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Increase of the α-amylase yield by Some *Bacillus* Strains*

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Abstract: In order to obtain high amounts of α-amylase enzyme from *Bacillus* bacteria a new medium was developed. In this medium, three strains of *Bacillus* bacteria were grown in the presence of different carbon, nitrogen and metal ions. The highest α-amylase yield was obtained by addition of Na-citrate, strach and sucrose for the strains of *B. subtilis, B. amyloliquefaciens* I and *B. amyloliquefaciens* II, respectively. As a nitrogen source, yeast extract was found to be effective on enzyme yield for *B. subtilis* and *B. amyloliquefaciens* II, while ammonium sulfate was found to be effective for *B. amyloliquefaciens* I. None of the metal ions increased the enzyme activity of all the strains. However, a slight increase was determined by increase of oxygen concentration. The optimum temperature of each bacteria for α-amylase was 55°C. The optimum pH of the *B. subtilis* enzyme was 7.0, while for both strains of *B. amyloliquefaciens* the optimum pH was 5.9. Enzymes were not stable at elevated temperatures and at low or high pH values. Inhibitory effects of Ag, Zn and Cu were observed in all *Bacillus* strains. Maltose and a small amount of glucose were determined as the split products of starch by thin layer chromatography (TLC) after incubation of starch with amylases of all the strains used in this study.

Key Words: *Bacillus,* α-amylase, production, characterization, thin layer chromatography.

Bazı *Bacillus* Suşlarında α-amilaz Veriminin Arttırılması

Özet: *Bacillis* suşlarından α-amilaz enziminin yüksek verimde elde etmek için yeni bir ortam geliştirildi. Bu ortamda üç *Bacillus* suşu farklı karbon, azot ve metal kaynakları varlığında üretildi. En yüksek alfa-amilaz verimi *B. subtilis*'te NA-sitrat, *B. amyloliquefaciens* I'de nisaşta, *B. amyloliquefaciens* II'de ise sukrozlu ortamda elde edildi. Azot kaynağı olarak *B. amyloliquefaciens* I için amonyum sülfat iyi bir kaynak iken yeast ekstrakt *B. subtilis* ve *B. amyloliquefaciens* II için enzim bakımından verimli bulundu. Hiç bir metal iyonu tüm suşların enzim aktivitesini arttırmadı. Diğer yandan enzim aktivitesinde az bir artış oksijen konsantrasyonunun arttırılması ile sağladı. α-amilaz için tüm suşlarda optimum sıcaklık 55°C'dir, *B. subtilis* için optimum pH 7, *B. amyloliquefaciens*'in her iki suşu için ise 5.9 olarak bulundu. Enzimin düşük ya da yüksek pH değerlerinde ve yüksek sıcaklıkta stabil olmadığı saptandı. Ag, Zn ve Cu'nun tüm suşlarda güçlü olarak inhibitör etki gösterdiği gözlenmiştir. Çalışmada kullanılan tüm suşlardan elde edilen amilazların nisaşta ile inkübasyonlarından sonra yapılan İnce Tabaka Kromatografileri ile nişastanın son ürünleri olarak az miktarda glukoz ve bir miktar maltoz saptanmıştır.

Anahtar Sözcükler: *Bacillus,* α-amilaz, Üretim, Karakterizasyon, İnce Tabaka Kromatografisi.

* This study is a part of a PhD thesis.

Introduction

The α-amylase enzyme (EC 3.2.1.1) is widely distributed in animals, plants and microorganisms. Microorganisms are very important for enzyme production, because of their short growth period. α-amylase catalyzes the hydrolysis of interlal α-glucosidal linkages in starch and other oligo-and polysaccharides, and shows varying action patterns. Amylase are widely used in the food and textile industries (1, 2).

Growth conditions and nutrients promote a high yield of α-amylase production (2, 3). Starch or other sugars as a carbohydrate source, and ammonium salts or complex organic compounds as a nitrogen source are needed for bacterial growth and enzyme production. On the other hand, all α-amylases are calcium metalloenzymes and thus need calcium or other metal ions for activity (4).

This study was firstly planned to modify a medium for a high production of α-amylase enzyme from some *Bacillus* strains isolated from Turkish soils. Later, the newly developed medium was tested for some carbon and nitrogen sources and metal ions to increase the enzyme yield and also enzymes were characterized.

Materials and Methods

Microorganisms

B. subtilis and two *B. amyloliquefaciens* strains were obtained from the enzyme producing plant (ORBA Biochemical, İstanbul). All strains were isolated from Turkish soils and were identified by the enzyme producing plant.

Media and culture conditions

Three kinds of enzyme production media of different contens (ORBA, Bezbaruah et al. 1991, modified medium) and bacterial growth media are given in Table 1. Enzyme production media were tested for the enzyme production acceptability of each strain. Firstly, bacteria were put in the bacterial growth medium for 20 hrs. Then, overnight cultures which adjustted OD_{600nm}=0.3 were inoculated with 1% in the enzyme production media (500 ml flasks with 150 ml medium) and were allowed to grow for *B. subtilis* at 30°C, and *B. amyloliquefaciens* strains at 37°C. Cultivation was carried out for 88 h on a rotary shaker at 150 rpm. Cells were removed by centrifugation (6000 rpm, 10 min.) and supernatants were used for determination of the enzyme activity while pellets were used for total protein. Because *B. subtilis* is a mucoidal strain and produces a large amount of mucous material during growth, we did not use supernatant containing mucous material.

Total protein was determined by biuret (5). α-amylase activity (Unit/ml) was assayed by the strach-iodine method according to Yoo et al. (1987) (6). One enzyme unit was defined as the amount of enzyme that 1 mg of starch (0.1%) hydrolyzed by enzyme in 10 min at 37°C and pH 5.9.

Table 1. Media for growth of *Bacillus* strains and α-amylase enzyme production.

Materials	Bacterial growth medium (%)	Enzyme production media		
		Bezbaruah et al (1991) (%)	ORBA Chemical (%)	Modified Medium* (%)
Starch	0.5	0.2	10.0	1.0
Peptone	0.5	0.5	–	0.5
Corn Steep	0.3	0.3	–	0.5
Liguor $(NH_4)_2SO_4$	0.4	–	0.6	0.8
$MgSO_4.7H_2O$	0.1	0.1	–	0.2
$(NH_4)_2HPO_4$	–	0.4	–	–
Soybean Fluor	–	–	3.0	–
$CaCl_4.2H_4O$	–	–	3.05	0.05
K_4HPO_4	–	–	1.4	1.4
KH_4PO_4	–	–	0.6	0.6
Citric Acid	–	–	0.32	–
pH	7.0	7.0	6.5	7.0

The effects of different carbon, nitrogen sources, metal ions and dissolved oxygen

In order to increase the enzyme productivity, sucrose, Na-citrate and glucose syrup were used as carbon sources instead of soluble strach, and yeast extract, soybean flour, peptone and ammonium sulfate were used as nitrogen sources instead of peptone+ammonium sulfate and Mg+Fe, Mg+Mn, Ca+Fe, Ca+Mn used as metal ion combinations in place of Ca+Mg in the modified medium.

Prepared concentraitons were 1%, 3%, 0.2%, 0.05%, 0.005% and 0.01% for carbon and nitrogen, metal ions of $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $MnCl_2.4H_2O$ and $FeSO_4$, respectively. The effects of dissolved oxygen in the modified medium were determined at different shaker speeds: 0, 50, 100 and 200 rpm.

Properties of the enzymes

The optimum temperatures of enzymes were established to be 37-100°C, and thermostability was determined to be 90°C for 2 hrs. The optimum pH was obtained between 4.0-9.0, and pH: 4.0 and pH: 9.0 were used for pH stability at 37°C for 24 hrs. The activity was accelerated in the presence of certain metal ions with a 5 mM concentration (Table 4). The activity was expressed as a percentage of the activity of the control, untreated with metal ions, which was taken as 100%. In addition, products of starch hydrolyzed by amylases were chromatographed on silica gel plates (0.25 mm thick and 20x20 cm in size) with butanol: ethanol: water (5:3:2, v/v/v) at room temperature, and they were visualized with sulfuric acid: methanol (1:3, v/v).

Results

The modified medium was the most conducive to higher enzyme activity, and therefore we chose it as a test medium for further investigations. This medium contained 10 times less strach than the ORBA test medium, and the absence of soya bean facilitated test measurement.

Table 2 shows that all strains grew very well on the ORBA Chemicals medium, but the maximum enzyme level of the strains was obtained in the modified medium at 72 h. In the medium of Bezbaruah et al. (7), maximal enzyme activity was reached in a short time. In this case, we think than it did not contain several elements in order to continue enzyme production.

Growth and enzyme production were differentt for each medium and strain. It has been reported that maximal enzyme activity was reached at the end of logaritmic phases (8), but we found that the bacteria grown in each medium also continued enzyme production until death phase. Therefore, we were unable to explain the relation between achieving maximal enzyme production and bacterial growth according to the data obtained.

The results related to carbon sources showed that all strains could produce more enzyms with different kinds of carbon sources, i.e., *B. subtilis* with Na-citrate, *B. amyloliquefaciens* I with soluble starch and *B. amyloliquefaciens* II with sucrose (Table 3). While the maximal growth was observed with Na-citrate for *B. subtilis,* the other strains were affected by soluble starch. *B. subtlis* had two times more growth with glucose syrup and Na-citrate than with other carbon sources. In contrast, *B. amyloliquefaciens* I had 150-50% growth with glucose syrup and strach, respectively. It was also determined that *B. subtilis,* which produces mucous material during the incubation period, did not produce any mucous material in the presence of Na-citrate.

Table 3 shows also *B. subtilis* and *B. amyloliquefaciens* II strains produced more enzyme with ammonium sulfate as nitrogen source, while *B. amyloliquefaciens* I produced more enzyme with yeast extract for this purpose. But *B. subtilis* has denser mucous material with ammonium sulfate. This created the greatest problem during centrifugation. When peptone was removed, mucous material was not observed.

Table 2. Maximal α-amylase production and bacteria growth in the different media.

	B. subtilis		*B. amyloliquefaciens* I		*B. amyloliquefaciens* II	
	Total Protein (mg/ml)	Enzyme activity (U/ml)	Total Protein (mg/ml)	Enzyme activity (U/ml)	Total Protein (mg/ml)	Enzyme activity (U/ml)
Bezbaruh et al. (1991)	2.8	5100	7.4	8726	4.4	12278
ORBA Chemical	16.4	11000	22.9	7500	24.6	15998
Modified	3.4	13600	10.0	14152	6.8	17570

While maximal growth and enzyme activity was obtained at 24-64 h in the Bezbaruah et al. (1991), others were determined 48-72 h. Each treatment was performed in triplicate.

Table 3. Effect of different carbon, nitrogen and metal ions sources on growth and enzyme production.

	B. subtilis		*B. amyloliquefaciens* I		*B. amyloliquefaciens* II	
	Total Protein (mg/ml)	Enzyme activity (U/ml)	Total Protein (mg/ml)	Enzyme activity (U/ml)	Total Protein (mg/ml)	Enzyme activity (U/ml)
Starch	3.4	13600	10.0	14152	6.8	17570
Glucose syrup	5.0	11100	9.4	12800	5.6	15384
Sucrose	4.8	11400	6.2	11692	5.2	17846
Na-citrate	6.4	16200	7.2	10460	4.0	16800
Yeast extract	4.2	13122	8.0	17846	8.4	18868
Peptone	6.6	13000	8.0	10500	6.6	15216
$(NH_4)2_2O_4$	4.8	16162	9.4	14090	5.6	22912
Soybean flour	9.0	15458	10.4	14152	7.2	15826
Mg+Fe	4.0	6152	4.2	9600	8.2	10104
Mg+Mn	4.4	3902	5.2	8292	6.6	9472
Ca+Fe	4.8	9230	7.6	13538	6.4	12324
Ca+Mn	3.4	9846	6.8	9998	8.8	11026

Dates shows maximal enzyme production at 72 hrs and maximal bacteria grown obtained at 40-72. Each treatment was performed in triplicate.

None of the additional metal ion combinations increased the enzyme activity of all strains (Table 3). The effects of dissolved oxygen are shown in Fig. 1. The enzyme production of all strains increased by shaking at 200 rpm under controlled pH conditions. This increase was 88% for *B. subtilis,* 70% for *B. amyloliquefaciens* I and 57% for *B. amyloliquefaciens* II according to 150 rpm (as control). Growth of *B. subtilis* increased at 200 rpm while those of other strains decreased.

Enzymatic properties of α-amylases

The optimum temperatures of all enzymes were 55°C (Fig. 2). Enzyme activity was decreased at the high temperature. This lost was 68%, 77%, 91% for *B. subtilis* amylase, 50%, 65%, 82% for *B. amyloliquefaciens* I amylase and 60%, 71%, 88% for *B. amyloliquefaciens* II amylase at 80°C, 90°C and 100°C, respectively. Thermostability tests of the enzymes at 90°C in 1 h showed that none of them were stable. The optimum pH of *B. subtilis* was 7.0 while that of other strains was 5.9 (Fig. 3).

The enzyme activity of *B. subtilis, B. amyloliquefaciens* I and *B. amyloliquefaciens* II lost 72%, 51%, 57% at pH 4.0. Loss in activity at pH 8.0 and 9.0 was 48%, 56%, 44% and 57%, 67%, 51%, respectively. The pH stability showed that they were more stable in the alkaline medium than in the acidic one, and were still active for 24 h after treatment with alkaline.

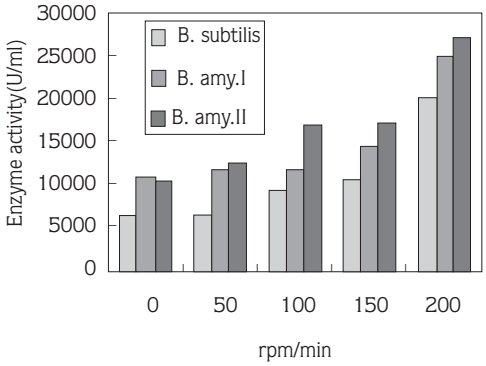

Figure 1. Effect of dissolved oxygen concentration on amylase activity. Each treatment was performed in triplicate.

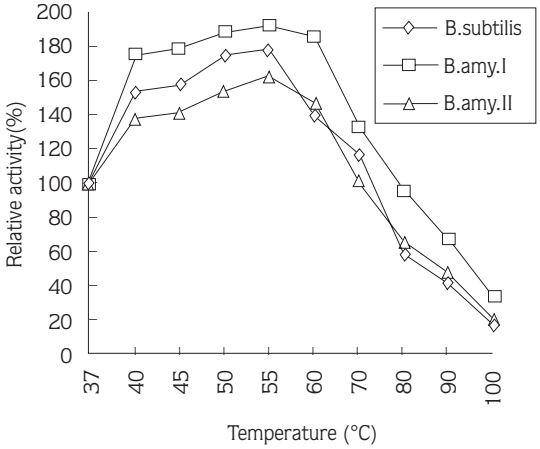

Figure 2. Effect of temperature on the α-amylases of three *Bacillus* strains. Each treatment was performed in triplicate.

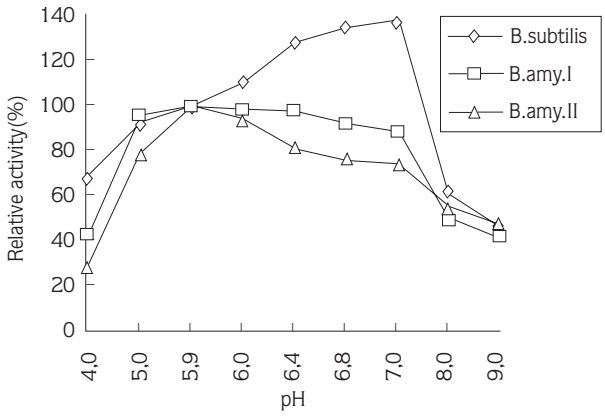

Figure 3. Effect of pH on the amylases. Each treatment was performed in triplicate.

α-amylase enzyme activity was tested in the presence of various metal ions (Table 4). We did not find any metal to have a stimulatory effect. It was found that Ag, Zn and Cu have inhibitory effects on all the amylases tested.

Fig. 4 shows split products of starch by α-amylases. The main product was maltose within maltooligosaccharides and a small amount of glucose was also determined.

Discussion

The effects of different carbon and nitrogen sources, metal ions and dissolved oxygen on α-amylase production showed that each bacteria behaved differently. Similar results have also been obtained by other researchers. Tonkova (9) reported that α-amylase production by *B. licheniformis* strain with citrate was fivefold greater than with glucose. Jamuna and Ramakrishna (10) determined that the enzyme production by *B. subtilis* was higher with glucose than with strach. However, Fang and Demain (11) have obtained greater production with starch as the sole carbon source.

As for nitrogen sources, Jin et al. (12) determined higher enzyme production from *B. subtilis* on yeast extract than peptone, and no effect of ammonium sulfate was recorded. Coleman and Elliott (13) stated that ammonium salts were stimulators of *B. subtilis* amylase enzyme production. In addition, Mai et al. (14) have reported that ammonium sulfate on the *B. stearothermophilus* enzyme yield. Carbon and nitrogen sources were very important for bacterial growth.

Both our results and those of other researchers have shown that the enzyme production patterns of *Bacillus* strains were very different, but the mechanism for this pattern is still not completely known.

Table 4. Effect of metal ions on α-amylase enzymes in *Bacillus* strains.

Metal ions (5 mM)	*B. subtilis* (%)	*B. amylo.* I (%)	*B. amylo.* II (%)
None	100	100	100
$FeSO_4$	96	13	11
$CaCl_2$	105	76	89
$MgSO_4$	122	98	102
$MnCl_2$	108	54	53
$ZnSO_4$	15	16	9
$BaCl_2$	103	68	62
$LiSO_4$	107	103	102
$CuCl_2$	40	34	42
$AgSO_4$	37	9	11

Each treatment was performed in triplicate.

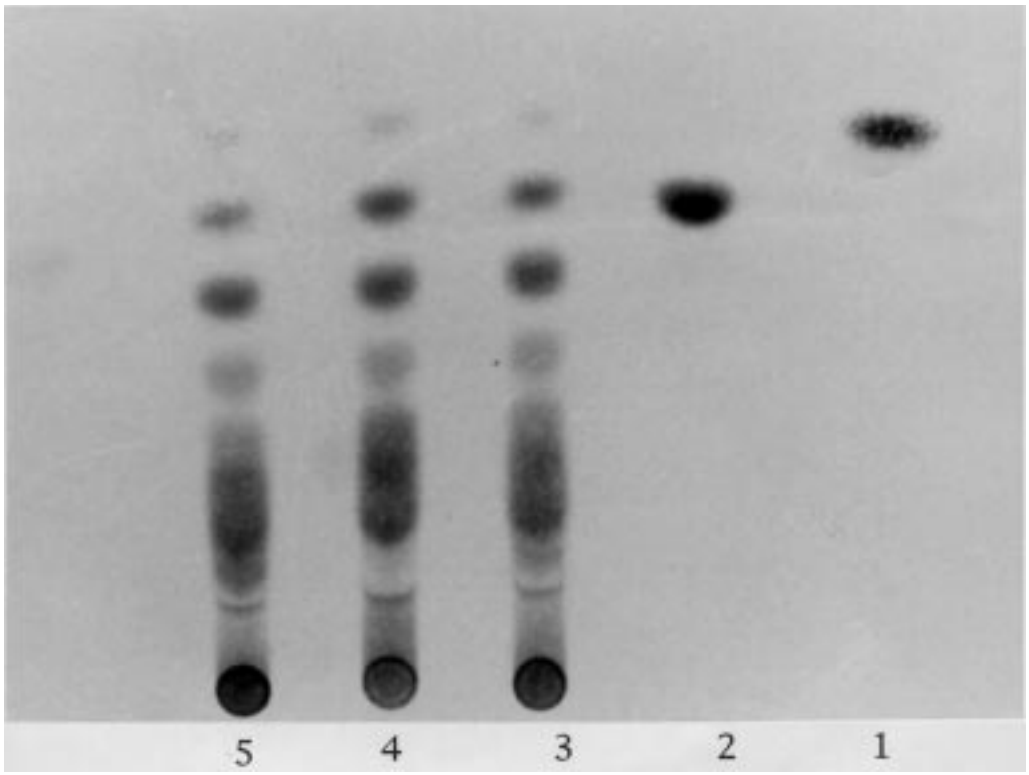

Figure 4. Hydrolysis products of starch with amylases by thin-layer chromatography. 1 Glucose and 2 Maltose as standards, hydrolysates of amylases of 3 *B. amyloliquefaciens* I, 4 *B. subtilis*, 5 *B. amyloliquefaciens* II.

We did not find a high increase with metal ions, but Kadrekar and Ramasarma (15) suggest that there is higher production with Ca+Mn. Manning and Campbell (16) and Hamada et al. and Despande and Cheryan (17, 18) have explained that Ca had significant effects on the metabolism and physiology of bacteria, and there was also found to be an effect on enzyme activity and stabilization in the defense against proteases. The importance of Ca ions has been reported by many researchers (19, 20). However, in our study, Ca did not have any effect on either growth or enzyme production.

Growth of *B. subtilis* increased at 200 rpm, while that of other strains decreased. A similar increase was found for the enzyme in controlled pH conditions have been also given (21).

The enzymatic properties of the α-amylases tested and of all the enzymes did not have stable temperature and pH. In general, *B. stearothermophilus* and *B. licheniformis* produced thermostability amylases (22, 23, 24). Fogarty (4) reported that the optimum pH value was 4.8-6.5, and the optimum temperature was 40°C-50°C for differet *Bacillus* strains.

The α-amylases produced maltose and glucose from soluble strach. Similar results have been reported by other researchers (25, 26, 2).

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References

1. Uhling, H., Industial enzymes and their applications. John Wiley & Sons. Inc., NewYork. 1998.
2. Yamamoto, T., Enzyme chemistry and molecular biology of amylases and related enzymes. CRC press, Tokyo. 1995.
3. Pazlarova, J., Baig, M.A., Votruba, J., Kinetics of α-amylase production in a batch and fed-batch culture of *B. subtilis* with caseinate as nitrogen source and starch as carbon source., Appl. Microbiol. Biotech., 20: 301-334, 1984.
4. Fogarty, W.M., Microbial enzymes and biotechnology Chapter 1 Microbial amylases. Applied Science Publisher, pp: 1-92, 1983.
5. Gurgun, V., Undersuchungen uber den anaeroben Dunkel Stoff weehsel einiger arter de phototrophen purpur bacterien., PhD thesis Gottingen-Germany, 1974.
6. Yoo, Y.J., Hong, J., Hatch, R.T., Comparison of α-amylase activities from different assay., Biotech. Method., Bioengn., vol: xxx, 147-151, 1987.
7. Bezbaruah, R.L., Gogoi, R.K., Pillai, K.R., Nigam, J.N., Amylase production by three *Bacillus* strains active at alkaline pH., J. Basic. Microbiol., 31: 13-20, 1991.
8. Priest, F.G., Extracellular enzyme synthesis in the genus *Bacillus*. Bacterial Rev., 41 (3): 711-735, 1977.
9. Tonkova, A., Effect of glucose and citrate on α-amylase production in *B. licheniformis*., J. Basic Microbiol., 31: 217-222, 1991.
10. Jamuna, R., Ramakrishna, S.V., Continuous synthesis of thermostable α-amylase by *Bacillus* cells immobilized in calcium alginate. Enzyme Microbiol. Techn. 14: 36-41, 1992.
11. Fang, A., Demain, A.L., A new chemicaly-defined medium for RAC-certified strains of *B. subtilis*., Appl. Microbiol. Biotech. 30: 144-147, 1989.
12. Jin, F., Cheng, X., Shi, Y., Zhang, C., Isolation of new thermophilic aerobic bacteria which produce thermostable α-amylase., J. Gen. Appl. Microbiol. 36: 415-424, 1990.
13. Coleman, G., Elliott, W.H., Studies on α-amylase formation by *Bacillus subtilis*., Biochem. J., 83: 256-263, 1962.
14. Mai, N.T., Giang, D.T., Minh, N.T.N., Thao, V.T., Thermophilic amylase producingbacteria from Vietnamese soils., World J. Microbiol. Biotech., 8: 505-508, 1992.

15. Kadrekar, S.N., Ramasarma, G.B., Amylase and protease production by *B. subtilis:* Studies on improvement of strain and medium., J. Fd. Sci. Technol., 27: 4-6, 1990.

16. Manning, G.B., Campbell, L.L., Thermostable α-amylase of *B. stearothermophilus.,* J. Biol. Chem., 236: 2952-2957, 1961.

17. Hamada, N., Fukumoto, J., Yamamoto, T., Glycosyl glycerophorsphoric acid and its polymer excreted by α-amylase-forming *B. subtilis.,* Agric. Biol. Chem. 35: 1052-1060, 1971.

18. Deshpande, S.S. and Cheryan, M., Effects of phytic acid, divalent cation and their interactions on α-amylase activity., J. Food Sci., 49: 516-519, 1984.

19. Kadziola, A., Abe, J.I., Svensson, B., Haser, R., Crystal structure of barley α-amylase., J. Mol. Biol., 239: 104-121, 1994.

20. Larson, S.B., Greenwood, A., Cascio, D., Day, J., McPherson, A., Refined molecular structure of pig prancreatic α-amylase at 2.1Å resolution., J. Mol. Biol., 235: 1560-1584, 1994.

21. Ishizaki, A., Ikeda, S., Shibai, H. and Hirose, Y., Influence of carbondioxide patrial pressure on pH in submerged cultures., Agric. Bio. Chem., 50 (3): 599-605, 1986.

22. Vihinen, M., Mäntsälä, P., Characterization of a thermostable *B. stearothermophilus* α-amylase, Biotech. Appl. Biochem., 12: 427-435, 1990.

23. Cordt, S., Hendrickx, M., Maesmans, G., Tobback, P., The Influence of polyalcohols and carbohydrates on the thermostability of α-amylase. Biotech. Bioengin., 43: 107-11, 1994.

24. Dobreva, E., Ivanova, V., Emanuilova, E., Effect of temperature on some characteristics of the thermostabile α-amylase from *B. licheniformis.*, World J. Microb. Biotech., 10: 547-550, 1994.

25. Kanno, M., A *B. acidocaldorius* α-amylase that is highly stable to heat under acidic conditions. Agric. Biol. Chem., 50: 23-31, 1986.

26. Takasaki, Y., An amylase producing maltotriose from *B. subtilis.*, Agric. Biol. Chem., 49: 1091-1097. 1985.