ISSN 1330-9862
(FTB-2247)

original scientific paper

Screening of Various Organic Substrates and the Development of a Suitable Low-Cost Fermentation Medium for the Production of α-Amylase by *Bacillus subtilis*

Sadin Özdemir[1], *Kemal Güven*[1]*, *Zübeyde Baysal*[2] *and Fikret Uyar*[1]

[1]Molecular Biology and Genetics Section, Science Faculty, Dicle University, TR-21280 Diyarbakır, Turkey

[2]Chemistry Department, Science Faculty, Dicle University, TR-21280 Diyarbakır, Turkey

Received: February 25, 2009
Accepted: June 17, 2009

Summary

The production of extracellular amylase by *Bacillus subtilis* has been studied in solid-state fermentation (SSF). In a sequential order, various process parameters were optimized for maximum amylase production. The tested process parameters were different solid substrates such as banana husk (BH), water melon husk (WMH), lentil bran (LB), wheat bran (WB), melon husk (MH) and maize oil cake (MOC), different incubation time (24–144 h), particle size (500–2500 μm), initial moisture content of the substrate (40–70 %, by mass per volume), inoculum size (10–60 %, by mass per volume) and inoculum concentration (10–60 %, by volume per mass). The maximum production of amylase (4857 U/mg) was achieved when 1 % starch (by mass, particle size of 1500 μm) was added to banana husk as the solid substrate, fermented for 72 h at 37 °C, at an inoculum level of 30 % (by volume per mass), and initial moisture content of 60 % (by volume per mass).

Key words: Bacillus subtilis, α-amylase, banana husk, solid-state fermentation

Introduction

α-Amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) is a widely distributed secretory enzyme (*1*). Amylases are enzymes which hydrolyse starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (*2*). Amylases also play a significant role in starch, detergent, beverage and textile industries and their commercial production from microorganisms represents 25–33 % of the world enzyme market (*3*).

Industrially important enzymes have traditionally been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH. However, industrial production of enzymes can be made economical by utilizing low-cost substrates such as agricultural byproducts in the production medium (*4*). Solid-state fermentation (SSF) constitutes an interesting alternative since the metabolites so produced are concentrated and purification procedures are less costly (*5*). SSF is generally characterized by the growth of microorganism on and/or within particles of a solid substrate in the presence of varying amounts of water. The solid substrate acts as a source of carbon, nitrogen, minerals and growth factors, and has a capacity to absorb water, necessary for microbial growth. As the microorganisms in SSF grow under conditions similar to their natural habitats (*6*), they may be able to produce certain enzymes and metabolites more efficiently than in submerged fermentation (*7*).

The major factors that affect microbial synthesis of enzymes in a SSF system include a selection of a suitable substrate and microorganism, particle size of the sub-

*Corresponding author; Phone: ++90 412 2488 550/3064; Fax: ++90 412 2488 300; E-mail: kemalg@dicle.edu.tr

strate, inoculum concentration and moisture level of the substrate. In SSF, the selection of a suitable solid substrate for the fermentation process is a critical factor and thus involves the screening of a number of agroindustrial materials for microbial growth and product formation (*8*). Agroindustrial residues are generally considered as the best substrates for the SSF processes and enzyme production in SSF (*9*). Solid-state fermentation (SSF) has been used for decades to convert moist agricultural polymeric substrates like wheat, rice, soy, cassava, *etc*. into fermented food products (*10*). Application of these agroindustrial residues in bioprocesses also solves pollution problems, which their disposal may otherwise cause (*5*). The use of agricultural wastes makes SSF an attractive alternative method (*11*). SSF is preferred to SmF because of simple technique, low capital investment, lower levels of catabolite repression and end product inhibition, low waste water output, better product recovery, high quality production and high specificity (*5,12–14*).

SSF technique is generally confined to the processes involving fungi. However, successful bacterial growth in SSF is known in many natural fermentations. *Bacillus* species are considered to be the most important sources of α-amylase and have been used for enzyme production in SSF (*4,11*). *Bacillus subtilis*, *B. polymyxa*, *B. mesentericus*, *B. vulgaris*, *B. coagulans*, *B. megaterium* and *B. licheniformis* have been used for α-amylase production in SSF (*15*).

Banana is one of the most consumed fruits in the world and it is grown extensively in tropical and subtropical countries. Its fruit stalk contains 56.8 % total sugar, 27 % starch, 4.65 % reducing sugar and 4.3 % protein on a dry mass basis. Most of the residual waste produced due to banana cultivation is discarded by farmers into nearby rivers, lakes and on roads, which causes a serious environmental concern (*16,17*).

The aim of the present study is to evaluate various organic substrates and to develop a suitable low-cost fermentation medium for the production of α-amylase by *B. subtilis*.

Materials and Methods

Selection and isolation of bacterial strain producing α-amylase

Bacillus subtilis, which was isolated from campus area of Dicle University, Diyarbakır, Turkey was used in the present study. Soil suspension in sterilised water was poured and spread onto nutrient agar plates, which were incubated at 37 °C for 24 h. The colonies that were found on the plates were transferred onto another nutrient agar consisting of 1 % soluble starch. These plates were also incubated at 37 °C for 48 h. Several amylase-producing bacterial colonies were selected after flooding the plates with iodine solution. The strain that yielded a high level of α-amylase was selected for further experiments.

Preparation of inoculum

A volume of 20 mL of nutrient broth taken in a 100-mL Erlenmeyer flask was inoculated with a loop full of cells from a 24-hour-old slant and kept at 37 °C in a rotary shaker (120 rpm). After 12 h of incubation, 1 mL of this culture was used as the inoculum. By a serial dilution and plating, the number of viable colonies in the inoculum was found to be $2 \cdot 10^8$ CFU/mL.

Solid-state fermentation

Banana husk (BH), water melon husk (WMH), lentil bran (LB), wheat bran (WB), melon husk (MH) and maize oil cake (MOC) were obtained from a local market in Diyarbakır, Turkey. These agroindustrials were used as solid substrates and their effect on the production of α-amylase was determined. The best solid substrate for α-amylase production was selected and used in subsequent experiments.

Dry substrates (3 g) which passed through the sieve of 1500 μm were placed into 100-mL Erlenmeyer flasks. To adjust moisture levels, 0.1 M Tris HCI buffer (pH=7.0) was added. After autoclaving at 121 °C for 15 min, and cooling to room temperature, the flasks were inoculated with 2 mL of spore suspension ($4 \cdot 10^8$ CFU/mL) and incubated at 37 °C and 120 rpm.

Enzyme extraction

Fermented substrates were mixed thoroughly with 0.1 M Tris-HCI buffer (pH=7.0). Their contents were mixed in a shaker at 120 rpm. The slurry was squeezed through a muslin cloth. The extract was centrifuged at 10 000 rpm for 10 min and then the clear supernatant was used as the crude enzyme.

Optimization of process parameters

SSF was carried out to study the effect of various physicochemical parameters required for the optimum production of α-amylase by *Bacillus subtilis*. The strategy was to optimize each parameter independently of the others and subsequently optimal conditions were employed in all experiments. The optimized parameters were incubation time (24, 48, 72, 96, 120 and 144 h), particle size (500, 1000, 1500, 2000 and 2500 μm), initial moisture content of the substrate (40, 50, 60, 65 and 70 %, by mass per volume), and inoculum size (10, 20, 30, 40, 50 and 60 %, by mass per volume). Studies were also performed to evaluate the influence of different carbon sources (starch, sucrose, mannose, arabinose, galactose and glucose at 1 % by mass) and nitrogen sources (ammonium sulphate, Bacto casamino acid, Bacto liver, methionine, ammonium nitrate, ammonium chloride and urea at 1 % by mass) when added to the fermentation medium.

Enzyme assay

α-Amylase activity was determined according to Bernfeld (*18*). A volume of 0.1 mL of enzyme solution and 0.2 mL of 0.5 % starch solution in 0.1 M Tris-HCl buffer (pH=6.0) were incubated for 30 min at 37 °C. After that, 0.4 mL of 3,5-dinitrosalicylic acid were added and the mixture was kept in boiling water bath for 5 min in order to stop the reaction. Then, it was diluted with 10 mL of distilled water, after which spectrophotometric measurement was conducted at 489 nm. One unit

of enzyme activity was defined as the amount of protein that produced 1 mg of reducing sugar per mL of enzyme solution in 30 min. Results are represented as mean±S.D. of at least three experiments.

Results and Discussion

A comparison of 16S rDNA sequence of this strain with other related bacteria showed that it had high similarity with *Bacillus subtilis.*

Effect of different substrates on α-amylase production

In SSF, the selection of a suitable solid substrate for the fermentation process is a critical factor (*10,12*), and thus involves the screening of a number of agroindustrial materials for microbial growth and product formation. Different SSF sources are tested here, such as banana husk (BH), water melon husk (WMH), melon husk (MH), wheat bran (WB), lentil bran (LB) and maize oil cake (MOC) for the production of α-amylase. As shown in Fig. 1, maximum amylase production (1010 U/mg) was obtained in a medium containing BH alone as the substrate. The order of substrates for enzyme production was BH>WMH>LB>WB>MH>MOC. The highest activity was observed with the banana husk, which was used as a substrate in later studies.

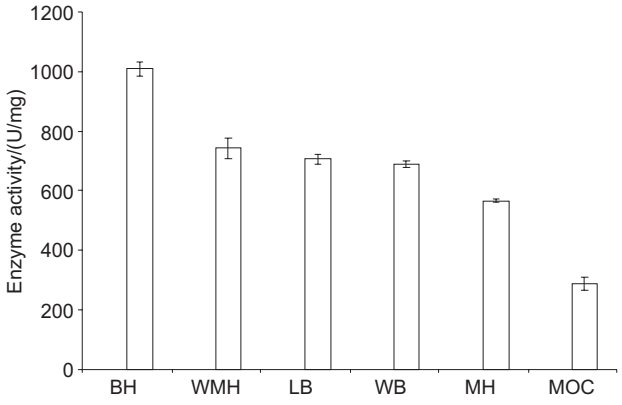

Fig. 1. Effect of different substrates on α-amylase production by *Bacillus subtilis* in solid-state fermentation. Process conditions: incubation time 48 h, inoculum size 20 % (by volume per mass), initial moisture content 50 % (by volume per mass), particle size 1500 μm, temperature 37 °C. Bars represent standard deviation

Effect of incubation time on α-amylase production

Incubation time depends on the characteristics of the culture, on growth rate and enzyme production (*19*). Fig. 2 shows the time course experiments of α-amylase production by *B. subtilis* using BH as a substrate. A gradual increase was seen in enzyme production from 24 to 72 h (2902 U/mg), after which a gradual decrease was observed. The decrease in enzyme yield after 72 h of incubation may be the result of denaturation or decomposition of α-amylase due to interaction with other components in the medium, as it is reported elsewhere (*20*). Moreover, the reaction for maximum enzyme production at 72 h could be due to the fact that the microorganism was in its exponential phase. At the later stage, when nutrients are depleted, it reaches its stationary phase and can start to produce secondary metabolites, thus resulting in a lower yield of enzyme (*21*). In subsequent experiments, therefore, 72 h was used as incubation time for the production of amylase.

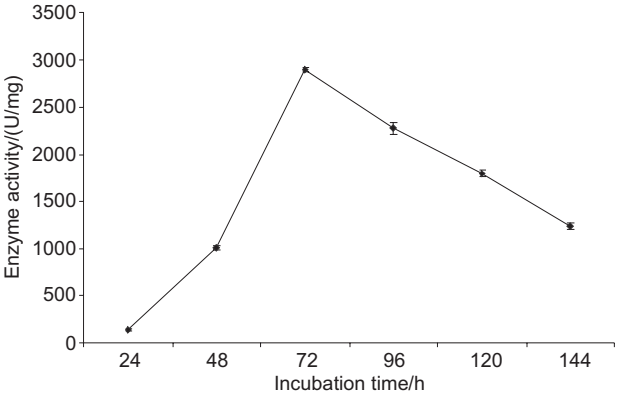

Fig. 2. Effect of incubation time on α-amylase production by *Bacillus subtilis* in solid-state fermentation using BH as substrate. Process conditions: inoculum size 20 % (by volume per mass), initial moisture content 50 % (by volume per mass), particle size 1500 μm, temperature 37 °C

Effect of inoculum size on α-amylase production

Smaller inoculum size requires longer time for the cells to multiply to a sufficient number to utilize the substrate and produce the desired product. An increase in the number of spores in the inoculum would ensure a rapid proliferation and biomass synthesis. A balance between the proliferating biomass and the available nutrient would yield an optimum at which the enzyme synthesis would be maximum (*21*). This was evident as the strain showed increased enzyme production with the increase in the inoculum size. The lowest enzyme production was obtained at the lowest value of 10 % inoculum, whereas the maximum enzyme activity (3285 U/mg) was obtained at 30 % inoculum (Fig. 3). With the increase of the inoculum level, the production of enzyme decreased

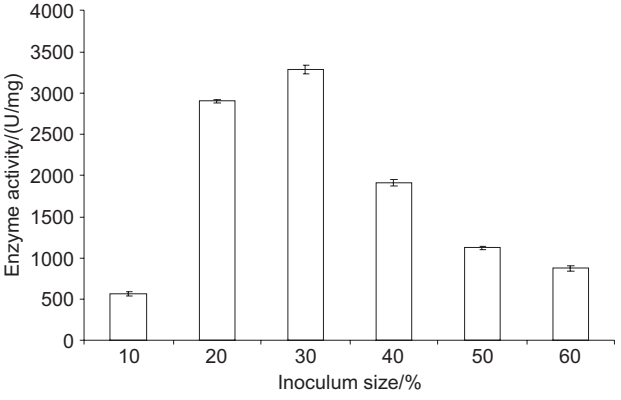

Fig. 3. Effect of inoculum size (by volume per mass) on α-amylase by *Bacillus subtilis* in solid-state fermentation using BH as substrate. Process conditions: incubation time 72 h, initial moisture content 50 % (by volume per mass), particle size 1500 μm, temperature 37 °C. Bars represent standard deviation

due to exhaustion of nutrients in the fermentation mash. In addition, the free excess liquid present in an unabsorbed form will give rise to an additional diffusional barrier together with that imposed by the solid nature of the substrate and lead to a decrease in enzyme production and growth (*22*).

Effect of initial moisture content of substrate on α-amylase production

Fig. 4 shows the effect of moisture content on the enzyme production. Optimal enzyme production was obtained at 60 % moisture content of the substrate (3427 U/mg). It has been reported that moisture levels in SSF processes vary between 30 and 85 % (*5*). Our results are in good agreement with these findings. Moisture content is a crucial factor in SSF that influences the growth of the microorganism and thereby enzyme production (*23*). Generally, bacteria require higher water activity for their growth. The necessary moisture in SSF exists in the absorbed or complex form within the solid matrix, which is likely to be more advantageous for growth because of the possible efficient oxygen transfer process (*5*). Lower moisture content causes reduction in solubility of the nutrients of the substrate, low degree of swelling and high water tension (*8*). On the other hand, higher moisture level decreases porosity, changes particle structure, promotes development of stickiness, reduces gas volume and decreases diffusion, which results in lowered oxygen transfer (*23*). In subsequent experiments, therefore, 60 % moisture content of substrate was used for the optimal amylase production.

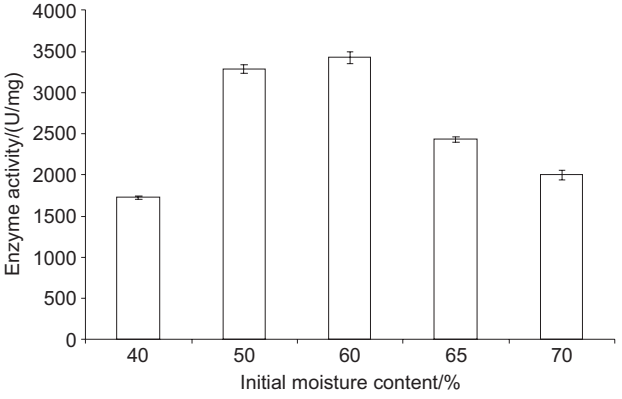

Fig. 4. Effect of initial moisture content (by volume per mass) on α-amylase production by *Bacillus subtilis* in solid-state fermentation using BH as substrate. Process conditions: incubation time 72 h, inoculum size 30 % (by volume per mass), particle size 1500 μm, temperature 37 °C. Bars represent standard deviation

Effect of substrate particle size on α-amylase production

The particle size is also very important for SSF. Fig. 5 shows the effect of substrate particle size on α-amylase production in SSF. Maximum enzyme production was found with 1500 μm particle size. Larger particles provide better respiration/aeration efficiency (due to increased interparticle space). In contrast, a small substrate particle may result in substrate accumulation, which may interfere with microbial respiration/aeration and therefore result in poor growth and enzyme production (*6,8*). In addition, with smaller particles, the surface area for growth is larger, but the interparticle porosity is lower. With larger sizes, the porosity is greater, but the saturated surface area is smaller. These factors lead to a decrease in the surface area, an increase in porosity and probably determine the values corresponding to optimum growth and enzyme production (*17*). Baysal *et al.* (*19*) reported that wheat bran and rice husk with particle size of 1000 and 500 μm, respectively, favoured α-amylase production. On the other hand, Balkan and Ertan (*8*) reported that corncob leaf, rye straw, wheat straw and wheat bran particles of ≥1 mm favoured α-amylase production. Thus, the particle size of substrate of 1500 μm was used for further studies.

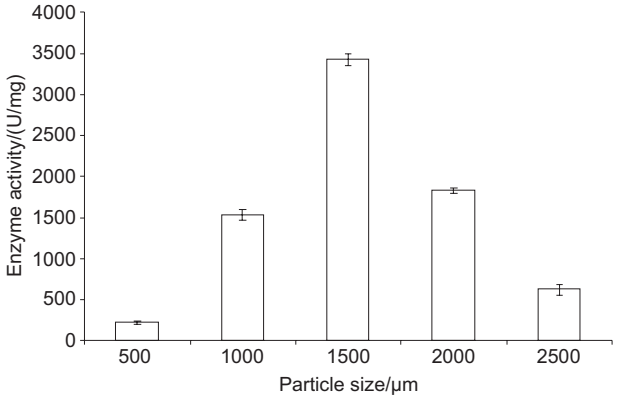

Fig. 5. Effect of substrate particle size on α-amylase production by *Bacillus subtilis* in solid-state fermentation using BH as substrate. Process conditions: incubation time 72 h, inoculum size 30 % (by volume per mass), initial moisture content 60 % (by volume per mass), temperature 37 °C. Bars represent standard deviation

Effect of carbon source supplementation on α-amylase production

The influence of supplementary carbon sources such as starch, sucrose, mannose, arabinose, galactose or glucose at 1 % (by mass) was studied. Of the carbon sources tested, starch increased α-amylase production the most (4857 U/mg), followed by sucrose (3804 U/mg) (Fig. 6), which was similar to the findings of Kunamneni *et al.* (*9*). Furthermore, Agger *et al.* (*24*) reported that starch was the best inducer for α-amylase production in TA1 strain of *Aspergillus nidulans*. Starch was also known to increase enzyme production in *Bacillus* sp. PS-7, *Bacillus subtilis* CBTK 106, *B. subtilis* IMG22 and *Bacillus* sp. I-3 (*17,25,26*). However, as shown in Fig. 6, a negative effect on α-amylase production is apparent by glucose. Mulimani and Patil (*27*) reported that supplemantion of 1 % glucose caused a negative effect on amylase production. It is well known that the synthesis of carbohydrate-degrading enzymes in most species of genus *Bacillus* is subject to catabolic repression by glucose (*28*). Other three compounds, *i.e.* mannose, arabinose and galactose,

did not have an effect on the enzyme synthesis. Similar results have been reported by Rajagopalan and Krishnan (*29*).

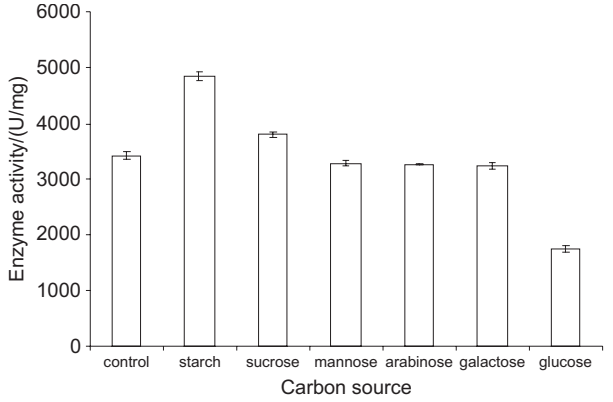

Fig. 6. Effect of carbon source (1 %) supplementation on α-amylase production by *Bacillus subtilis* in solid-state fermentation using BH as substrate. Process conditions: incubation time 72 h, inoculum size 30 % (by volume per mass), initial moisture content 60 % (by volume per mass), particle size 1500 μm, temperature 37 °C. Bars represent standard deviation

Effect of nitrogen source supplementation on α-amylase production

Studies on supplementation of nitrogen sources such as Bacto casamino acid, Bacto liver, methionine, ammonium sulphate, ammonium nitrate, ammonium chloride or urea at 1 % concentration to the BH showed various effects on enzyme production by *B. subtilis*. Among the nitrogen sources, ammonium sulphate considerably increased amylase production (4424 U/mg), followed by Bacto casamino acid (3917 U/mg) and Bacto liver (3724 U/mg) (Fig. 7). In the previous studies, ammonium sulphate had been found to increase enzyme production in different microorgaminsms (*17*,*24*,*26*). Methionine was found to have no effect on amylase production compared to the control. However, ammonium nitrate, ammonium chloride and urea had a negative effect on amylase production, as clearly shown in Fig. 7. Ramachandran *et al.* (*21*) have already reported that supplementation of urea at 1 % concentration resulted in a decrease in amylase production.

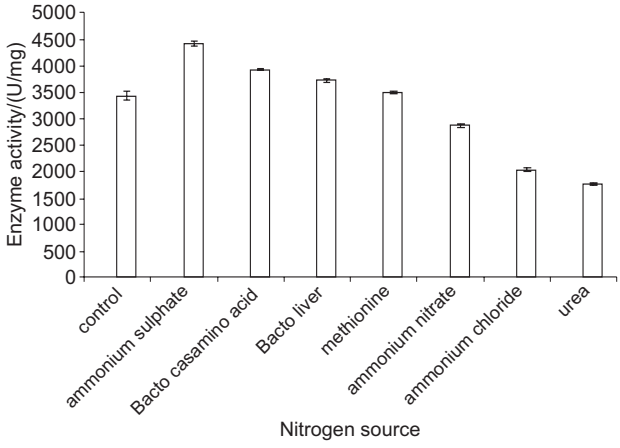

Fig. 7. Effect of nitrogen source (1 %) supplementation on α-amylase production by *Bacillus subtilis* under solid-state fermentation using BH as substrate. Process conditions: incubation time 72 h, inoculum size 30 % (by volume per mass), initial moisture content 60 % (by volume per mass), particle size 1500 μm, temperature 37 °C. Bars represent standard deviation

Conclusions

In the present study, the use of different solid agro-industrial materials as substrates and the effect of various fermentation parameters such as incubation time, inoculum size, initial moisture content, particle size, carbon sources and nitrogen sources for the production of α-amylase by *Bacillus subtilis* under solid-state fermentation were tested. These studies showed that among various substrates used, banana husk (BH) was found to be the best substrate for the production of α-amylase in *Bacillus subtilis*. Evidently, BH provided necessary nutrients for the microorganism to grow and synthesize the enzyme. In addition, supplementation with 1 % starch and 1 % ammonium sulphate to BH positively enhanced the enzyme synthesis.

References

1. R. Gupta, P. Gigras, H. Mohapatra, V.K. Goswami, B. Chauhan, Microbial α-amylases: A biotechnological perspective, *Process Biochem. 38* (2003) 1599–1616.
2. M. Asgher, M.J. Asad, S.U. Rahman, R.L. Legge, A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing, *J. Food Eng. 79* (2007) 950–955.
3. G. Rajagopalan, C. Krishnan, α-Amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate, *Bioresour. Technol. 99* (2008) 3044–3050.
4. A. Pandey, C.R. Soccol, P. Nigam, V.T. Soccol, Biotechnological potential of agro-industrial residues. I: Sugarcane bagasse, *Bioresour. Technol. 74* (2000) 69–80.
5. D. Gangadharan, S. Sivaramakrishnan, K.M. Nampoothiri, A. Pandey, Solid culturing of *Bacillus amyloliquefaciens* for alpha amylase production, *Food Technol. Biotechnol. 44* (2006) 269–274.
6. A. Pandey, P. Selvakumar, C.R. Soccol, P. Nigam, Solid state fermentation for the production of industrial enzymes, *Curr. Sci. 77* (1999) 149–162.
7. A. Pandey, Solid-state fermentation, *Biochem. Eng. J. 13* (2003) 81–84.
8. B. Balkan, F. Ertan, Production of α-amylase from *Penicillium chrysogenum* under solid-state fermentation by using some agricultural by-products, *Food Technol. Biotechnol. 45* (2007) 439–442.
9. A. Kunamneni, K. Permaul, S. Singh, Amylase production in solid state fermentation by the thermophilic fungus *Thermomyces lanuginosus*, *J. Biosci. Bioeng. 100* (2005) 168–171.
10. Y.S.P. Rahardjo, S. Sie, F.J. Weber, J. Tramper, A. Rinzema, Effect of low oxygen concentrations on growth and α-amylase production of *Aspergillus oryzae* in model solid-state fermentation systems, *Biomolecular Eng. 21* (2005) 163–172.
11. H. Anto, U. Trivedi, K. Patel, Alpha amylase production by *Bacillus cereus* MTCC 1305 using solid-state fermentation, *Food Technol. Biotechnol. 44* (2006) 241–245.

12. M.S. Tanyildizi, D. Özer, M. Elibol, Production of bacterial α-amylase by *B. amyloliquefaciens* under solid substrate fermentation, *Biochem. Eng. J. 37* (2007) 294–297.

13. H. Xu, L. Sun, D. Zhao, B. Zhang, Y. Shi, Y. Wu, Production of α-amylase by *Aspergillus oryzae* As 3951 in solid state fermentation using spent brewing grains as substrate, *J. Sci. Food Agric. 88* (2008) 529–535.

14. F. Ertan, H. Yagar, B. Balkan, Optimization of α-amylase immobilization in calcium alginate beads, *Prep. Biochem. Biotechnol. 37* (2007) 195–204.

15. K.R. Babu, T. Satyanarayana, α-Amylase production by thermophilic *Bacillus coagulans* in solid state fermentation, *Process Biochem. 30* (1995) 305–309.

16. M.P. Shah, G.V. Reddy, R. Banerjee, P.R. Babu, I.L. Kothari, Microbial degradaton of banana waste under solid state bioprocessing using two lignocellulolytic fungi (*Phylosticta* spp. MPS-001 and *Aspergillus* spp. MPS-002), *Process Biochem. 40* (2005) 445–451.

17. C. Krisha, M. Chandrasekaran, Banana waste as substrate for α-amylase production by *Bacillus subtilis* (CBTK 106) under solid-state fermentation, *Appl. Microbiol. Biotechnol. 46* (1996) 106–111.

18. P. Bernfeld: Amylases, α/β. In: *Methods in Enzymology, Vol. 1*, S.P. Colowick, N.O. Kaplan (Eds.), Academic Press, New York, USA (1955) pp. 149–154.

19. Z. Baysal, F. Uyar, C. Aytekin, Solid state fermentation for production of α-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water, *Process Biochem. 38* (2003) 1665–1668.

20. M.V. Ramesh, B.K. Lonsane, A novel bacterial thermostable alpha-amylase system produced under solid state fermentation, *Biotechnol. Lett. 9* (1987) 501–504.

21. S. 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, *Bioresour. Technol. 93* (2004) 169–174.

22. N.D. Mahadik, U.S. Puntambekar, K.B. Bastawde, J.M. Uhire, D.V. Gokhale, Production of acidic lipase by *Aspergillus niger* in solid state fermentation, *Process Biochem. 38* (2002) 715–721.

23. S. Sivaramakrishnan, D. Gangadharan, K.M. Nampoothiri, C.R. Soccol, A. Pandey, α-Amylases from microbial sources – An overview on recent developments, *Food Technol. Biotechnol. 44* (2006) 173–184.

24. T. Agger, J.B. Petersen, S.M. O'Connor, R.L. Murphy, J.M. Kelly, J. Nielsen, Physiology characterisation of recombinant *Aspergillus nidulans* strains with creA genotypes expressing *A. oryzae* α-amylase, *J. Biotechnol. 92* (2002) 279–285.

25. H.K. Sodhi, K. Sharma, J.K. Gupta, S.K. Soni, Production of a thermostable α-amylase from *Bacillus* sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production, *Process Biochem. 40* (2005) 525–534.

26. N. Goyal, J.K. Gupta, S.K. Soni, A novel raw starch digesting thermostable α-amylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch, *Enzyme Microb. Technol. 37* (2005) 723–734.

27. V.H. Mulimani, G.N. Patil, α-Amylase production by solid state fermantation: A new practical approach to biotechnology courses, *Biochem. Educ. 28* (2000) 161–163.

28. L.L. Lin, C.C. Chyau, W.H. Hsu, Production and properties of a raw-strach-degrading amylase from thermophilic and alkaliphilic *Bacillus* sp. TS-23, *Biotechnol. Appl. Biochem. 28* (1998) 61–68.

29. G. Rajagopalan, C. Krishnan, Optimization of medium and process parameters for a constitutive α-amylase production from a catabolite derepressed *Bacillus subtilis* KCC103, *J. Chem. Technol. Biotechnol. 83* (2008) 654–661.