Production and Optimization of α-Amylase from *Bacillus circulans* ATCC 4516 with Solid State Fermentation

Katı Faz Fermantasyonu ile *Bacillus circulans* ATCC 4516' dan α-Amilazın Üretimi ve Optimizasyonu

Research Article

Besi Serin[1], Nurullah Akcan[2], Fikret Uyar[1]*

[1]Dicle University, Faculty of Science, Department of Biology, Diyarbakır, Turkey

[2]Siirt University, Siirt Health High School, Siirt-Turkey

ABSTRACT

The aim of this paper is to study influence of the certain production parameters of α-amylase by Bacillus circulans ATCC 4516. Optimization parameters of α-amylase were carried out with solid state fermentation (SSF). Solid waste from rice bran used as the basic nutrient source. Supplementation with carbon and metal salt sources decrased the enzyme production. Certain fermentation parameters involving incubation time, incubation temperature, inoculum level, initial pH and extraction medium were studied separately. Maximal amount of α-amylase production (2716.9 ± 35.9 U/mg) was obtained inoculum level 25%, initial pH 7.5 at 37ºC for 48 h with supplementation of ammonium chloride.

Key Words

Bacillus circulans ATCC 4516, rice bran, α-amylase, solid state fermentation

ÖZET

Bu çalışmanın amacı *Bacillus circulans* ATCC 4516 tarafından üretilen α-amilazın belirli üretim parametrelerini incelemektir. α-Amilazın optimizasyon parametreleri katı faz fermantasyonu ile gerçekleştirilmiştir. Katı atık besin kaynağı olarak pirinç kepeği kullanılmıştır. Karbon ve metal tuz kaynakları eklenmesi ile enzim üretimi azalmıştır. İnkübasyon zamanı, inkübasyon sıcaklığı, inokülüm düzeyi, başlangıç pH ve ekstraksiyon ortamı gibi belirli fermantasyon parametreleri ayrı ayrı incelenmiştir. Maksimum α-amilaz üretim miktarı (2716.9 ± 35.9 U/mg) amonyum klorür eklenmesi ile 48. saatte 37ºC'de inokülüm düzeyi %25, başlangıç pH'ı 7.5 olarak elde edilmiştir.

Anahtar Kelimeler

Bacillus circulans ATCC 4516, pirinç kepeği, α-amilaz, katı faz fermantasyon

Article History: Received April 03, 2012; Revised July 05, 2012; Accepted May 3, 2012; Avaliable Online: August 17, 2012.

Correspondence to: Fikret Uyar, Faculty of Sciences, Dept. Biology, Dicle University, Diyarbakır, TURKEY

Tel: +90412 24 88 550 /3060 Fax: +90 412 248 83 00 E-Mail: fuyar@dicle.edu.tr

INTRODUCTION

In recent years the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms [1]. α-Amylases (endo-1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.1) are extracellular enzymes that randomly cleave the 1,4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. Among various extracellular enzymes, α-amylase ranks first in terms of commercial exploitation [2]. α-Amylase is an important industrial enzyme. Interest in amylases has increased in recent years and there are a number of reports on *Bacillus* amylases with diverse properties suitable for industrial application [3]. α-Amylase widely used enzymes whose spectrum of applications has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification they also find applications in food, baking, brewing, detergent, textile, paper, and distilling industry [4]. In spite of the wide distribution of raw starch digesting enzymes, microbial sources have many advantages for the industrial production such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization [5]. Currently, a large number of microbial amylases are available commercially and they have almost completely replaced the chemical hydrolysis of strach in the starch processing industry [6].

SSF has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water; however, the substrate must possess enough moisture to support growth and metabolism of the organism [7,8]. Traditionally, amylase has been produced by submerged fermentation (SmF) and used in a one-way process in solution. In recent years, however, solid state fermentation (SSF) process have been increasingly utilized for the production of this enzyme [6]. SSF offers numerous oppurtunities in processing of agro-industrial residues and SSF reproduces the natural microbiological processes like composting and ensiling. On one hand by utilizing the low cost agricultural residues SSF adds on to economic feasibility of the process and on other hand it solves the problem of its disposal which otherwise cause pollution [7,8]. SSF has many advantages over SmF, including superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery and lack of foam build up and reported to be the most appropriate process for developing countries [9]. In the SSF process, the solid substrate not only supplies the nutrients to the cultures but also serves as an anchorage for the microbial cells [10]. In SSF, the solid material can serve as a physical support and as a source of carbon and nutrients to sustain microbial growth or only as an inert physical support to which nutrients and the carbon source are added. This solid material generally is a natural compound consisting of agricultural and agroindustrial by-products and residues, urban residues or a synthetic material [11]. The use of agricultural wastes makes SSF an attractive alternative method. The major factors that affect microbial synthesis of enzymes in a SSF system include selection of a suitable substrate and microorganism, particle size of the substrate, inoculum concentration and moisture level of the substrate [12,13].

The aim of the present study was to evaluate the feasibility of easily available substrate in SSF for the production of α-amylase by *Bacillus circulans* ATCC 4516. In this study, the effects of incubation time, incubation temperature, extraction medium, agitation speed, inoculum level, initial pH, supplementation with various carbon, nitrogen, and metal salt sources were investigated with rice bran.

MATERIALS AND METHODS

Microorganism

α-Amylase producing *B. circulans* ATCC 4516 which was purchased from MicroBioLogics, Inc. was used as biological material. *Bacillus circulans* ATCC 4516 was grown on nutrient agar at 37°C for 24 h for inoculum preparation. A loopful of the growth was transferred to Luria broth (LB) liquid medium (1% yeast extract, 0.5% peptone, 0.5% NaCl, (w/v), pH 7.0).

Substrate

Rice bran was used to be solid substrate. This is cheap and readily available in the local market, Diyarbakır, Turkey. The substrate was ground into coarse powder with a blender.

Solid State Fermentation

SSF was carried out by taking 3 g of rice bran (dry substrate) in a 100 mL erlenmeyer flask to which distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121°C for 15 min. Flasks with inoculated with 3.0 ml of spore suspension (2.10^7 CFU/mL) were shaken at 150 rpm at 37°C for 144 h. The contents of the flasks were harvested and assayed every 24 h.

Enzyme Extraction

The enzyme from the fermented bacterial bran was extracted twice with tap water. The slurry was squeezed through damp cheesecloth. Extracts were pooled and centrifuged at 4°C for 15 min at 10.000 rpm to separate small particles of different substrates, cells and spores. The brown, clear supernatant was used in enzyme assays.

Enzyme Assay

α-Amylase activity was determined by the procedure of Bernfeld using soluble starch as a substrate [14]. The reaction mixture containing 200 µl of 1% substrate (w/v) in 0.1 M phosphate buffer (pH: 7.0) and 150 µl of enzyme solution was incubated for 30 min at 37°C. The reaction was stopped by adding 400 µl of 3,5-dinitrosalicylic acid solution followed by heating in a boiling water bath for 5 min and cooling at room temperature and then 8 ml of deionized water was added. Absorbance of each solution containing the brown reduction product was measured at 489 nm in a UV-Visible spectrophotometer.

One unit (U) of α-amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar as maltose per minute, under assay conditions and expressed as U/g of dry substrate.

All the experiments are independent of each other. Results are represented as mean ± S.D. of at least three experiments.

Protein Determination

The protein amount was determined by Lowry method by using bovine serum albumin (BSA) as standart [15].

Effect of Process Parameters on α-Amylase Production in SSF

Various process parameters influencing enzyme production during SSF were optimized. The strategy was to optimize each parameter independently of the others and subsequently optimal conditions were employed in all experiments. The rice bran was employed for further optimization of process parameters, namely incubation time (24-72 h), incubation temperature (30, 37, 40, 45 and 50 °C), extraction medium (distilled water, tap water, 50 mM NaCl, 0.1 M phosphate buffer (pH 7.0), 1% solution of various detergents like Tween 40, CHAPS (3-[(3-Cholamido-propyl)-dimethylammonio]-propane- sulfonate), sodium dodesil sulfate (SDS) and Triton X-100), agitation speed (60, 100, 120, 150, 180 and 200 rpm), inoculum size (5-80%), initial pH of the medium (pH 5.0-9.0) were optimized. To study the efficacy of various inducers, the medium was supplemented independently with 1% mannose, xylose, lactose, sucrose, fructose, galactose, glucose, and arabinose. While nutrient supplementation such as inorganic nitrogen sources 1% (by mass) (ammonium nitrate, sodium nitrate, ammonium chloride and ammonium sulphate), organic nitrogen sources (peptone, tryptone, yeast extract, beef extract, urea, and casein), and added metal salts 0.1% (by mass) $FeSO_4.7H_2O$, $MgSO_4.7H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$ and $CaCl_2$ were optimised. For each experimental variable all other parameters were kept at their optimal level.

RESULTS AND DISCUSSION

Bacillus species are considered to be the most important sources of a-amylase and have been used for enzyme production using SSF [16]. In SSF, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation [6].

In this study in order to reduce the cost of α-amylase production, agro-industrial residue or cheap material was choosen as culture media. The composition of rice bran is approximately 10% protein, 10% moisture, 20% crude fiber, 15% crude fat and 45% carbohydrates [17]. Maximum α-amylase production was obtained with rice bran as the substrate.

Temperature plays a significant role in development of the biological process as it influences protein denaturation, enzyme inhibition and cell growth [18]. Investigation of the effect of temperature on enzyme production during fermentation showed that the optimum temperature for maximum yield of α-amylase was 37°C for rice bran with 1440 ± 73.5 U/mg (Figure 1). Previously similar results were reported for production of α-amylase [3,12,19]. 37°C is necessary for maximum enzyme titre. It has also been reported that the metabolic heat generated during microbial cultivation in SSF exerts harmful effects on the microbial activity and thus the initial set temperature is vital [20].

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. The *Bacillus circulans* ATCC 4516 produced high production of α-amylase (1705.3 ± 47.3 U/mg) at 48 h (Figure 2). The production of enzyme decreased after 48 h for rice bran. Enzyme production is related to growth of the microorganism. Growth of the organisms would have reached a stage (due to insufficient nutrients) that indirectly stimulates production of secondary metabolites [21].

To obtain the enzyme extraction from fermented biomass is important. The medium used for the extraction of crude enzyme from the fermented matter was found to have a profound effect on the enzyme yield [20]. Results from Figure 3 show that maximum enzyme yield (2006 ± 59.7 U/mg) was observed when tap water was used.

Enzymes are susceptible to mechanical force, which may disturb the elaborate shape of complex molecule to such a degree that denaturation occurs [9]. The optimal agitation speed for maximum α-amylase production (2081.2 ± 45.3 U/mg) was obtained at 150 rpm (Figure 4). Almost similar observations were evident in other studies where increase of α-amylase yield by some *Bacillus* strains was observed [9, 12, 22]. The decline in enzyme production observed before and after 150 rpm of agitation rates might be due to happen that low agitation speed could cause improper mixing of nutrients whereas high agitation speed could mechanically damage microbial cells by exerting high sheer stress which ultimately affected enzyme yield [23].

The inoculum level was also an important factor for the production of α-amylase. The highest enzyme production (2135.4 ± 52.6 U/mg) was obtained at an inoculum level of 25% (v/w). High inoculum levels are inhibitory in nature. A higher inoculum size may increase moisture content and lead to a decrease in growth and enzyme production; this may be due to the limiting nutrients at higher inoculum size and a lower inoculum size may require a longer time for fermentation to form the desired product [20,24,25]. The results from this study indicate that 25% inoculum size was optimal, balancing enzyme and biomass production (Figure 5).

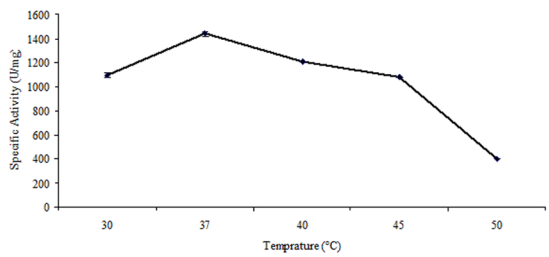

Figure 1. Effect of incubation temperature on the production of α-amylase by *Bacillus circulans* ATCC 4516. Inoculum size 25% (by volume per mass), Initial pH 7.5, Agitation speed 150 rpm, Incubation time 48 h.

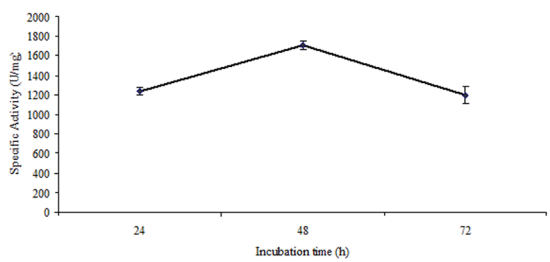

Figure 2. Effect of incubation time on the production of α-amylase by *Bacillus circulans* ATCC 4516. Inoculum size 25% (by volume per mass), Initial pH 7.5, Agitation speed 150 rpm, Incubation temperature 37°C.

Among the physicochemical parameters, the pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme secretion [21]. Figure 6 depicted that pH played a sensitive role in enzyme production and growth of *B.circulans* ATCC 4516. The enzyme production was maximum when initial medium pH was 7.5, which yielded 2191.2 ± 15.9 U/mg enzyme units for rice bran. Further increase in pH resulted in decrease of α-amylase production by the *B.circulans* ATCC 4516. Haq *et al.* (2003) reported pH 7.5–8.0 to be the best for the production of alpha amylase by *Bacillus subtilis* [26]. Variations in pH result from the substrate consumption (*e.g.* protein hydrolysis) and/or metabolite production (*e.g.* organic acids). They are indicators of changes in metabolic activity [27]. Generally, agro industrial wastes possess unique buffering action and have advantages for enzyme production. Therefore, in the subsequent experiments, the initial pH of the fermentation medium was adjusted to 7.5.

The supplementation of rice bran with the different carbon sources; mannose, xylose, lactose, sucrose, fructose, galactose, glucose, and arabinose at 1% (w/v) concentration on the production of α-amylase by *B.circulans* ATCC 4516 was investigated in order to obtain a suitable medium for industrial enzyme production. In our studies, as shown in Table 1 in comparison with the control (2154.8 ± 10.3 U/mg), there was no significant increase in enzyme yield in the case of the supplementation of carbon sources. All carbon sources supported good growth of the microorganism. On the contrary, the production of α-amylase by *B. circulans* ATCC 4516 was suppressed when the bacterium was grown on readily metabolizable sugars, since a low basal activity of α-amylase was detected in the culture medium in the presence of mannose, xylose, lactose, sucrose, fructose, glucose, and arabinose. A similar result obtained that carbon sources such as glucose, maltose did not enhance α-amylase production by *B. coagulans* in solid-state fermentation using wheat bran [28]. Galactose had no impact on enzyme production while the other carbon sources exhibited repressive effect. Easily metabolizable carbohydrates may result in the better growth of the bacteria along with reduction in the enzyme formation [29].

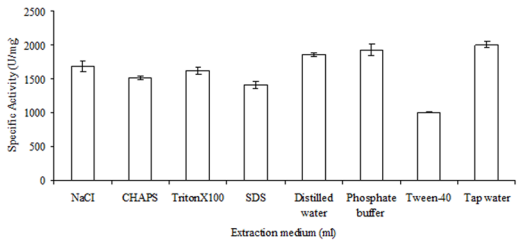

Figure 3. Effect of extraction medium on the production of α-amylase by *Bacillus circulans* ATCC 4516. Inoculum size 25% (by volume per mass), Initial pH 7.5, Agitation speed 150 rpm, Incubation temperature 37°C, Incubation time 48 h.

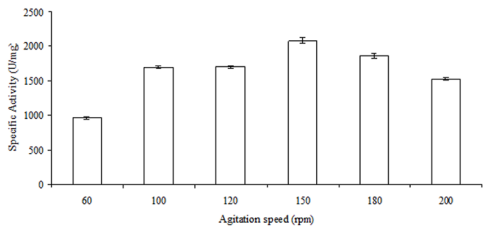

Figure 4. Effect of agitation speed on the production of α-amylase by *Bacillus circulans* ATCC 4516. Inoculum size 25% (by volume per mass), Initial pH 7.5, Incubation temperature 37°C, Incubation time 48 h.

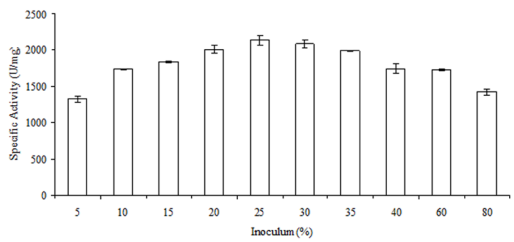

Figure 5. Effect of inoculum size on the production of α-amylase by *Bacillus circulans* ATCC 4516. Initial pH 7.5, Agitation speed 150 rpm, Incubation temperature 37°C, Incubation time 48 h.

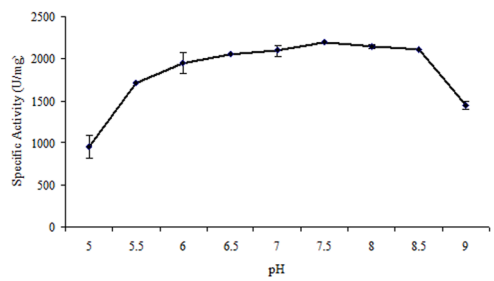

Figure 6. Effect of initial pH on the production of *Bacillus circulans* ATCC 4516 α-amylase. Inoculum size 25% (by volume per mass), Agitation speed 150 rpm, Incubation temperature 37°C, Incubation time 48 h.

Table 1. Effect of carbon sources on the production of α-amylase by Bacillus circulans ATCC 4516. Inoculum size 25% (by volume per mass), Agitation speed 150 rpm, Initial pH 7.5, Incubation temperature 37°C, Incubation

Carbon source (1%)	Specific Activity (U/mg)
Control*	2154.8±10.3
Mannose	1413.8±13.2
Arabinose	1234.9±18.6
Sucrose	1527.4±63.5
Glucose	631.6±17.0
Galactose	2014.8±12.7
Fructose	1174.1±15.2
Lactose	1288.0±36.6
Xylose	1397.0±16.9

*Control contains only rice bran and tap water

Table 2. Effect of nitrogen sources on the production of α-amylase by *Bacillus circulans* ATCC 4516. . Inoculum size 25% (by volume per mass), Agitation speed 150 rpm, Initial pH 7.5, Incubation temperature 37°C, Incubation time 48 h.

Nitrogen source (1%)	Specific Activity (U/mg)
Control*	2154.8±10.3
Sodium nitrate	1522.3±6.0
Ammonium sulphate	2119.9±100.5
Ammonium nitrate	2155.9±57.1
Ammonium chloride	2716.9±35.9
Beef extract	1508.0±11.3
Tryptone	1100.5±58.3
Peptone	1186.0±57.6
Yeast extract	1351.2±27.4
Urea	1459.2±14.2
Casein	1082.3±16.3

*Control contains only rice bran and tap water

Table 3. Effect of metal salt sources on the production of α-amylase by *Bacillus circulans* ATCC 4516. Inoculum size 25% (by volume per mass), Agitation speed 150 rpm, Initial pH 7.5, Incubation temperature 37°C, Incubation time 48 h.

Metal salts source (0.1%)	Specific Activity (U/mg)
Control*	2154.8±10.3
$MgSO_4$	1869.3±131.1
$ZnSO_4$	320.5±28.2
$FeSO_4$	2091.3±15.9
$CuSO_4$	290.8±39.3
$CaCl_2$	2095.8±20.2

*Control contains only rice bran and tap water

Addition of organic nitrogen sources such as casein, peptone, tryptone, yeast extract, beef extract, urea, and casein, and inorganic nitrogen source such as ammonium nitrate, sodium nitrate, ammonium chloride and ammonium sulphate to the medium were investigated. As shown in Table 2, in comparison with the control (2154.8 ± 10.3 U/mg), there was significant increase in enzyme yield in the case of the supplementation of ammonium chloride (2716.9 ± 35.9 U/mg) which proved to be the best among all the nitrogen sources. Pedersen and Nielsen (2000) and Ramanchandra et al. (2004b) also reported that nitrate was inferior to ammonia in α-amylase production [30,31]. Supplementation of additional nitrogen sources in general has been reported to be inhibitory for α-amylase production by microorganisms [26,32]. Ammonium chloride had been found to be suitable with rice bran when used in SSF.

Addition of metal salts source such as $FeSO_4$, $MgSO_4$, $CuSO_4$, $ZnSO_4$ and $CaCl_2$ to the medium were also investigated. Comparison with the control (2154.8 ± 10.3 U/mg), the production of α-amylase by *B.circulans* ATCC 4516 was suppressed when the bacterium was grown on metal salts source (Table 3). Although there are many reports indicating an enhancement of α-amylase production by salts [33,34]. The salt requirement for production of this particular enzyme was apparently provided by the nature of rice bran. These are important in terms of the cost of production of enzyme.

CONCLUSION

Commercial α-amylase production is usually produced by submerged fermentation; however, SSF appear promising due to the natural potential and advantages they offer. Based on the present study, it appears that rice husk, which is inexpensive and readily available agricultural substance, could replace the commercial and more expensive substances in the development of a suitable economic fermentation medium for obtaining high yields of α-amylase. However, the present study was entirely a laboratory-scale study, and it has to be further improved for a large-scale SSF.

REFERENCES

1. S. Alva, J. Anupama, J. Savla, Y.Y. Chiu, P. Vyshali, M. Shruti, B.S. Yogeetha, D. Bhavya, J. Purvi, K. Ruchi, B.S. Kumudini, K.N. Varalakshmi, Production and characterization of fungal amylase enzyme isolated from Aspergillus sp. JGI 12 in solid state culture, Afr. J. Biotech., 6 (2007) 581.
2. R.C. Ray, S. Kar, S. Nayak, M.R. Swain, Extracellular α-amylase production by Bacillus brevis MTCC 7521, Food Biotechnol., 22 (2008) 234.
3. G. Rajagopolan, C. Krishnan, Optimization of medium and process parameters for a constitutive α-amylase production from a catabolite derepressed Bacillus subtilis KCC103, J. Chem. Technol., 83 (2008) 654.
4. S. Ramachandran, A.K. Patel, K.M. Nampoothiri, S. Chamdran, G. Szakacs, C.R. Soccol, A. Pandey, α-Amylase from a fungal culture grown on oil cakes and its properties, Braz. Arch. Biol. Techn., 47 (2004) 309.
5. H. Sun, X. Ge, L. Wang, P. Zhao, M. Peng, Microbial production of raw starch digesting enzymes, Afr. J. Biotechnol., 8 (2009) 1734.
6. A. Kunamneni, K. Permaul, S. Singh, Amylase production in solid state fermentation by the thermophilic fungus Thermomyces lanuginosus, J. Biosci. Bioeng., 100 (2005) 168.
7. R.R. Singhania, A.K. Patel, C.R. Soccol, A. Pandey, Recent advances in solid state fermentation, Biochem. Eng. J., 44 (2009) 13.
8. A. Pandey, Solid-state fermentation, Biochem. Eng. J., 13 (2003) 81.
9. M.Ş. Tanyıldızı, D. Özer, M. Elibol, Production of bacterial α-amylase by B. amyloliquefaciens under solid substrate fermentation, Biochem. Eng. J., 37 (2007) 294.
10. S. Kar, T.K. Data, R.C. Ray, Optimization of thermostable α-amylase production by Streptomyces erumpens MTCC 7317 in solid state fermentation using cassava fibrous residue, Braz. Arch. Biol. Techn., 53 (2010) 301.
11. E.B.N. Graminha, A.Z.L. Gonçalves, R.D.P.B. Pirota, M.A.A. Balsalobre, R. Da Silva, E. Gomes, Enzyme production by solid state fermentation: application to animal nutrition, Anim. Feed Sci. Tech., 144 (2008) 1.
12. H. Anto, U. Trivedi, K. Patel, Alpha amylase production by Bacillus cereus MTCC 1305 using solid state fermentation, Food Technol. Biotechnol., 44 (2006) 241.
13. A. Pandey, P. Selvakumar, C.R. Soccol, P. Nigam, Solid state fermentation for the production of industrial enzymes, Curr. Sci., 77 (1999) 149.
14. P. Bernfield, Amylases, α and β. In: Methods in Enzymology, Academic Press, 1 (1955) 149.
15. O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin-phenol reagents, J. Biol. Chem., 48 (1951) 17.
16. A. Pandey, P. Nigam, C.R. Soccol, V.T. Soccol, D. Singh, R. Mohan, Advances in microbial amylases, Biotechnol. Appl. Biochem., 31 (2000) 135.
17. J.G.N. Amissah, W.O. Ellis, I. Oduro, J.T. Manful, Nutrient composition of bran from new rice varieties under study in Ghana, Food Control, 14 (2003) 21.
18. B. Balkan, F. Ertan, Production and properties of α-amylase from Pencillium chrysogenum and its applications in starch hydrolysis, Prep. Biochem. Biotechnol., 35 (2005) 169.
19. N. Akcan, High level production of extracellular α-amylase from Bacillus licheniformis ATCC 12759 in submerged fermentation, Rom. Biotech. Lett., 16 (2011) 6833.
20. D. Gangadharan, S. Sivaramakrishnan, K.M. Nampoothiri, A. Pandey. Solid culturing of Bacillus amyloliquefaciens for alpha amylase production, Food Technol. Biotechnol., 44 (2006) 269.
21. F. Febe, A. Sabu, K.M. Nampoothiri, G. Szakacs, A. Pandey, Synthesis of α-amylase by Aspergillus oryzae in solid-state fermentation, J. Basic Microbiol., 42 (2002) 320.
22. A. Nusrat, S.R. Rahman, Comparative studies on the production of extracellular α-amylase by three mesophilic Bacillus isolates Bangladesh, J. Microbiol., 24 (2007) 129.
23. M. Nadeem, J.I. Qazii, S. Baig, Effect of aeration and agitation rates on alkaline protease production by Bacillus licheniformis UV-9 mutant, Turk J. Biochem., 34 (2009) 89.
24. Z. Baysal, F. Uyar, Ç. Aytekin, Solid state fermentation for production of α-amylase by a thermotolerant Bacillus subtilis from hot-springwater, Process Biochem., 38 (2003) 1665.
25. P. Kashyap, A. Sabu, A. Pandey, G. Szakas, C.R. Soccol, Extracellular L-glutaminaseproduction by Zygosaccharomyces rouxii under solid state fermentation, Process Biochem., 38 (2002) 307.
26. I. Haq, H. Ashraf, J. Iqbal, M.A. Qadeer, Production of alpha amylase by Bacillus licheniformis using an economical medium, Bioresource Technol., 87 (2003) 57.
27. B.V. Maurel, O. Orliac, P. Christen, Sensors and measurements in solid-state fermentation: A review, Process Biochem., 38 (2003) 881.
28. K.R. Babu, T. Satyanarayana, Amylase production by thermophilic Bacillus coagulans in solid state fermentation, Process Biochem 30 (1995) 305.
29. R. Rama, S.K. Srivastav, Effect of various carbon substrates on α-amylase production from Bacillus species, J. Microb. Biotechnol., 10 (1995)76.

30. H. Pedersen, J. Nielsen, The influence of nitrogen sources on the alpha amylase productivity of Aspergillus oryzae in continuous cultures, Appl. Microbiol. Biotechnol., 53 (2000) 278.
31. Ramachandran, A.K. Patel, K.M. Nampoothiri, F. Francis, V. Nagy, G. Szakacs, A. Pandey, Coconut oil cake a potential raw material for the production of α-amylase, Bioresource Technol., 93 (2004) 169.
32. F. Ertan, B. Balkan, S. Balkan, T. Aktaç, Solid state fermentation for the production of α-amylase from Penicillium chrysogenum using mixed agricultural by-products as substrate, Biologia, 61 (2006) 657.
33. M.V. Ramesh, B.K. Lonsane, Solid state fermentation for production of α-amylase by Bacillus megaterium 16 M, Biotechnol. Lett., 11 (1989) 49.
34. W.X. Wu, J. Mabinadji, T.F. Betrand, Effect of growth culture conditions on production of an extracelluler thermostable alpha-amylase from an isolate Bacillus sp, Agric. Life Sci., 45 (1999) 404.