

Full Length Research Paper

Production of α -amylase from some thermophilic *Aspergillus* species and optimization of its culture medium and enzyme activity

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This paper aimed to evaluate the thermophilic and hyperthermophilic fungi isolated from extreme conditions capable of secreting α -amylase, an enzyme that has a commercial value especially in the production of bread in the food industry and also to ensure their optimization. In this study, thermostable amylase activities of some thermophilic *Aspergillus* species were evaluated. The suitable medium and microorganisms for α -amylase synthesis were selected. Subsequently, the α -amylase activity of the microorganism was researched. In the measurements made on the 7th day of production on respectively *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus terreus* cultures produced at mycological, stock basal medium (SBM) and starch yeast extract liquid medium in order to determine a more efficient medium for amylase activity, the α -activity observed was 1.83, 1.66 and 0.75 U/ml in the mycological liquid medium, 2.37, 2.28 and 1.69 U/ml in the SBM medium and 6.8, 5.9 and 5.1 U/ml in the starch yeast extract liquid medium. The optimum temperature (65°C) and the optimum pH 8, respectively, and activity of α -amylase enzyme obtained from *A. niger* showed the highest activity level.

Key words: *Aspergillus*, enzyme activity, thermophilic fungi, α -amylase.

INTRODUCTION

Many processes which have significance in industrial terms take place much more easily through biotechnological and enzymatic processes. Therefore, the use of enzymes in industry has been inevitable. Through the use of enzymes in industry, the elimination of the conditions that require energy such as high pressure and temperature provide economic benefits (Çetin, 1983). The quantity of enzymes used in food industry is about 1.4. billion USD across the world and

it is one of the most common consumption areas with an increase in market network, over 10% per year and sales growth at a rate of 4-5%. 75% of the industrial enzyme production takes place within the food industry (Cowan, 1996). Today, many enzymes secreted by microorganisms are used in the food industry. The food industry sector in which amylases are most widely used is bread production. Amylases are one of the important commercial enzymes occupying about 25 to 33% of the

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world's enzyme market. With the use of thermostable α -amylase, the risk of bacterial and viral contamination is minimized at high temperatures used in processes (Fujiwara, 2002). Today, the emphasis placed on characterizing the amylolytic enzymes increases day by day for both their biotechnological potentials and quality of being a good model for use in both thermostability studies (Leveque et al., 2000).

α -Amylase ("3.2.1.1"), the 1-4 alpha glucosidic links oligosaccharides and polysaccharides (Nielsen and Borchert, 2000). Generally, amylases are classified into three categories. These are exoamylases (β -amylase), endoamylases (α -amylase) and the amylase enzymes (pullulanase, isoamylase) which do not show branching (Sahnoun et al., 2012). The α -amylase enzyme allows for the formation of glucose, maltose, maltotriose and α -limit dextrins by splitting off the α -1,4 links. Glucoamylase breaks the α -1,3, α -1,4 and α -1,6 links and converts them into glucose molecule (Haki and Raksit, 2003).

The α -amylase enzyme was first industrially produced in Japan in 1939 using *Bacillus subtilis* strain. In 1970, *B. subtilis* and *Bacillus licheniformis* were widely used for the production of alpha-amylase enzyme (Sahnoun et al., 2012). All α -amylases of thermophilic and hyperthermophilic origin are monomeric enzymes and their molecular weights range from 42 to 68 kD (Leveque et al., 2000). The fact that thermostable α -amylases do not require calcium ions for thermostability and activity makes thermophilic amylases different from the mesophilic α -amylases (Laderman et al., 1993). Today, many enzymes of microbiological origin are used in the food industry. The field of the food industry in which the amylases are most commonly used is the bakery sector. It is recommended to use α -amylases which are thermostable and resistant to bread-baking temperature (Nguyen et al., 2002).

MATERIALS AND METHODS

Microorganisms used in the study

A. niger, *A. oryzae*, *A. terreus*, fungi included in the class of Ascomycetes were used in this study. In order to ensure the continuity of the stock cultures of microorganisms; starch yeast extract agar (starch: 5.0 g/l; yeast extract: 2.0 g/l; KH_2PO_4 : 1.0 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.5 g/l; agar: 15 g/l) medium was used for amylase production. Stock fungal cultures were transferred to starch yeast extract agars and Petri dishes every 3 weeks and the fungal cultures produced after 5 days of incubation at 30°C were stored at 4°C for use in the study.

Preparation of production medium for amylase enzyme

In our study conducted to determine the more efficient medium for amylase activity, three different starchy media were tested, including mycological liquid medium, stock basal medium (SBM) and starch yeast extract liquid medium. SBM and stock mineral medium (SMM) suggested by Forney and Reddy (1979) and Kirk (1981) were used. SBM medium was prepared using (in g/100 ml)

KH_2PO_4 : 0.02, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.05, $\text{NH}_4\text{H}_2\text{PO}_4$: 0.05, yeast extract: 0.001, Starch: 1. The content of the SMM medium was however used as 0.14% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and as 0.1% FeSO_4 was added at a rate of 0.1% to the SBM medium. The pH of the media prepared was adjusted to pH: 6. The sterilization of SBM media was performed in the autoclave for 25 min at 110°C. In the sterilization of SMM medium, the millipore filtration technique was employed.

During the other studies we conducted to determine the more efficient medium for amylase activity, mycological liquid medium recommended by Paice et al. (1989) was used in addition to the SBM medium. The components of the mycological liquid medium were prepared using 1% Bacto-soytone, 4% Bacto-dextrose. In this medium, the pH value was adjusted to pH 6 and was sterilized in the autoclave for 15 min at 121°C.

The modified starch yeast extract liquid medium showing a high-productivity and activity was sterilized in the autoclave at 121°C for 15 min and at pH: 7 (Saleem and Mohsen, 2014) and used in the studies of microorganism cultivation and enzyme production.

Production of α -amylase enzyme through flask-shaking method and determination of its culture and activity optimization

Effect of incubation period

Following the planting process of the discs of 10 mm in diameter cut from 7-day fungal cultures in 50 ml of starch yeast extract liquid medium (pH 6), they were incubated in the shaking incubator and determined by looking at different incubation period (in the 2nd, 3rd, 4th, 5th, 6th, 7th and 8th day). To this end, the cultures were centrifuged at 10000 g, 4°C for 10 min and then the supernatant obtained by filtration was used in enzyme activity tests (Saleem and Mohsen, 2014).

Effect of temperature

Effect of temperature was determined after 7 days of incubation at 5, 20, 25, 30, 35, 40, 50, 65, 70°C in 50 ml of starch yeast extract liquid medium (pH 6) (Saleem and Mohsen, 2014).

Effect of pH

Effect of pH was determined after 7 days of incubation at 30°C at a pH value of 4, 5, 6, 7, 8 and 9 (Saleem and Mohsen, 2014).

Amylase activity assay

Amylase activity was measured according to the method of identifying reducing sugars released by enzymatic hydrolysis of raw starch with dinitrosalicylic acid (DNS) according to Miller (1959). A unit of amylase hydrolyzing the raw starch was defined as the amount of enzyme releasing 1 μmol of reducing sugar (glucose) per minute in standard test conditions (Singh et al., 2014). One milliliter (1 ml) of starch suspended in acetate buffer of 1% 0.1 M, pH 5.0 and 0.5 mL of crude enzyme were put on the sampling tube. Following their incubation in a shaking water bath at 50°C for 30 min, the tubes were kept in boiling water for 5 min in order to stop the reaction and centrifuged for 5 min by 3000 rev/min. After being soaked in boiling water for 5 min, 8 ml of distilled water was added to them. The OD values of sampling and control tubes were read against the blank tube in a spectrophotometer set at 550 nm wavelength (UV/VIS 1800 SHIMADZU). The absorbance value of the control tube was subtracted from the absorbance value of the sampling tube and thus, the OD values of reducing sugars released

Table 1. Amylase activities (by day) of the *Aspergillus* species produced in different media.

| Medium | Day | <i>A. niger</i> amylase activity (U/ml) | <i>A. oryzae</i> Amylase activity (U/ml) | <i>A. terreus</i> Amylase activity (U/ml) |
|--------------|-------------|--|---|--|
| Mycological | 2 | 0.18±0.04 | 0.11±0.04 | 0.10±0.04 |
| | 3 | 0.21±0.03 | 0.19±0.03 | 0.14±0.03 |
| | 4 | 0.38±0.04 | 0.28±0.04 | 0.25±0.04 |
| | 5 | 1.58±0.03 | 1.38±0.03 | 0.58±0.03 |
| | 6 | 1.82±0.01 | 1.66±0.01 | 0.75±0.01 |
| | 7 | 1.83±0.01 | 1.66±0.01 | 0.75±0.01 |
| | Stock basal | 2 | 0.81±0.01 | 0.79±0.01 |
| 3 | | 1.04±0.02 | 1.01±0.02 | 1.09±0.02 |
| 4 | | 1.42±0.02 | 1.22±0.02 | 1.22±0.02 |
| 5 | | 2.21±0.03 | 2.11±0.03 | 1.47±0.03 |
| 6 | | 2.36±0.02 | 2.27±0.02 | 1.67±0.02 |
| 7 | | 2.37±0.01 | 2.28±0.01 | 1.69±0.01 |
| Starch yeast | | 2 | 1.13±0.02 | 1.03±0.02 |
| | 3 | 2.18±0.01 | 2.08±0.01 | 1.98±0.01 |
| | 4 | 3.82±0.02 | 3.22±0.02 | 2.99±0.02 |
| | 5 | 5.35±0.02 | 5.25±0.02 | 4.37±0.02 |
| | 6 | 6.79±0.01 | 5.88±0.01 | 4.99±0.01 |
| | 7 | 6.8±0.01 | 5.9±0.01 | 5.1±0.01 |

The values given in the table are the average of 3 trials. Standard deviations are given by their sides.

by the enzyme were determined. Also, the enzyme activity was calculated as unit/ml using the glucose standard graph previously issued by the DNS at 550 nm.

Optimization of enzyme activity

Enzyme pH stability

In order to determine enzyme pH stability, substrate solutions with 1% starch were prepared using buffers Na-Fosfat (pH 6.0-10.0), Glisin-NaOH (pH 8.5-10.5) and Boraks-NaOH (pH 11.0-13.0). After 0.5 ml enzyme was incubated for 1 h in a water bath at temperatures ensuring the production of enzymes by mixing substrate solution at different pHs into tubes at a volume of 0.5 ml, they were boiled for 5 min by putting an equal volume of DNS reagents into the samples (1 ml enzyme + substrate, 1 ml DNS). Following this process, the samples were cooled and read at 550 nm in the spectrophotometer. The blank consists of an equal volume of substrate and DNS mixture (Arkan, 2008; Shanmughapriya et al., 2010).

Enzyme thermal stability

In order to determine enzyme thermal stability, a pre-incubation process was performed to the enzymes for 1 h at different temperatures between 30-100°C. Following the pre-incubation process, 0.5 ml from the enzyme pre-treated at different temperatures and 0.5 ml from the substrate samples prepared at the pH which allows for the optimum activity were mixed and submitted to 1 h of incubation at the temperature of optimum activity. At the end of incubation, standard activity was determined (Arkan et al., 2003; Arkan, 2008).

RESULTS

In this study, first, the selection of the media and microorganisms suitable for amylase suitable was performed. Then, an analysis was performed on the amylase activity effectiveness of the microorganisms. In the measurements performed on the 7th day of the production of *A. niger*, *A. oryzae* and *A. terreus* cultures produced in mycological, SBM and starch yeast extract liquid medium, an α -amylase activity was observed respectively as 1.83, 1.66 and 0.75 U/ml in mycological liquid medium, 2.37, 2.28, 1.69 in SBM and 6.8, 5.9 and 5.1 U/ml in starch yeast extract liquid medium (Table 1).

After 7 days of incubation, it was found that, for the production and activity of α -amylase enzyme obtained from the *A. niger* showing the highest activity, the optimum temperature was 65°C and the optimum pH was 8 (Table 2).

DISCUSSION

In this study, the potential of thermotolerant amylase enzyme derived from thermophilic fungi which can be used in the food sector as well as many other sectors was studied. In terms of enzyme activity, different characteristics were observed among different species of the same genus of fungi.

Table 2. The effect of the ambient pH and temperature on the activity of amylase obtained from *Aspergillus niger*.

| pH | Enzyme activity (U/ml) | Temperature (°C) | Enzyme activity (U/ml) |
|----|------------------------|------------------|------------------------|
| 6 | 5.99 ± 0.01 | 30 | 4.98 ± 0.02 |
| 7 | 7.79 ± 0.01 | 40 | 6.86 ± 0.02 |
| 8 | 8.15 ± 0.03 | 50 | 6.91 ± 0.03 |
| 9 | 8.09 ± 0.01 | 55 | 7.89 ± 0.01 |
| 10 | 7.05 ± 0.02 | 60 | 8.10 ± 0.02 |
| 11 | 7.00 ± 0.02 | 65 | 8.25 ± 0.01 |
| 12 | 5.10 ± 0.03 | 80 | 8.00 ± 0.02 |
| 13 | 3.10 ± 0.01 | 100 | 7.99 ± 0.01 |

The values given in the table are the average of 3 trials. Standard deviations are given by their sides.

Because of diminishing natural resources, the microorganisms are today regarded as a potential for many production areas and extensive studies are conducted in this regard. Among these groups of microorganisms, fungi also have an important place. They have biotechnological potentials for use in thermostability studies. As well, protein engineering studies are carried out with the aim of developing enzymes in a more unusually thermostable feature using the mechanisms of thermostability (Leveque et al., 2000).

Until the early 1970s, plant and animal materials were considered to be the best source of enzymes. However nowadays, the importance of microbial enzymes steadily increases in terms of their technical and economic advantages. So far, more than 2000 enzymes have been identified, and although about 100 of these enzymes were found to be suitable for commercial use, only 18 of them are used for industrial purposes. α -Amylase enzyme was first produced, on an industrial scale, in 1939 in Japan using *B. subtilis*. In general, the immobilized enzymes with thermostability are used in industrial applications. The application area of α -amylases is reasonably expanded and diversified. These enzymes are used in starch liquefaction, bread production, textiles and in paper and fruit juice industry as well as in the alcohol fermentation (Uçar, 2011).

The CO₂ gas is what ensures the leaven of the bread and allows it to gain volume during both fermentation and baking in bread making. Performing the yeast fermentation depends, above all, on the presence of a sufficient amount of fermentable sugar in the medium. However, flour has a very low amount of fermentable sugar necessary for yeast activity and a very high amount of starch. Amylase enzymes lyse the bruised starch (4-10%) and gelatinized starch available in the flour and form fermentable sugar (Elgün and Ertugay, 1995).

In this study, isolation of thermophilic fungi producing amylase from water and soil samples obtained from the area where the thermal springs ranging from 55 to 90°C around Afyon and Eskisehir in Turkey. Thermostable

amylase activity of *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus terreus* were evaluated.

The determination of our fungal isolates showing the maximum alpha amylase enzyme activity which is important in bread production among the isolates isolated from extreme conditions; the provision of material to the microbial gene banks and the use of thermostable fungal enzymes in the industrial applications, economy and the food industry will be among the outputs of this research.

Conclusion

In our study, the production of the α -amylase enzyme obtained from *A. niger*, *A. oryzae*, and *A. terreus* fungus through flask-shaking method and their culture and activity optimization was performed. It was found that, for the production and activity of α -amylase enzyme obtained from the *A. niger* showing the highest activity, the optimum temperature was 65°C and the optimum pH was 8. This study is important in terms of providing a basis for future studies on thermostable and immobilized fungal α -amylase enzyme to be obtained from the strains of domestic thermophilic fungi for use in food and bread-making industry as well as in other sectors.

Conflict of interests

The author has not declared any conflict of interest.

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