARNET & FERGUS, 1971).

So far some strains of the thermophilic fungus *Thermomyces lanuginosus* have been investigated, but there has not been any comprehensive screening for evaluation of the amylolytic enzyme production of the individual strains. In the present study, we have collected and screened as many strains as possible and determined the optimal conditions for amylolytic enzymes.

Materials and methods

All chemicals used were of analytical grade and purchased either from Merck, Sigma, Reanal or other companies.

Micro-organisms

Thermomyces lanuginosus strains were originated from various culture collections. The investigated strains are the following: IMI 084400/ATCC 22070, IMI 110803, IMI 158749, ATCC 38905, ATCC 46882, IMI 140524, IMI 096213, CBS 218.34, CBS 224.63, CBS 288.54, CBS 395.62, ATCC 28083, ATCC 16455, IMI 131010, ATCC 34626, ATCC 36350, ATCC 44008 (RM-B), DSM 5828.

Maintenance of stock cultures

The strains were maintained in yeast-powder/soluble starch (YPSS) agar medium (COONEY & EMERSON, 1964), and stored under refrigeration.

Iodine vapour method for detecting amylolytic activities

The screening experiments were first carried out by the iodine vapour method based on diameter measurements. The clear zones around the colonies indicated starch degrading capability. The YPSS medium was poured into Petri dishes. The fungus was inoculated onto the surface of the media and, after cultivation of the microbe, the diameters of the colonies and the clear zones, indicated by iodine vapour, were determined. The ratio of the clear zone to the colonies was calculated for the evaluation of the results.

Cultivation and production of amylolytic enzymes by Thermomyces lanuginosus

A three-stage cultivation technique was used. In the first stage, the fungus was grown on YPSS slant agar for 8 to 10 days at 50 °C in humidified incubation. In the second stage a suspension of spores was prepared using 0.1% Triton X-100 solution. Five ml of it was added to 100 ml of glucose-asparagine medium, pH 6.0, to initiate the cultivation at 50 °C and 220 r.p.m. in an orbital shaker for 1 to 2 days to obtain a homogeneous mycelium growth. In the third stage, 10 ml of the mycelial suspension was used as inoculum for initiating the production of amylolytic enzymes in 150 ml of starch-asparagine medium (soluble starch 40 g, L-asparagine 4 g, KH_2PO_4 3 g, K_2HPO_4 2 g, $MgSO_4.7H_2O$ 0.5 g and 1 ml of VOGEL's (1956) trace elements solution was dissolved in 1 l of distilled water). Samples (10 ml) were taken under laminar box from duplicate flasks at varying times. The samples were filtered and the α-amylase and glucoamylase activities were assayed in the filtrate.

Enzyme assays

α-amylase. A reaction mixture containing 1 ml of 0.1 mol sodium-acetate buffer (pH 5.0) and 1 ml of 0.5% (w/v) soluble starch solution was mixed and pre-incubated at 50 ^{o}C for 10 min before adding 1 ml of appropriately diluted culture filtrate as an enzyme source. After 5 min the reaction was stopped by adding 1 ml of 0.5 mol HCl. The unhydrolysed starch in this aliquot was estimated by the iodine method described below. One unit of α-amylase activity was defined as the amount of enzyme that hydrolyses 1mg of soluble starch in 1 min.

Glucoamylase. One ml of reaction mixture containing 0.25 ml of 0.1mol phosphate buffer (pH 4.6) and 0.25 ml of 1% (w/v) soluble starch solution was pre-incubated at 50 ^{o}C for 10 min. Half a ml of appropriately diluted culture filtrate as an enzyme source was added, and the incubation was continued for further 15 min. The reaction was stopped by placing the tubes in a boiling bath for 30 min. After cooling the released glucose concentration was estimated by the glucose-oxidase/peroxidase method (McCOMB & YUSHOK, 1957; WOOD & BHAT,

1988). One unit of glucoamylase activity was defined as the amount of the enzyme that releases 1 μmol of glucose in 1 min.

The iodine method for starch estimation

An aliquot of the sample (starch hydrolysate) was mixed with 1 ml of iodine reagent and this mixture was adjusted up to 2 ml by distilled water. The iodine reagent contained 0.02% (w/v) of iodine and 0.2% (w/v) of KI in 0.5 N HCl. Five ml of distilled water was added to this mixture and the colour developed was read at 590 nm against blank. The amount of starch was estimated using a standard curve prepared with potato soluble starch (Merck) under the same conditions.

Results and discussion

Morphological observations of different *Thermomyces lanuginosus* strains

All investigated *Thermomyces lanuginosus* strains were found to be capable of growing in the following culture media: PDA, OA, YPSS, PSA, MA2, NUT and PYS (ATCC, 1984). The morphology of individual strains showed deviations within the range of the species. Their colonies developed in PDA and reached diameters of 6-8 cm at 45 ^{o}C within 3 days. The structures and the colours of their surfaces were very different. Pigments diffusing into the nutrient agar varied in colour from amber to purple. Variation of colony morphology was observed both among the different strains and during the growth of some strains; their fructification properties have segregation (ATCC 36350, IMI 084400). With respect to micro-morphological properties, the tested strains showed only slight differences. Their mycelia consisted of branched, partitioned hyphae with diameters between 1.5-4.0 μm in the nutrient agar. The aerial hyphae rise at right angles to the basal hyphae, which run along the surface of the medium. The unicellular spherical asexual spores appeared at the tips of the air hyphae.

Rapid screening of different strains of *Thermomyces lanuginosus*

Based on the results of iodine vapour method, all investigated strains showed a ratio of clear zone-to-colonies higher than 1(Fig. 1). This indicated that all of them produced amylolytic enzymes. The strains ATCC 34626, ATCC 44008 (RM-B) and IMI 084400, which have ratios higher than 2, showed clear differences between clear zone and colony. To confirm these results, shaken flask experiments were carried out and their results (see later) validating the previous findings.

Screening of *Thermomyces lanuginosus* strains for the production of amylolytic enzymes

The production of α-amylase and glucoamylase activities were evaluated at different times. The activity measurements were done at 50 ^{o}C and at pH 5.0 (50 mmol sodium-acetate buffer). The maximum activities reached are shown in Fig. 2. The production of α-amylase was in the range of 4.6-46.6 U ml^{-1}. With respect to α-amylase activity the strains ATCC 28083 and

ATCC 34626 looked promising (Fig. 2A). In the case of glucoamylase activity two strains (ATCC 44008 and ATCC 34626) were selected (Fig. 2B). They showed higher activities than 1.0 U ml^{-1}. Based on the experimental results three strains ATCC 28083, ATCC 34626 and ATCC 44008 deserve a more detailed study. It should be stressed that *Thermomyces lanuginosus* ATCC 34626 strain performed well with respect to both α-amylase and glucoamylase.

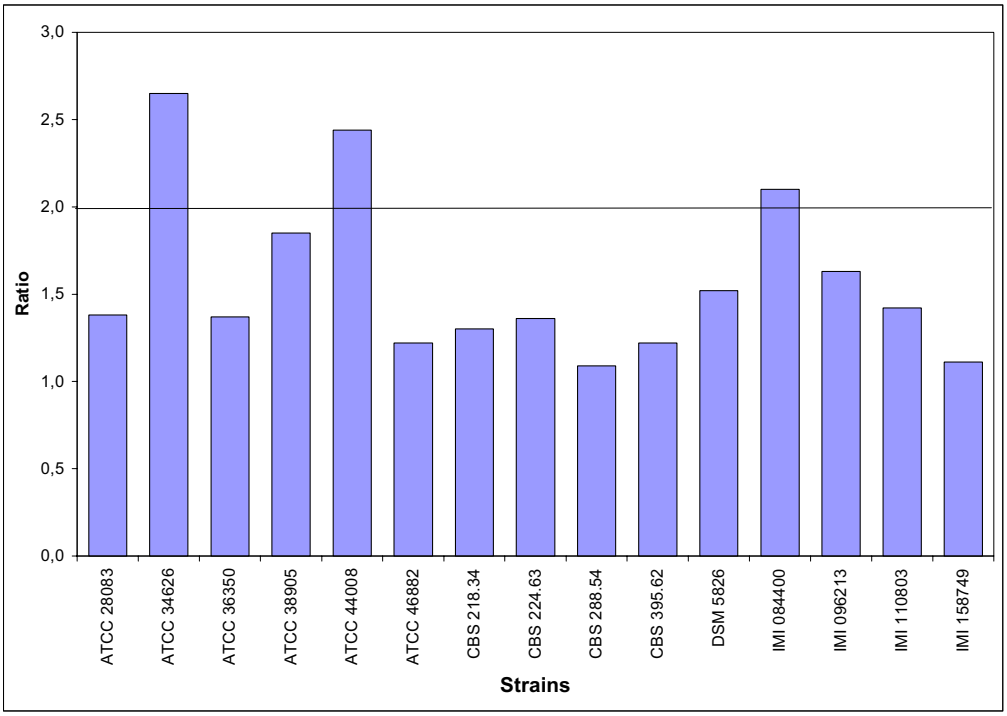

Fig. 1. Amylolytic activity of *Thermomyces lanuginosus* strains determined by iodine vapour rapid screening (YPSS medium, 50°C)

Determination of optimum parameters for activity assays

The effects of pH and temperature on the activities of α-amylase and glucoamylase were investigated. The optimum pH was determined to be in the range of 4.0-5.5 (0.1 mol sodium-acetate buffer) in the case of glucoamylase activity. 0.1 mol sodium-acetate buffer, pH 4.0-5.6, and 0.1 mol citrate/Na_2HPO_4 buffer, pH 5.6-7.0, were needed in order to determine the pH optimum of α-amylase. The optimum temperature was defined in the range of 50-85 ^oC at the optimum pH of glucoamylase and α-amylase activities.

On the basis of activity assays the pH optima of α-amylase and glucoamylase activity were 5.0 and 4.6, respectively (Fig. 3 A, B). TAYLOR and co-workers (1978) showed that glucoamylase from *Thermomyces lanuginosus* can be completely stable in the pH range 5-10 for 24 h at room temperature while losing activity at pH 4.0. Our results are consistent with their findings.

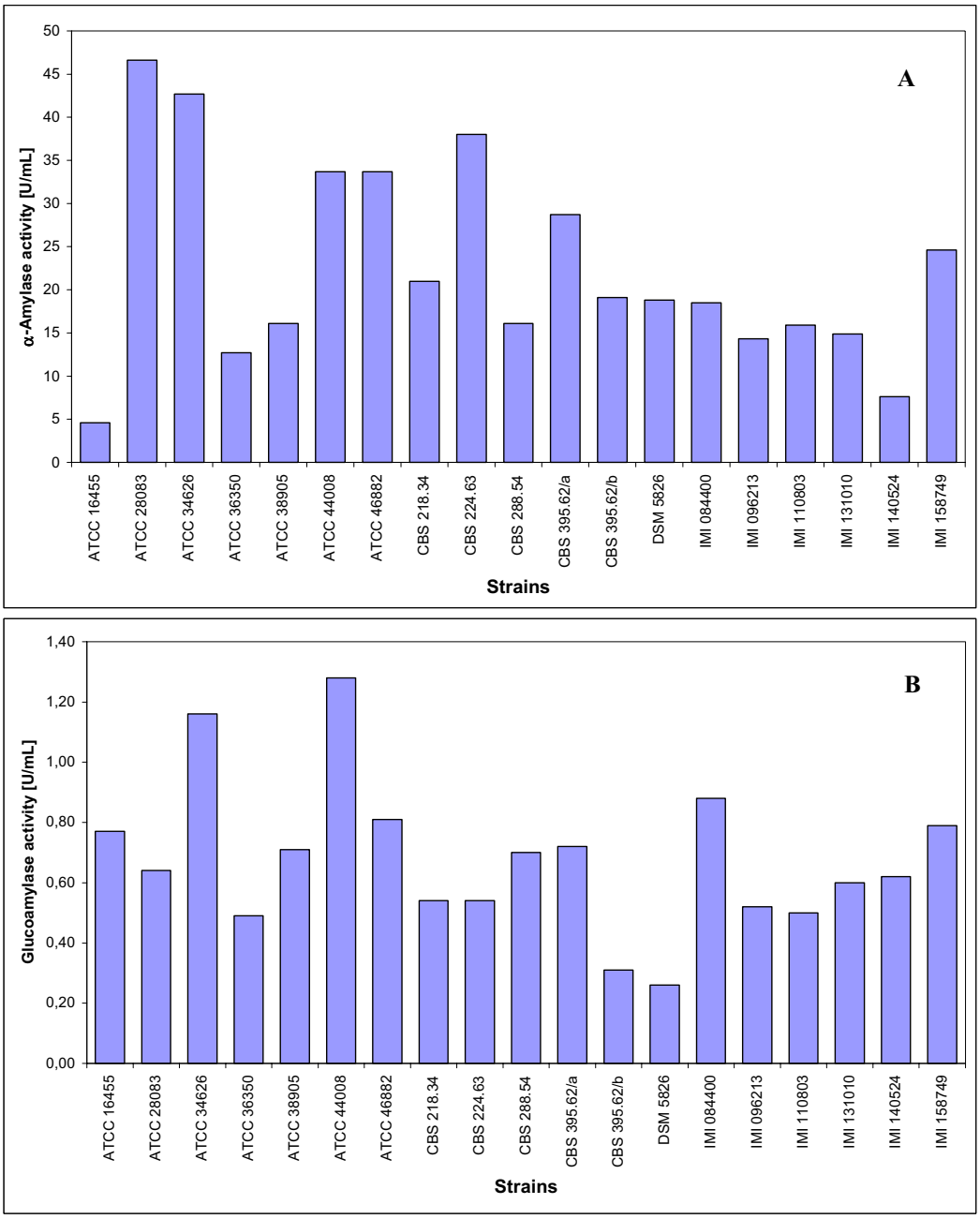

Fig. 2. Production of individual amylolytic enzymes by *T. lanuginosus* strains (with basic parameters: pH 5.0 and temperature 50 °C) A: α-amylase; B: glucoamylase

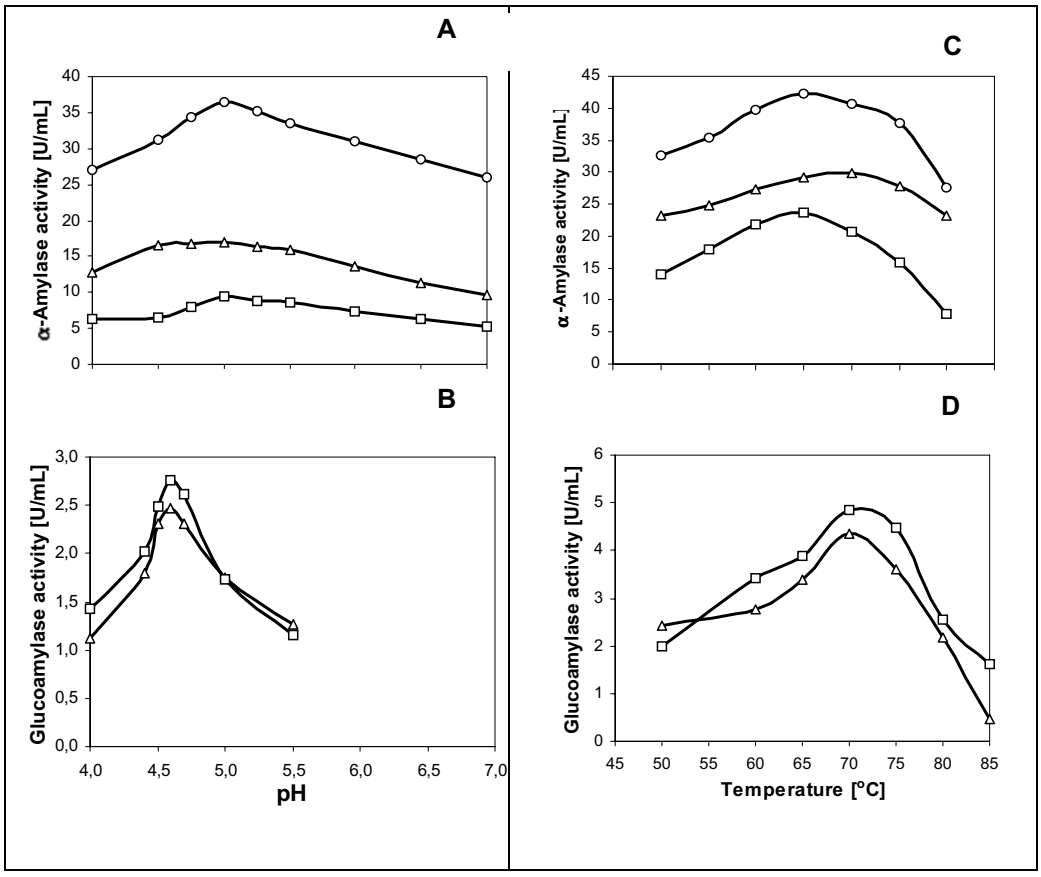

Fig. 3. Effect of pH (A, B) and temperature (C, D) on amylolytic activities from *T. lanuginosus*. :ATCC 28083; :ATCC 34626; : ATCC 44008

The crude ferment broth from different strains of *Thermomyces lanuginosus* showed different optimal temperature values at pH optimum. In case of α-amylase the optimal temperature was between 65-70 °C (Fig. 3 C), depending on the strain used. With respect to glucoamylase it was 70 °C (Fig. 3 D). This means that the optimal temperature of glucoamylase originating from *Thermomyces lanuginosus* is 10 °C higher than that from *Aspergillus niger* (60 °C, NIGAM & SINGH, 1995). Some glucoamylase preparations produced from *Aspergillus niger* have an optimal temperature at 70 °C (SAHA & ZEIKUS, 1989), but they are not very stable (MUNCH & TRITSCH, 1990). This optimum was exactly the same as that found by BASAVESWARA RAO and co-workers (1981) and HAASUM and co-workers (1991). The activities of both amylolytic enzymes have decreased drastically above 75 °C.

Applying the optimal parameters in the activity assays resulted in 30% higher and 2.0-2.5 times higher activity in case of α-amylase and glucoamylase, respectively.

Effect of Ca^{2+} ion on α-amylase activity

In investigating the effect of Ca^{2+} ion on α-amylase activity, $CaCl_2$ was applied in different concentrations ranging from 25 mmol to 200 mmol in the buffer used. The experiment was carried out under optimal conditions. The activity of α-amylase was almost twice higher than in the control without Ca^{2+} ion and reached the maximum value at 75 mmol (data not shown). These results indicated the existence of α-amylase in the ferment broth. These results are in agreement with JENSEN and co-workers (1987), who successfully separated extracellular amylolytic enzymes (α-amylase, glucoamylase and α-glucosidase) from a ferment broth of *Thermomyces lanuginosus*.

*

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Appendix 3

Optimisation of composition of media for the production of amylolytic enzymes by *Thermomyces lanuginosus* ATCC 34626

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Summary

The composition of media for the production of amylolytic enzymes by *Thermomyces lanuginosus* was optimised in different ways. Effects of various carbon and nitrogen sources were investigated. *Thermomyces lanuginosus* grown on starch, maltodextrin, dextrin, maltose, amylopectin, glucose and dextran substrates showed good α-amylase (92-125 U/mL) and glucoamylase (6-13 U/mL) activities. Among the tested nitrogen sources L-asparagine was the best one. The optimum pH of fermentation medium was found and fixed at 4.9 by using 100 mM citrate buffer for the production of amylolytic enzymes. Response Surface Method (RSM) was applied for searching the optimum concentration of components of media for amylolytic enzyme production. A second-order polynomial model was fitted at significance level 95 % ($P<0.05$) for both α-amylase and glucoamylase. The developed composition of media was checked in respect of the production of amylolytic enzymes. The concentration of L-asparagine was adjusted to 0.75 %. The concentration of starch was set at 6.5 % for α-amylase and 2 % for glucoamylase.

Key words: Thermomyces lanuginosus, glucoamylase, α-amylase, fermentation, Response Surface Method

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Introduction

Amylolytic enzyme preparations (α-amylase EC 3.2.1.1. and glucoamylase EC 3.2.1.3.) are now commercially produced by *Bacillus, Aspergillus* and *Rhizopus* species for processing starch. Barnett and Fergus *(1)* reported that extracellular amylolytic enzymes were produced by *Thermomyces lanuginosus* grown in a starch-based medium. Since then several authors worked on the screening of microorganisms for enzyme production *(2)*, isolation and production of extracellular amylases *(3,4)*, and purification and characterisation of the enzymes *(5,6)*.

The media optimisation is a relevant aspect to be considered in the development of fermentation technology. However, there are only a few reports concerning the comprehensive optimisation of medium composition. Gupta and Maheshwari *(7)* reported on the relationship between growth (dry mass) of *T. lanuginosus* ATCC 44008 and pH of the medium. Arnesen *et al. (8)* stimulated the secretion of α-amylase by adding Tween 80 to the growth medium

The traditional one-factor at a time approach to optimisation is time-consuming and unsuitable for reaching the true optimum mainly because of interaction of the factors. Response Surface Method (RSM), as an experimental strategy for seeking the optimum conditions of a multivariable system, is a much more efficient methodology for optimisation. It has been successfully applied for optimising the media composition and operating conditions in many bioprocesses.

In the present report the effects of pH, nitrogen and carbon sources, K_2HPO_4 and KH_2PO_4 on the production of amylolytic enzymes were studied.

Materials and Methods

Microorganism used

Thermomyces lanuginosus strain ATCC 34626 originated from American Type Culture Collection and kindly supplied by Dr. Bhat (Institute of Food Research, Norwich, UK).

Maintenance of stock culture

The strain was maintained on Yeast-Pepton/Soluble Starch (YPSS) agar medium *(9)* or on Potato Dextrose Agar (PDA), and stored in refrigerator.

Cultivation and production of amylolytic enzymes by Thermomyces lanuginosus

A three-stage cultivation technique was used. In the first stage, the fungus was grown on YPSS or PDA slant agar for 8 to 10 days at 50 °C in a humidified thermostat. In the second stage a suspension of spores was prepared using 0.1 % of Triton X-100 solution. A volume of 5mL of the suspension was added to 100mL glucose-asparagine medium (pH=6.0) to initiate the cultivation at 50 °C and 220 rpm in an orbital shaker for 1 to 2 days to obtain a homogeneous mycelium growth. In the third stage, 10mL of the mycelial suspension was used as inoculum for initiating the production of amylolytic enzymes in 150mL starch-asparagine medium (soluble starch: 40g, L-asparagine: 4g, KH_2PO_4: 3g, K_2HPO_4: 2g, $MgSO_4.7H_2O$: 0.5g, Vogel's trace elements solution *(10)*: 1mL was dissolved in 1L distilled water). Samples

(10mL) were taken under a laminar box from duplicate flasks at varying times. The samples were filtered and the α-amylase and glucoamylase activities were assayed in the filtrate.

Determination of dry mass of growing mycelia

A volume of 10mL of homogeneous mycelium suspension was harvested, washed and, after drying, weighed.

Analytical chemicals were from Sigma, Reanal, Fluka and Merck.

Enzyme assays

α-amylase. A reaction mixture containing 1mL of 0.1 M sodium-acetate buffer pH=5.0 and 1mL 0.5 % (w/v) soluble starch solution was mixed and pre-incubated at 50 ^{o}C for 10 min before adding 1mL of appropriately diluted culture filtrate as an enzyme source. After 5min the reaction was terminated by adding 1mL of 0.5 M HCl. The unhydrolysed starch in this aliquot was estimated by the iodine method described below. One unit of α-amylase activity was defined as the amount of enzyme that hydrolyses 1mg soluble starch in 1min under relevant conditions.

Glucoamylase. A volume of 1mL of reaction mixture containing 0.25mL of 0.1 M phosphate buffer pH=4.6 and 0.25mL of 1 % (w/v) soluble starch solution was pre-incubated at 50 ^{o}C for 10 min. A volume of 0.5mL of appropriately diluted culture filtrate as an enzyme source was added and the incubation continued for further 15min. The reaction was terminated by placing the tubes in a boiling bath for 30min. After cooling, the released glucose concentration was estimated by glucose oxidase/peroxidase *(11)* using a standard glucose curve prepared under the same conditions. One unit of glucoamylase activity was defined as the amount of enzyme that releases 1μmol glucose in 1min under relevant conditions.

Iodine method for starch estimation

An aliquot of the sample (starch hydrolysate) was mixed with 1mL of iodine reagent in a total volume of 2mL (distilled water). This reagent contained 0.02 % (w/v) iodine and 0.2 % (w/v) KI in 0.5 N HCl. To this mixture 5mL distilled water was added and the developed colour was measured at 590nm against blank. The amount of starch was estimated using a standard potato soluble starch (Merck, Darmstadt) curve prepared under the same conditions.

Experimental design

In order to optimise the medium composition for amylolytic enzymes production, various carbon-sources and N-sources modelling industrial fermentation technology were investigated in respect of the amylolytic enzymes. Experimental design of Response Surface Method (RSM) was also applied. At the same time the effect of buffer capacity (KH_2PO_4 and K_2HPO_4) of the medium was checked as well. In all experiments enzyme activities were assayed. A full polynomial model obtained by a multiple regression technique for two factors using SPSS 8.0 for Windows (Copyright © by SPSS Inc., 1994-1997) was used to determine the optimum composition of the medium.

Results and Discussion

Effects of pH on production of enzymes

Based on the preliminary experimental results, it was confirmed that the pH of the ferment broth reached a very high value (pH=8.0-9.5) at the end of the fermentation (after 82 h) and this may cause inactivation of the enzymes. The effect of pH on the productivity of amylolytic enzymes in fermentation was studied. The media of these experiments were prepared with 10 0mM of citrate-buffer with different pH values.

Due to the higher buffer capacity of the media a pH control was partly achieved. The experiments were run up to 168 h. Fermentation was followed by α-amylase and glucoamylase assays, and pH measurement. The activities of both amylolytic enzymes (α-amylase and glucoamylase) on all trials were increased and reached maximum values at about 96th hour of fermentation. After that, these activities were decreased (data not shown). The maximum values of activities are presented in Table 1. From the analysis of the changes of pH values during fermentation can be concluded that the pH in the reference run increased to a great extent. The application of a buffer system could restrict the increase of pH. The results revealed that the pH has a strong influence on the α-amylase and glucoamylase activities. When the initial pH of the media was adjusted to 4.9 with citrate-buffer, the glucoamylase activity was 3.5 times and the α-amylase activity 1.7 times higher than that of the reference. Reducing the initial pH of the media both amylolytic activities decreased drastically.

In case of the buffered conditions the profile of the dry mass and the amylolytic activities showed inverse function with the initial pH of the medium. In this way it was possible to repress the mycelium growth and favour the enzyme production. It should be noticed, that if the initial pH was 3.0 or less, no growth was observed. Further experiments are needed to clear up the function of the pH and glucoamylase activity. Taylor *et al. (12)* reported that *Humicola lanuginosa* (present name is *Thermomyces lanuginosus*) produces two forms of glucoamylase, one with pH optimum of 4.9 and another of 6.6. This demonstrates that the highest activities were measured when the final pH was between 4.8 and 5.7.

Table 1 Relationship between initial pH of the media, the yield of mycelium and amylolytic enzyme activities

Initial pH of medium	Final pH of ferment broth	w (dry mass) %	Relative α-amylase activity (%)	Relative glucoamylase activity (%)
Reference (pH=6.3*)	8.8	0.81	100	100
4.9	5.7	0.57	173	364
4.5	4.8	0.73	124	165
4.0	4.1	0.89	20	41
3.5	3.5	1.28	16	7
3.0	n.g.			
2.5	n.g.			

* prepared with water, n.g.: no growth was detected

Effect of different carbon sources on the production of amylolytic enzymes

The effects of various carbon sources on the production of α–amylase and glucoamylase by *Thermomyces lanuginosus* ATCC 34626 strain are shown in Table 2.

Starch is a generally accepted nutritional component for induction of amylolytic enzymes. This material was applied as reference. To get detailed information about the synthesis of amylolytic enzymes of *Thermomyces lanuginosus* various carbon-sources were used. When growing fungus on glucose, maltose, maltodextrin, amylopectin, dextran and dextrin the amylolytic activities were higher than on starch. In case of α-amylase the maltodextrin was found to be the best carbon source. The α-amylase activity was approximately 25 % higher than that of the control (with starch). In the case of glucoamylase the dextrin proved to be the best, giving two times higher activity than starch.

Table 2 Effects of various carbon sources on the production of amylolytic enzymes

Carbon source	α-amylase (U/ml)	Glucoamylase (U/ml)	pH
Starch	92.53	6.15	5.53
Glucose	103.76	6.36	5.03
Maltose	110.42	9.92	5.30
Maltodextrin	124.22	10.56	5.30
Amylopectin	92.43	7.76	5.22
Amylose	59.40	4.32	5.23
Dextran	104.50	9.39	5.24
Dextrin	111.16	12.66	5.21

All values in table were measured at 96th hour of fermentation

Effects of different nitrogen sources on production of amylolytic enzymes

In the investigation of the effects of various nitrogen-sources on amylolytic enzyme production (Table 3) L-asparagine was found to be the most promising one. In case of α-amylase activity yeast extract seemed to be suitable as well, but in case of glucoamylase the enzyme productivity was just half of that on L-asparagine. This result contradicts the observation of Haasum *et al. (4)* who found outstanding glucoamylase activity obtained from fermentation on yeast extract. This difference can be caused by the deviation in pH and the strain applied during the fermentation. When inorganic nitrogen sources were used, the fungi grew excellently, but very poor enzyme activities were achieved.

Table 3 Effects of various nitrogen sources on α-amylase and glucoamylase production

Nitrogen source	α-amylase (U/mL)	Glucoamylase (U/mL)	pH
L-asparagine	89.87	6.29	5.16
Yeast extract	72.84	3.18	5.19
CH_3COONH_4	24.21	0.72	5.28
NH_4NO_3	26.55	1.60	4.87
$NaNO_3$	10.54	0.00	5.06
$(NH_4)_2SO_4$	36.11	3.14	4.53
$NH_4H_2PO_4$	28.43	3.27	4.47

All values in table were measured at 96th h of the fermentation

Experimental design of Response Surface Method

Central composite design (CCD) was adopted as an efficient way to find a culture medium with optimum composition for amylolytic enzymes production. Starch and L-asparagine were selected as carbon and nitrogen sources for independent variables. Based on preliminary experiments 2 % concentration change in starch and 0.2 % in L-asparagine have significant effects on enzyme activities. The full second-order polynomial model was used to fit to the dependent variables using the following equation:

$$Y = b_0+b_1.x_1+b_2.x_2+b_3.x_1^2+b_4.x_2^2+b_5.x_1.x_2$$

where Y (α-amylase or glucoamylase activities that were measured at 96th h of fermentation) is the dependent variable to be modelled, b_i are regression coefficients of model, and x_1 (starch), x_2 (L-asparagine) are independent variables (Table 4).

Table 4 Experiment design and results

Trial*	w (starch) %	w (L-asparagine) %	α-amylase (U/mL)	Glucoamylase (U/mL)	Final pH
1	2	0.2	30.12	2.64	5.55
2	2	0.6	91.34	7.33	5.74
3	6	0.2	72.23	4.49	5.15
4	6	0.6	113.19	6.11	5.16
5	1.16	0.4	44.08	3.96	5.86
6	6.84	0.4	104.21	5.89	5.20
7	4	0.116	45.86	2.8	5.15
8	4	0.684	107.43	6.96	5.14
9	4	0.4	89.87	6.29	5.16
10	4	0.4	87.99	6.17	5.16
11	4	0.4	88.48	6.05	5.22
12	4	0.4	87.36	6.15	5.18

* media were prepared with 100 mM citrate buffer (pH=4.9), all enzyme activities and pH values in the table were measured at 96th hour of the fermentation

The data of Analysis of Variance (ANOVA) table prove that the applied model fits the experimental values (Table 5).

Table 5 Summarised data of analysis of variance of α-amylase and glucoamylase models

Mode	Sum of squares		Degree of	Mean of squares		F		Significant	
	AA*	GA**	freedom	AA	GA	AA	GA	AA	GA
Regression	7769.47	25.84	5	1553.89	5.169	103.19	43.78	0.00	0.00
Residual	90.35	0.71	6	15.06	0.118				
Total	7859.82	26.55	11						

*AA α-amylase activity **GA glucoamylase activity

The t-values of the estimated coefficients showed that all coefficients gained by regression analysis have significant ($P<0.05$) effects on both amylolytic activities (Table 6). The increase of the concentration of L-asparagine as nitrogen source has a positive effect on the production of both amylolytic enzymes. In case of starch, the increase of the concentration showed a positive effect on Ŭ-amylase production, and a negative effect on glucoamylase production (Figure 1).

Table 6 Coefficients of regression analysis for the prediction of α-amylase and glucoamylase production

Model	Coefficients		t		Significant (P)	
	AA*	GA**	AA	GA	AA	GA
B_0	-65.520	-5.34	-5.565	-5.12	0.001	0.002
B_1	27.925	2.09	7.590	6.43	0.000	0.001
B_2	279.518	26.90	7.579	8.26	0.000	0.000
B_3	-1.696	-0.14	-4.447	-4.14	0.004	0.006
B_4	-138.558	-14.52	-3.634	-4.30	0.011	0.005
B_5	-12.662	-1.92	-2.610	-4.47	0.040	0.004

*AA α-amylase activity **GA glucoamylase activity

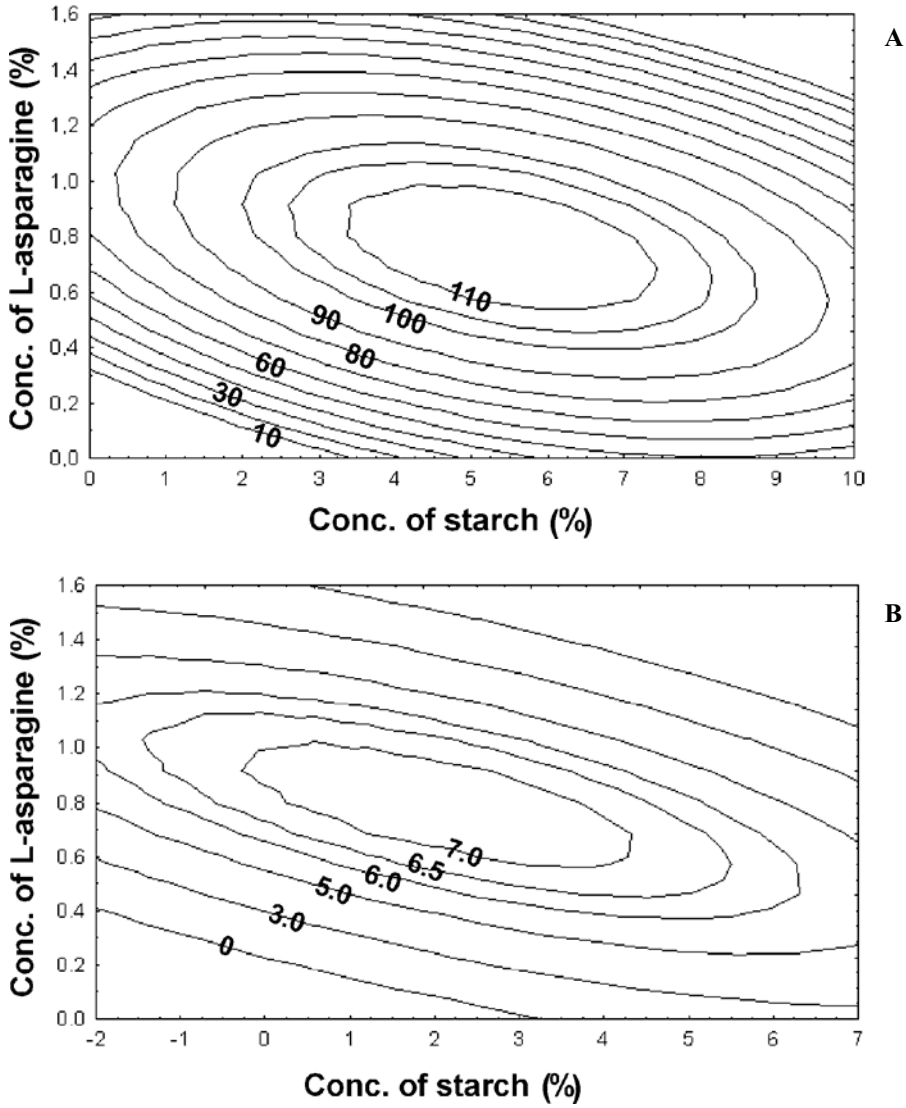

Figure 1 Contour draw of fitted second-order polynomial models (a) α-amylase, (b) glucoamylase

The interaction between the two factors was also significant at 95 % level. According to fitted models, the optimum concentration for production of Ŭ-amylase is 5.5 % for starch and 0.75 % for L-asparagine, for production of glucoamylase 2.2 % of starch and 0.75 % of L-asparagine.

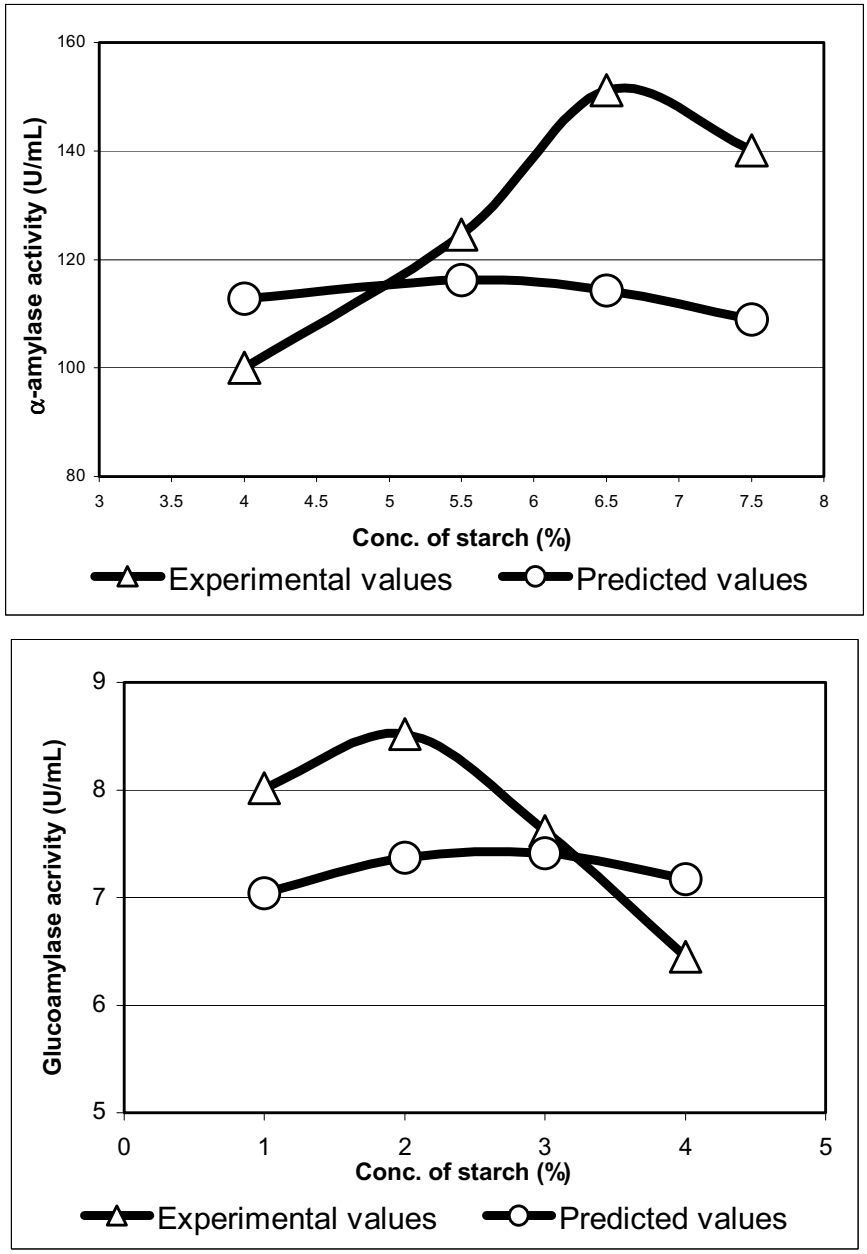

Fig. 2. Effect of starch on production of amylolytic (α-amylase and glucoamylase) enzymes by *Thermomyces lanuginosus* ATCC 34626; (a) α-amylase, (b) glucoamylase; the medium contained 0.75 % (w/v) L-asparagine, 0.3% K_2HPO_4, 0.2 % (w/v) KH_2PO_4, 0.05 % (w/v) $MgSO_4.7H_2O$ and 1 mL Vogel's trace-element solution experimental values; predicted values

The predicted maximum enzyme activities from the models are 119 U/mL and 7.5 U/mL in case of α-amylase and glucoamylase, respectively. The set of experiments with media containing different starch concentrations was carried out to check how the real data fit the data predicted by modelling. At 6.5 % starch concentration the maximum of α-amylase activity was reached with the value of 151 U/mL and the maximum activity of glucoamylase was 8.4 U/mL at 2 % of starch concentration (Fig. 2). The measured values of amylolytic activities were still within the confidence interval (95 %) of predicted values according to SPSS. The fitting of the experimental and predicted data was verified and approved. The enzyme activities were increased by approximately 50 % in case of α-amylase and by 30 % in case of glucoamylase when using the newly developed media.

Effects of the concentrations of KH_2PO_4 and K_2HPO_4 on production of amylolytic enzymes

To improve the amylolytic enzyme production the effect of the concentration of KH_2PO_4 and K_2HPO_4 was investigated at different times as well. Based on the previous results the effect of inorganic phosphates on the production of α-amylase and glucoamylase was separately investigated. The results at 94th h of fermentation are presented in Table 7.

The reduction of concentration of KH_2PO_4 and KH_2PO_4 leads to improvement of productivity of both amylolytic enzymes. The maximum activities were reached at 0.15 % of KH_2PO_4 and 0.1 % of K_2HPO_4.

Table 7 The effect of the fraction of KH_2PO_4 and KH_2PO_4 of the growth media on amylolytic activities

ɔ (KH_2PO_4) g/L	ɔ (K_2HPO_4) g/L	α-amylase			Glucoamylase		
		w (starch) %	Activity (U/mL)	pH	w (starch) %	Activity (U/mL)	pH
3.00	2.00	6.5	184.72	5.73	2.0	17.48	5.30
3.00	0.00	6.5	66.48	4.99	2.0	12.30	5.72
0.00	2.00	6.5	241.23	6.11	2.0	9.48	5.88
1.50	1.00	6.5	259.75	5.64	2.0	22.43	5.67
0.75	0.50	6.5	233.15	5.41	2.0	18.57	5.83
0.00	5.75	6.5	35.85	6.90	2.0	7.16	5.98
4.50	0.00	6.5	32.77	5.02	2.0	8.46	5.90
0.00	3.90	6.5	104.23	6.58	2.0	5.36	5.77
6.10	0.00	6.5	27.07	4.85	2.0	7.70	5.19

Conclusions

Adjusting the initial pH of the medium with 100mM citrate-buffer an improvement was achieved in the productivity of the amylolytic enzymes. In the investigated range pH=4.9 was found to be the most suitable one.

L-asparagine was the most promising among 5 investigated nitrogen sources. Yeast extract would be beneficial for α-amylase as well, but for glucoamylase the enzyme activity was decreased to half of that gained on L-asparagine.

The fungus grown on glucose and all tested glucose polymers showed considerable amylolytic activities. The production of amylolytic enzymes on glucose as a sole carbon source indicates a constitutive synthesis of the α-amylase and glucoamylase.

Response Surface Method was used to find optimum concentration of medium components. Second-order polynomial models were applied. All estimated parameters had significant level higher than 95 % and some higher than 99 %. The predicted values were verified experimentally.

Based on the results of the optimisation experiments the proposed compositions of the fermentation media (w/v) are in case of α-amylase production: soluble starch 6.5%, L-asparagine 0.75%, KH_2PO_4 0.15%, K_2HPO_4, 0.1 %, $MgSO_4.7H_2O$ 0.05%, Vogel's trace elements 0.1 mL and in case of glucoamylase production soluble starch 2.0 %, L-asparagine 0.75%, KH_2PO_4 0.15%, K_2HPO_4, 0.1 %, $MgSO_4.7H_2O$ 0.05%, Vogel's trace elements 0.1 mL. Both media are prepared with 100 mM citrate buffer (pH=4.9).

To reach the maximum amylolytic activities, 96-hour fermentation time is needed.

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Appendix 4

Purification and characterisation of amylolytic enzymes from thermophilic fungus *Thermomyces lanuginosus* strain ATCC 34626

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Abstract

Amylolytic enzymes (α-amylase and glucoamylase) from *Thermomyces lanuginosus* ATCC 34626 were purified to electrophoretic homogeneity. The molecular mass of purified α-amylase and glucoamylase were 61 and 75 kDa, respectively. Their p*I* values were calculated to be 3.5 - 3.6 and 4.1 - 4.3. The amylolytic enzymes from *T. lanuginosus* exhibit pH optima in the range 4.6 – 6.6 in the case of α-amylase and 4.4 - 5.6 in the case of glucoamylase. Both purified enzymes have temperature optima at 70 °C. Zn^{2+} ions strongly inhibit both enzyme activities. Mn^{2+} and Fe^{2+} ions are activators in the case of glucoamylase; Ca^{2+} and Ba^{2+} are activators in the case of α-amylase. With half-life times longer than 1 day at 60 °C both enzymes prove to be thermostable in the pH range 4.5 - 8.5. The amylolytic enzymes from *T. lanuginosus* loose activities rapidly when incubated at temperature higher 80 °C or at pH lower than 4.0. Both enzymes are found to be glycosylated; 8.5 % carbohydrate in the case of α-amylase and 3.3 % in the case of glucoamylase. The K_m and V_{max} of α-amylase on soluble starch were 0.68 mg/ml and 45.19 U/mg, respectively. The K_m values of glucoamylase on maltose, maltotriose, maltotetraose, maltopentose and soluble starch were 6.5, 3.5, 2.1, 1.1 mM and 0.8 mg/ml, respectively. The first 37 residues of *N*-terminal of the purified α-amylase of *T. lanuginosus* ATCC 34626 were sequenced. Almost complete homology with the α-amylase from *Aspergillus oryzae* and *Emericella nidulans* was observed.

Keywords: α-Amylase, Glucoamylase, *T. lanuginosus*, Enzyme purification and kinetics

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Introduction

Enzyme preparations containing α-amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3) play an important role in the degradation of starch. They are produced commercially in bulk from micro-organisms (*Bacillus*, *Aspergillus* sp.) and represent about 25 - 33 % of the world enzyme market, in second place after proteases. Their main application is in the production of High Glucose Syrup (HGS) from starch [1] and furthermore in the production High Fructose Corn Syrup (HFCS) [2]. Barnett & Fergus [3] suggested that *T. lanuginosus* could be an excellent test organism to study thermostable amylolytic enzymes. This fungus secretes high levels of enzymes and is thermophilic. Therefore, it may provide novel enzyme variants with high temperature optima and a long "shelf-life", which are desirable characteristics for world-wide commercial application of enzyme [4]. Several research groups have studied this organism as a potential thermostable enzyme source. The results published so far show that the physicochemical properties of amylolytic enzymes from *T. lanuginosus* vary from strain to strain. Jensen et al.[5] showed that the α-amylase from strain 1457 had molecular mass of 45-57 kDa by Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)**.** Mishra & Maheshwari [6] found that the α-amylase from *T. lanuginosus* IISc 91 gave about 24 kDa by SDS-PAGE and about 74 kDa by gelfiltration. In the case of glucoamylase the situation is very similar [5],[6],[7]. This means that different strains of *T. lanuginosus* may secrete different forms of amylolytic enzymes.

Our previous papers dealt with the screening of strains [8] and optimisation of the fermentation medium [9] for production of amylolytic enzymes. The purification and characterisation of amylolytic enzymes as well as some kinetic data from *T. lanuginosus* ATCC 34626 are presently reported.

Materials and Methods

Materials

The Sepharose CL-6B, DEAE Sepharose Fast Flow, Q Sepharose Fast Flow and Superose 12 were from Pharmacia (Pharmacia, Uppsala, Sweden). All other chemicals were of analytical grade and purchased from Sigma, Reanal, Merck and other companies.

Micro-organism

T. lanuginosus strain ATCC 34626 originated from American Type Culture Collection and was kindly supplied by Dr. Bhat (Institute of Food Research, Norwich, UK). This strain was maintained on Potato Dextrose Agar (PDA) slant and grown at 50 °C for 10 days and stored at 4 °C until use.

Media and culture conditions for enzyme production

Conidia suspension (5 ml) prepared by using 0.1 % Triton X-100, was added to 100 ml of glucose-asparagine medium (glucose 2 g, L-asparagine 0.4 g, KH_2PO_4 0.3 g, K_2HPO_4 0.2 g, $MgSO_4.7H_2O$ 0.05 g and 0.1 ml of Vogel's trace elements solution [10] in 100 ml distilled water, pH 6.0) to initiate cultivation. The inoculum culture was prepared in orbital shaker at 47 ^oC and

220 rpm for 2 days. The enzyme production was carried out in 500-ml Erlenmeyer flasks containing 150 ml medium using 5 ml of inoculum culture. The medium consisted of 40 g soluble starch, 7.5 g L-asparagine, 3 g KH_2PO_4, 2 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$ and 1 ml of Vogel's trace elements solution in 1000 ml 100 mM citrate buffer pH 5.0. The initial pH of the medium was adjusted to 5.0 and not further controlled. After the fermentation was completed, the fungus was harvested by filtration and the enzyme activities were assayed in the culture filtrates.

Enzyme Assays

Glucoamylase activity A 1 ml reaction mixture containing 0.25 ml 0.1 M sodium-acetate buffer pH 4.6 and 0.25 ml 1 % (w/v) soluble starch solution was pre-incubated at 50 ^oC for 10 min. Appropriately diluted culture filtrate (0.5 ml) as an enzyme source was added, and the incubation continued for further 15 min. The reaction was terminated by placing the tubes in a boiling bath for 30 min. After cooling the released glucose concentration was estimated by glucose oxidase/peroxidase (GOD/POD) method [11] using a standard glucose curve prepared under same conditions. One unit of glucoamylase activity was defined as the amount of enzyme that releases 1 μmol glucose in 1 min under relevant conditions.

α-Amylase activity A reaction mixture containing 1 ml 0.1 M sodium-acetate buffer pH 5.0 and 1 ml 0.5 % (w/v) soluble starch solution was mixed and pre-incubated at 50 ^oC for 10 minutes before adding 1 ml of appropriately diluted culture filtrate as an enzyme source. The buffer contained 7.5 mM Ca^{++} ion. After 5 minutes, the reaction was terminated by adding 1 ml 0.5 M HCl. The unhydrolysed starch in this aliquot was estimated by the iodine method described below. One unit of α-amylase activity was defined as the amount of enzyme that hydrolyses 1 mg soluble starch in 1 min under relevant conditions.

Iodine Method for Starch Estimation

An aliquot of the sample (starch hydrolysate) was mixed with 1 ml of iodine reagent in a total volume of 3 ml (distilled water). This reagent contained 0.02 % (w/v) iodine and 0.2 % (w/v) KI in 0.5 N HCl. To this mixture 5 mL distilled water was added and the colour that developed was read at 590 nm against blank. The amount of starch was estimated using a standard potato soluble starch (MERCK) curve prepared under the same conditions.

Determination of reducing sugars

The reducing sugar concentration was determined by copper-bicinchoninate described by Waffenschmidt & Jaenicke [12] and Fox & Robyt [13] with some minor modifications. Two stock solutions were prepared: solution A consisted of 150 mg of 4,4'-dicarboxy-2,2'-biquinoline dissolved in 100 ml of solution containing 7.16 g anhydrous sodium carbonate. The final volume was adjusted to 115 ml by using distilled water. Solution B was consisted of 3.5 g of aspartic acid and 1.09 g of copper sulphate dissolved in 140 mL of solution containing 5 g of sodium carbonate. The final volume (150 mL) was obtained by adding distilled water. The

working reagent was prepared by mixing 23 ml of solution A, 1 mL of solution B and 6 mL of 96 % ethanol. This was kept in the dark for 2 hours before use. The microtiter plate protocol was the following: 40 µl of sample containing reducing sugar was added to 160 µl of working reagent in wells. The plate was covered with wrap and incubated at 80 ^{o}C for 30 min. After incubation the plate was cooled to room temperature for 15 min and the absorbance was read at 540 nm. The reducing sugar concentration was quantified by using sa
tandard curve prepared with maltose.

Determination of protein concentration

The protein concentration was estimated by measurement of 280nm absorbance and/or by Modified Lowry Method using Sigma Diagnostics Protein Assay Kit following the instruction given by Sigma (USA).

Enzyme purification

Amylolytic enzymes were purified from ferment broth produced by *T. lanuginosus* ATCC 34626 grown on starch-based medium given above. All purification steps were carried out at 4 ^{o}C using an FPLC system (Pharmacia, Uppsala, Sweden). The collected fractions were monitored for protein content (A_{280}) and enzyme activity. Details of purification protocol are given in Result and Discussion part.

Electrophoretic analysis

The protocols are described by Laemmli [14]. The proteins were stained with 0.25 % Coomassie brilliant blue G-250 or 0.4 % silver nitrate solution (Bio-Rad, USA). In case of the molecular mass estimation the following markers (LMW, Pharmacia, Uppsala, Sweden) were used: phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soyan trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Agarose gel (1 %) and polyacrylamide gel (6 %) were used for estimation of p*I* of α-amylase and glucoamylase by isoelectric focusing at 4 ^{o}C in the pH range 2.5 - 10. The p*I* calibration kit from Pharmacia (Pharmacia, Uppsala, Sweden) and ampholine carried ampholytes from Bio-Rad (BioRad, USA) were used. The proteins were stained with 0.1 % Serva blau G in case of the agarose gel and 0.15 % Coomassie brilliant blue G-250 in case of the acrylamide gel. To calculate the molecular mass and p*I* of enzymes, the gel analysis system running 1-D-analysis software v2.4b (Signum/Biotech Fisher GMBH, Germany) was used.

Ultrafiltration

For concentration and dialysis of enzyme solution the Amicon (USA) ultrafiltration units with PM-10 membranes were used.

Effect of pH and temperature on enzyme activities

Sodium acetate buffers (50 mM) pH from 3.5 to 5.6, 50 mM phosphate-citrate buffers pH from 4.0 to 7.0 and 50 mM Tris-maleate buffers pH from 7.0 to 8.6 were used. Temperature effects

on enzyme activities were studied at different temperatures (40 °C to 85 °C) at pH 5.6 in the case of α-amylase and pH 4.6 in the case of glucoamylase.

Effects of metal ions and some other compounds on enzyme activities

Metal salts ($ZnSO_4$, $CoCl_2$, $MnSO_4$, $FeSO_4$, $BaCl_2$, $CaCl_2$) (10 mM), disodium-EDTA (10 mM) and urea (10 mM) were dissolved in 50 mM sodium acetate buffer and applied to assay enzyme activities described above.

Effects of pH and temperature on enzyme stability

The one-variable-at-a-time approach (OVAT) is used very frequently to study the effects of pH and temperature on enzyme stability. This method does not guarantee the real optima under operating conditions. Moreover it ignores the interactive effects among different variables. To overcome this disadvantages, the response surface method (RSM), which includes the factorial design and regression analysis, gives proper solution in the technological optimisation [15],[16]. In our case the pH and temperature were selected as independent factors (variables). The various pH levels were adjusted by application of different buffer systems: 50 mM sodium acetate buffer (pH 3.5 – 5.6), 50 mM phosphate-citrate buffer (pH 4.0 - 8.0) and 50 mM Tris-HCl buffer (pH 7.5 - 9.0). Appropriate amounts of enzymes were incubated in 50 mM buffers pH from 3.5 to 9.0 with 0.5 stepwise and temperatures from 50 °C to 85 °C with 5 °C stepwise. Samples were taken in different times and assayed for activities at standard conditions described above. The matrix of half-life time with two dimensions (pH and temperature) was built and the half-life time values in matrix were maximised conventionally as 1440 minutes (one day). The RSM was applied to evaluated matrix data using SPSS 10.0 for MS Windows statistical software package (Copyright © 1994-2000 by SPSS Inc.).

Determination of carbohydrate content of enzymes

Enzyme samples (280 ml containing 50 µg purified protein) were hydrolysed by adding 120 µl of 100 % trifluoracetic acid (TFA) and incubation at 100 °C for 4 h. After cooling to room temperature the mixture was concentrated in vacuo. To remove the excess of TFA three times 1 ml distilled water was added and evaporated before carbohydrate analysis. The sugars were quantified by determination of reducing sugar and by high pressure anion exchange liquid chromatography (HPAELC) using CarboPack 10 column (DIONEX) connected to a DIONEX system and a PAD detector (DIONEX). Elution was done with 0.1 M NaOH.

Determination of kinetic parameters of amylolytic enzymes

The kinetic parameters of α-amylase for soluble starch were investigated in 50 mM sodium acetate buffer system pH 5.6 containing 7.5 mM Ca^{2+}. The reducing sugar concentration was determined by method described above. Based on the results of preliminary experiments, 10 U (3.384 µg protein) of enzyme was applied in 1 mL of total volume of reaction mixture.

In the case of glucoamylase, different substrates (maltose, maltotriose, maltotetraose, maltopentaose and soluble starch) were used. These were dissolved in 50 mM sodium acetate

buffer system pH 5.0 and 0.5 U (8.33 μg protein) of glucoamylase was applied. The released glucose concentration was determined by GOD/POD method. The reactions were carried out at 65 ^{o}C and the Hanes-Woolf method [17] was applied to evaluate experimental data. All experiments were done in triplicates and the average data were used to calculate the K_m and V_{max}.

N-terminal amino-acid sequence

The purified α-amylase enzyme was applied onto Applied BioSystem 476A pulsed liquid sequenator (Foster City, Ca, USA) to determine the primary sequence of the peptide chain. The primary sequence was analysed using the BLAST database (http://pbil.univ-lyonl.fr/BLAST/).

Results and discussion

Purification of α-amylase and glucoamylase

The purification scheme is shown in Fig. 1. The crude enzyme solution that was obtained from fermentation broth after removal of mycelia by filtration, was precipitated with $(NH_4)_2SO_4$ at 90 % saturation and kept at 4 °C overnight. The precipitated proteins were collected by centrifugation at 55220 x g for 1 h and dissolved in a minimal amount of 30 mM sodium acetate buffer (pH 5.0). The undissolved pellets were removed by centrifugation at 7396 x g for 15 min. Then, the sample was loaded onto DEAE Sepharose Fast Flow column (2.5 cm x 50 cm) equilibrated with 50 mM sodium acetate buffer pH 5.0 containing 0.2 % sodium azide. The column was connected to a FPLC system (Pharmacia, Uppsala, Sweden). The bound proteins were eluted with a NaCl salt gradient (0-0.7 M) in the same buffer. The flow rate was 1.75 mL/min. Two peaks of amylolytic activity were separated, each was collected separately and concentrated by ultrafiltration. Then, a gelfiltration was carried out on Sepharose CL-6B column (5 cm x 70 cm). Sodium acetate buffer (20 mM) pH 5.0 containing 0.2 % NaN_3 was used at a flow rate of 0.25 mL/min.

The α-amylase fraction was applied onto a Q Sepharose Fast Flow column (1 cm x 30 cm) equilibrated with 50 mM sodium acetate buffer pH 5.6 and eluted with a NaCl gradient (0-0.5 M) in the same buffer at a flow rate of 1.25 mL/min. The fractions showing α-amylase activities were collected and concentrated by ultrafiltration.

Finally, the enzyme was loaded onto Superose 12 column (2.5 cm x 80 cm) and eluted with 20 mM sodium acetate buffer pH 5.6 containing 0.2 % NaN_3. The apparently pure α-amylase enzyme was collected, concentrated and dialysed against water before lyophylisation. In the case of glucoamylase fraction, the sample was submitted onto Q Sepharose Fast Flow column (1 cm x 30 cm) equilibrated with 50 mM sodium acetate buffer pH 4.5 and eluted by a NaCl gradient (0-0.4 M) in the same buffer. The flow rate was 1.25 mL/min. The fractions showed glucoamylase activities were collected, concentrated and applied onto Superose 12 column (2.5 cm x 80 cm) equilibrated with 20 mM sodium acetate pH 4.5. Elution with the same buffer yielded pure glucoamylase, which after concentration and dialysis against distilled water was freeze-dried. All purification steps were carried out at 4 ^{o}C.

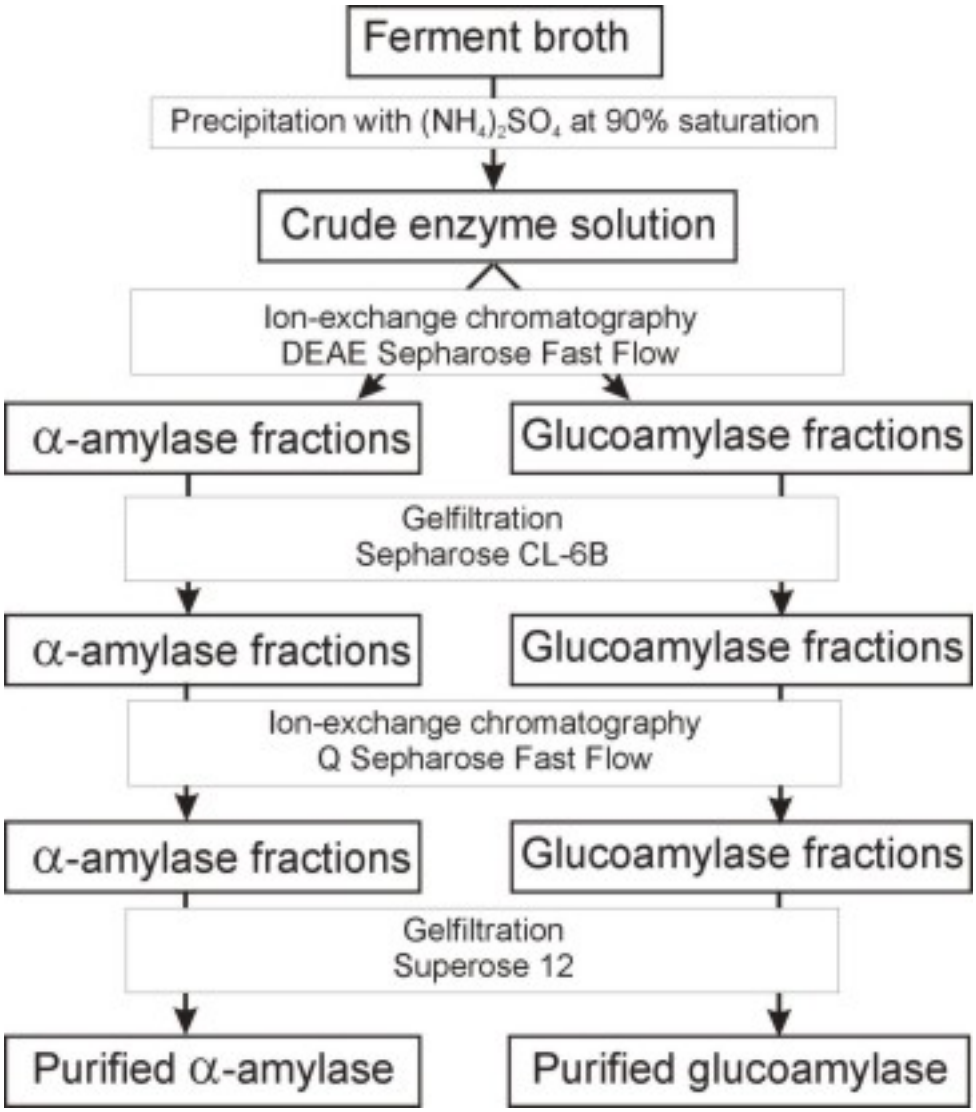

Figure 1 Purification protocol of extracellular amylolytic enzymes (α-amylase and glucoamylase) from *T. lanuginosus* ATCC 34626

The result gained by Phadebas test (Pharmacia) confirm that the pure enzyme was α-amylase. The results gained during the purification process are summarised in Table 1. The purification of amylolytic enzymes from *T. lanuginosus* ATCC 34626 yielded about 27 % of α-amylase and 23 % of glucoamylase. The purified enzymes exhibit very high specific activity: 3384 U/mg protein in the case of α-amylase and 60 U/mg protein in the case of glucoamylase. The purification factors were 16.7 for α-amylase and 8.6 for glucoamylase.

Table 1 Purification of extracellular amylolytic enzymes from *T. lanuginosus*[a]

Steps	Total proteins [mg]		Total activity [U]		Recovery [%]		Specific activity [U/mg]		Fold of purification	
	AA	GA	AA	GA	AA	GA	AA	GA	AA	GA
Ferment broth	488		99000	3413	100		203	7	1.0	
$(NH_4)_2SO_4$	349		87300	2849	88		250	8	1.2	
DEAE Sepharose FF	151	90	50029	2118	51	62	331	24	1.6	3.4
Sepharose CL-6B	102	61	45186	1828	46	54	443	30	2.2	4.3
Q Sepharose FF	16	24	36834	1335	37	39	2302	56	11.3	8.0
Superose 12	8	13	27070	782	27	23	3384	60	16.7	8.6

[a]AA: α-amylase GA: glucoamylase

Physicochemical properties of amylolytic enzymes from T. lanuginosus

Showing single protein species on SDS-PAGE staining with 0.25% Coomassie brilliant blue G-250, both enzymes were purified to apparent homogeneity. From these electrophoretic results the molecular masses were estimated to be 75 and 61 kDa for glucoamylase and α-amylase, respectively. In 1988 Jensen et al. [5] have successfully purified amylolytic enzymes from *T. lanuginosus* strain No. 1457 (Denmark) and reported that the glucoamylase has molecular weight about 70 - 76kDa and 54 - 57 kDa for α-amylase. They also proposed that the glucoamylase might have two forms: one has molecular weight about 70 kDa and another has about 76 kDa. Taylor et al. [19] also showed that glucoamylase from *T. lanuginosus* has two isoforms GI and GII. Mishra & Maheshwari [6] reported that the glucoamylase and α-amylase from IISc 91 (India) have molecular weights 45 and 42 kDa, respectively. The glucoamylase from strain No. A236 (China) has molecular weight 72 kDa [7] and from strain No. F1 (Germany) has 70 kDa [20]. The α-amylase from strain No. F1 [20] has molecular mass 55 kDa. In our case, the α-amylase has higher molecular mass than that reported so far. The p*I* of α-amylase was in range of 3.5 - 3.6 and glucoamylase was in range of 4.1 - 4.3. These results agree with the results reported by Jensen et al. [5], Li et al. and [7] Obido & Ulbrich-Hofmann [20].

Carbohydrate contents

Both amylolytic enzymes from *T. lanuginosus* ATCC 34626 are glycoproteins with carbohydrate contents of about 8.46 % of carbohydrate in the case of α-amylase and 3.27 % of carbohydrate in the case of glucoamylase. After total hydrolysis the major monosaccharides in α-amylase were chromatographically characterised as mannose (48 %), NAcglucosamine (15%) and NAcgalactosamine (36%). Mannose (62 %), glucose (2 %), NAcglucosamine (33 %) and NAcgalactosamine (3 %) are the major sugars present in the glucoamylase.

Effect of pH and temperature on enzyme activities

Optimum α-amylase activity is found in the pH range 4.6-6.6 (Fig. 2A) with changes less than 10 %. Enzyme activity decreased drastically at pH below 4.0 or above 7.0. In the case of glucoamylase the optimum range was from 4.4 to 5.6 (Fig. 2B).

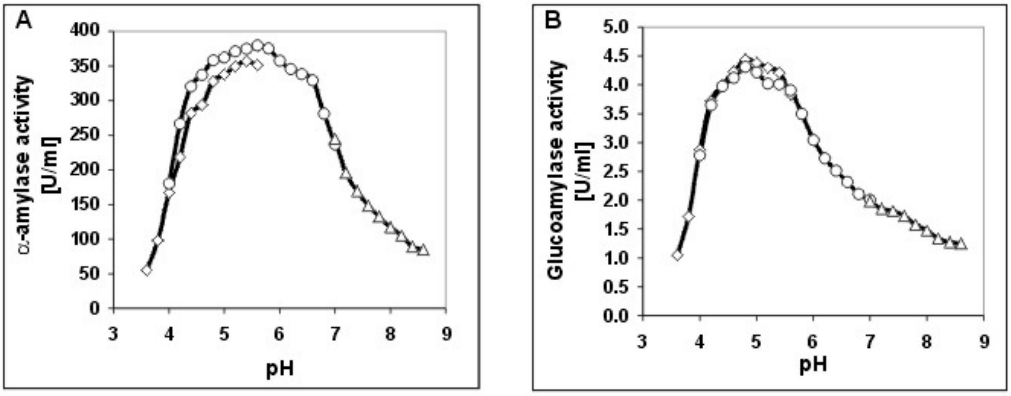

Figure 2 Effects of pH on amylolytic enzyme activities A: α-amylase, B: glucoamylase. The experiments were carried out at 70 °C and the 50 mM buffers (◇ sodium acetate ○ phosphate-citrate △ Tris-maleate) were applied.

Both enzymes exhibit temperature optima at 70 °C. These results agree with those reported by Basaveswara Rao *et al.* [21], Li *et al.* [7] and Odibo & Ulbrich-Hofmann [20] for other strains of *T. lanuginosus*. At temperature higher than 75 °C, the activities decrease drastically (Fig. 3).

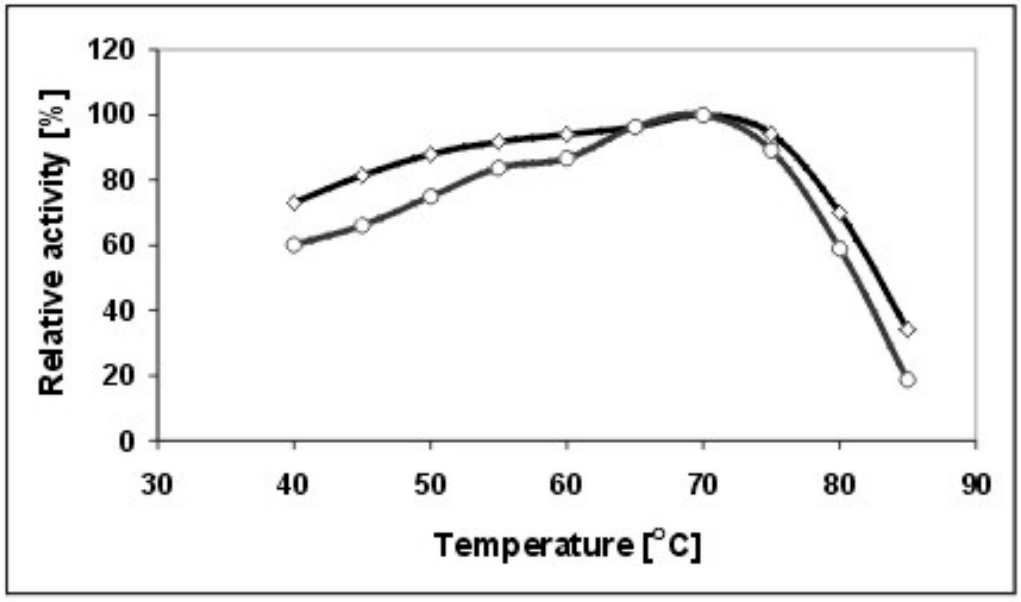

Figure 3 Effects of temperature on amylolytic enzyme activities
◇ α-amylase (incubated time 5 min) ○ glucoamylase (incubated time 15 min)

Effects of metal ions on enzyme activities

The activities of amylolytic enzymes from *T. lanuginosus* decreased significantly by adding 10 mM of Zn^{++} ion to reaction mixture (Table 2). In the case of α-amylase, Co^{++} showed the inhibitor and the Ca^{++} and Ba^{++} activator effects. The role of Ca^{++} ion was investigated by

Mishra & Maheshwari [6] and Rónaszéki *et al.* [8]. In the case of glucoamylase all investigated ions except Zn^{++} showed positive effects on enzyme activity. The Mn^{++} was the best activator; at 10 mM of this ion the glucoamylase activity increased 1.5 times.

Table 2 Effects of metal ions and other compounds on amylolytic enzyme activities (Concentration of all added compounds was 10 mM)

	Relative activity [%]	
	α-amylase	Glucoamylase
Control	100	100
$ZnSO_4.7H_2O$	24	84
$CoCl_2.6H_2O$	92	128
$MnSO_4.H_2O$	100	149
$FeSO_4.7H_2O$	99	122
$BaCl_2.2H_2O$	112	109
$CaCl_2$	135	117
EDTA	103	114
Urea	109	120

Effects of pH and temperature on enzyme stability

To investigate the effects of pH and temperature on enzyme stability, the pH and temperature were selected as independent factors and half-life time was chosen as dependent factor. The least square method was adopted to evaluate the data. Both investigated factors (pH and temperature) have significant effects on enzyme stability.

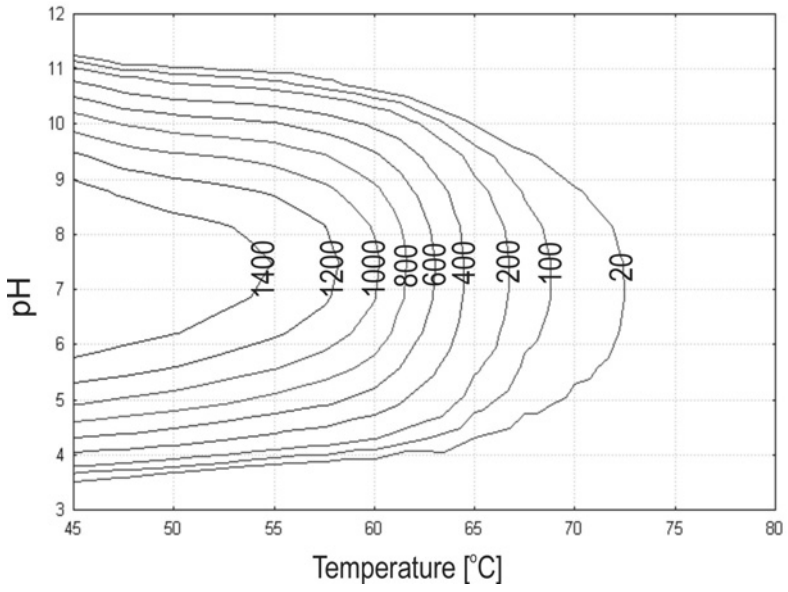

Figure 4 Effects of pH and temperature on stability of α-amylase. Contour draw of half-life time

As shown the data fit the model for α-amylase stability for at least one day at 55 °C in the pH range 5.0 to 8.5 (Fig. 4). The enzyme looses its activity rapidly at temperature higher than 75 °C in the investigated pH range. In this case the half-life time is less than 20 minutes. In the presence of Ca^{++} ion the half-life time increased by 5 times at 70 °C and pH 7.0 (from 50 min. up to 250 min.). These results meet those reported by Mishra & Maheshwari [6]. They found that in the presence of Ca^{++} ion the stability of α-amylase from *T. lanuginosus* strain IISc 91 was eight times higher than in absence of Ca^{++} ion.

The results of RSM analysis of glucoamylase prove that the enzyme is stable at 50 °C in the pH range 4.4 to 7.5 (Fig. 5). The enzyme lost its activity very fast when increased the temperature. At 70 °C enzyme lost 50 % of its activity in 30 minutes. When temperature was higher than 80 °C enzyme lost its total activity in 5 minutes.

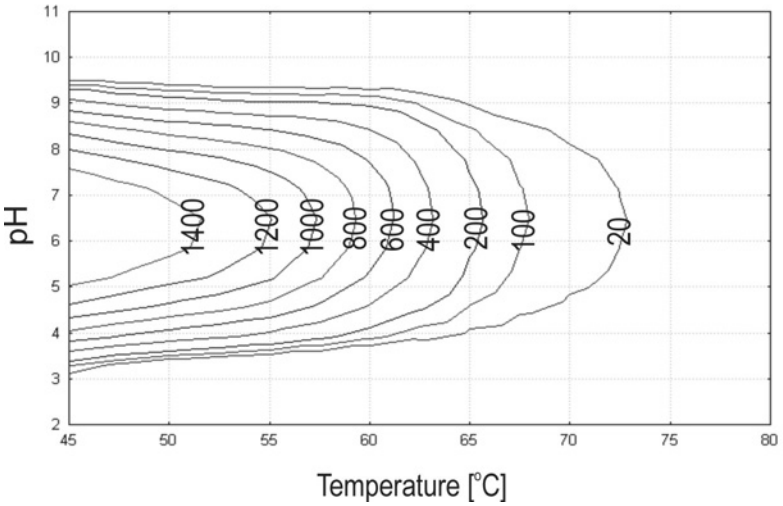

Figure 5 Effects of pH and temperature on stability of glucoamylase. Contour draw of half-life time

To investigate the effects of the presence of α-cyclodextrin and Mn^{++} ion on the stability of glucoamylase, the experiments were carried out with 0.5 % (w/m) of α-cyclodextrin and 10 mM of Mn^{++} in 50 mM phosphate buffer pH 6.0 and incubated at 70 °C. The results showed that the Mn^{++} ion did not have significant effects on glucoamylase stability. With 0.5 % α-cyclodextrin the half-life time can increase to about 3 times (from 100 min. up to 300 min.). These results proved that in the presence of substrate or substrate homologue protects the enzyme.

Kinetic studies

The kinetic parameters of both purified enzymes were studied on different substrates. In the case of glucoamylase the enzyme cleaved maltose, but it is very slowly as corroborated by the kinetic data (Table 3). For the higher maltooligosaccharides k_{cat}/K_m values increase steadily meaning that the catalytic efficiency increases with substrate chain length.

Table 3 Kinetic parameters of glucoamylase from *T. lanuginosus* ATCC 34626

Substrate	K_m [mM]	V_{max} [U/mg]	k_{cat} [s^{-1}]	k_{cat}/K_m
Maltose	6.5	7.7	9.6	1.5
Maltotriose	3.5	18.8	23.5	6.6
Maltotetraose	2.1	29.2	36.6	17.4
Maltopentose	1.1	29.3	36.7	33.6
Soluble starch (MERCK)	0.82 (mg/ml)	75.11	94.0	114.8

In the case of α-amylase the K_m and V_{max} were 0.68 mg/ml and 45.19 (U/mg protein), respectively. The K_m value agreed very well with the K_m value (0.67 mg/ml) of α-amylase from *T. lanuginosus* F1 studied by Obido & Ulbrich-Hofmann [20] and higher than the K_m value (0.5 mg/ml) of α-amylase from *T. lanuginosus* ATCC 34626 (wild strain) reported by Petrova and co-workers [22]. k_{cat} and the rate of k_{cat}/K_m were 42.18 s^{-1} and 61.78 ml/mg.s, respectively.

N-terminal amino acid sequences of α-amylase

N-terminal amino-acid analysis showed that the first 37 residues from *N*-terminal of α-amylase from *T. lanuginosus* ATCC 34626 are ATPDEWKAQaIYFMLTDRFARTDXSTTAPXD-XTAGkY (the residues written in lowercase are uncertain according to sequencing). Using database search (http://pbil.univ-lyonl.fr/BLAST/) the segment shows strong homology with sequences of α-amylase isolated from *Emericella nidulans* and *Aspergillus species* (Fig 6).

Figure 6 Sequence alignment of α-amylase from *T. lanuginosus* with other α-amylases (database ID numbers are on right side). Amino acid residues found in all sequences are demonstrated in bold letter.

The alignment suggests that the ambiguous alanine residue should be serine whereas presence of the residue lysine is seemed to be confirmed. This conclusion agreed with the sequence patented by Michelsen and Rasmussen [23], who published the sequence of α-amylase from *T. lanuginosus*. All of the other α-amylase demonstrated in the Fig 6 are belongs to glycosyl hydrolase family 13 (http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf_13.html) and the high similarity of α-amylase from *T. lanuginosus* suggests that this α-amylase enzyme also belongs to glycosyl hydrolase family 13.

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Curriculum Vitae

Quang D. Nguyen was born on July 14th, 1970 in Nghi Duc, Nghe an, Vietnam. After finishing higher school he took admission to Hanoi Agricultural University. Based on the result of admission and the Cultural and Educational Agreement between Hungarian and Vietnamese Governments he won the state scholarship to Hungary. In 1990 he started his studies at GödöllŐ Agricultural University (Hungary), but in 1991 he moved to University of Horticulture and Food, Faculty of Food (Budapest, Hungary). During the university studies, in 1994 he participated the Competition for students of Hungarian Agricultural Universities about informatics and won second place medal. In 1995 he won the extra-prize medal with his thesis entitled "Modelling of canned heating" at XXII. OTDK. In 1995 he started diploma works at Department of Brewing and Distilling. The results of his works were demonstrated at MÉTE OTDK and the first place medal was awarded to him. One year later in 1997, he took the second place medal at XXIII. OTDK. He obtained Master of Science (MSc) degree in 1997 from University of Horticulture and Food, Faculty of Food. In this year he took admission to PhD programme of Food Engineering and Sciences under supervisor of Prof. Dr. Ágoston Hoschke. His PhD topic is "study of fungal enzymes". In 1998 thanks for INCO-COPERNICUS Programme in frame of exchange of researchers he spent 3 months in Institute of Food Research, Reading Laboratory, UK, and here he has done the research works dealing with β-fructofuranosidase synthesised by *Aspergillus niger*. Three years later, he spent 6 months in Ghent University, Department of Microbiology, Physiology and Biochemistry, Laboratory of Biochemistry (Belgium). There he did the works dealing with kinetics of amylolytic enzymes from *Thermomyces lanuginosus* as well as determination of *N*-terminal sequence of α-amylase.

Since 2001 he works as assistant lecturer at Department of Brewing & Distilling, Szent István University.

The results of his research works were published in various scientific periodicals as well as demonstrated at different international and Hungarian scientific conferences.

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