Middle-East Journal of Scientific Research 7 (3): 407-412, 2011
ISSN 1990-9233

Isolated of Halotolerant Penicillium Strains from the Howz Soltan Lake to Produce α-amylase

[1]*Akhavan Sepahy Abbas,* [1]*Navabzadeh Ali,*
[2]*Larypoor Mohaddeseh and* [3]*Navabzadeh Bahman*

[1]Department of Microbiology, Islamic Azad University of North Branch, Tehran, Iran
[2]Department of Mycology, Faculty of Veterinary,
Science and Research Branch, Islamic Azad University, Tehran, Iran
[3]Shahid Beheshti University of Medical Sciences, School of Dentisty, Tehran, Iran

Abstract: It has been found that some filamentous fungi can survive and grow in high concentration of salt. We try to isolate and identify halotelorant Penicillium that produce α-amylase. After that a total of 100 water samples were collected from howz Soltan soil, their were tested for the present of fungi by using of the purplate technique. Of a total of 100 soil samples, 65samples were isolated as 9 Species of penicillium. The best species was selected to produce α-amylase. Early, Penicillium was optimized in a submerged cultivation. Maximum enzyme production in preliminary experiments was obtained after two days of incubation in a medium containing 10% (w/v) NaCl, in addition to $MgSO_4.7H_2O$ 1% and 1% (w/v) of starch. The optimum pH for growth and enzyme production was 5, activity determined about 37(u/ml). After that it was found to produce maximum amount of α-amylase from Filamentous fungi, Solid-state fermentation (SSF)was prepared. SSF was carried out using wheat bran (WB) as substrates for α-amylase production by a fungal culture of *Penicillium chrysogenum.* Result showed that used SSF medium could increase the α-amylase activity to ten fold, in comparison with subaro broth as submerged fermentation (SMF).The effects of moisture level, inoculums concentration, salt concetration, temperature range (25-45), pH range(4-9), carbon and nitrogen source on enzyme synthesis from *Penicillium chrysogenum* were investigated and media for optimal production of α-amylase by *Penicillium chrysogenum* has been showed in this study.

Key words: *Penicillium chrysogenum* · α-amylase · Howz Soltan lake

INTRODUCTION

α-amylases (EC 3.2.1.1, 1,4-a-D-glucan glucanohydrolyase) hydrolyse starch to a range of products such as glucose and maltose or to specific malto-oligosaccharide or mixed malto-oligosaccharides). α-amylases is secreted as a primary metabolite and its secretion is growth associated [1]. They are employed in industries for different purposes; α-amylases find application in baking, brewing, detergent, textile, paper and distilling industry [2]. A search for highly active amylolytic enzymes with novel properties is necessary to improve biotechnological processes. Thermostable [3], hydrolyzed native starch [4], stable-to-high salt concentrations [5] and also alkaline amylases attract particular attention, because most of the known and widely used industrial fungal amylases are active in the acidic medium.

Glucose and maltose-forming α-amylases in alcohol fermentation and sugar syrup formulation and maltooligosaccharide forming α-amylases in food processing [6].

Although α-amylase can be derived from several sources, including plants, animals and microorganisms, once produced by microorganisms generally meet industrial demands.

Currently, a large number of microbial amylases are available commercially, having almost completely replaced the chemical hydrolysis of starch in the starch-processing industry fungal α -amylase are preferred over other

Corresponding Author: Mohaddeseh Larypoor, Department of Mycology, Faculty of Veterinary, Science and Research Branch, Islamic Azad University, Tehran, Iran, E-mail:mlarypoor@yahoo.com.

microbial sources due to their more accepted GRAS status[7]. Traditionally, α-amylase is produced by SMF. In recent years, however, SSF processes have been utilized more and more for the production of this enzyme [8]. SSF has many advantages over SMF, such as simple technique, superior productivity, 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]. Bacteria, yeasts and fungi can grow on solid substrates and find applications in SSF processes. Filamentous fungi are the best adapted for SSF. The hyphal mode of fungal growth and their good tolerance to low water activity and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* species and some species of Penicillium [2,10,11].

The cost of enzyme production in submerged fermentation is high; this leads to search of alternative methods. The use of agroindustrial residues make solid state fermentation more economic [12]. Baysal *et al.* 2003 reported amylase production using wheat bran as substrate. The effects of the starch, protein and soluble oligosaccharides contents in wheat bran on the production of extracellular amylase were reported in Penicillium decumbens by Sun *et al.* 2007. Strains of *Penicillium* sp. were isolated from the coastal soil of the howz Soltan lake habitat. One isolate produced extracellular α-amylase which was confirmed by observe Environs halo and by identification of the products activity obtained by starch hydrolysis. Since this natural isolate produced low concentration of amylase, attempts were made to increase the productivity by optimizing the cultural conditions. It was believed that microbial communities at high salinities are dominated exclusively by archaea and bacteria and one eukaryotic species, the alga Dunaliella salina. studies on the microbial diversity in hypersaline environments revealed the presence of melanized fungi, 'considered as a new group of eukaryotic halophiles' and several other fungi including *Penicillium* spp. [13-15].

MATERIALS AND METHODS

Microorganism and Culture Conditions: Penicillium sp. were isolated from the soil (Faculty of Science, Azad University) on glucose-peptone medium containing 100 g/l of NaCl, . For comparison amylase production, starch agar medium contain 10% concentration of sea salt used to compared halotolerante penicellium and produced α-amylase. Studied growing different species of penicillium by metered wide diameter clear zone around coloni and wide colony diameter after 5 day of growing period. The amylolytic activities of 3 isolates growing on agar medium were assessed by the formation of a clear zone around the colonies. The isolate resulting in clear zone the widest diameter was picked up, subcultured, purified and identified with molecular technique as *P. chrysogenum* and then used for the following experiments.

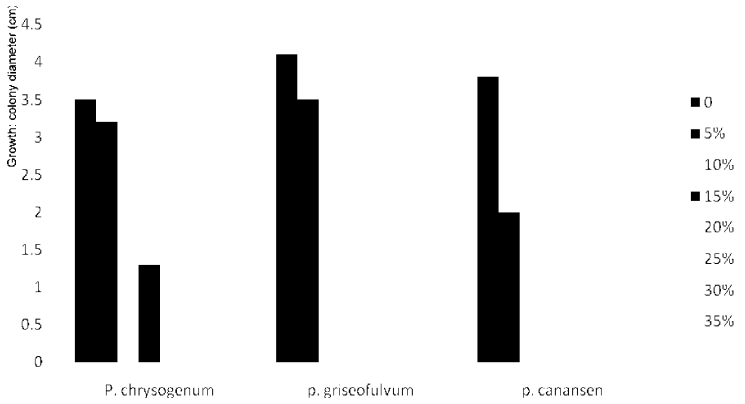
Isolation and Screening of Alpha Amylase-producing Isolates: In the recent years, there has been a growing interest in isolation of microorganisms from various sources for production of industrially valuable microbial product. The present study showed that three fungal isolates could be produced amylase in hypersalin environmet effectively. The obtained suspension was vortexed and 1.0 ml of this suspension was then plated on peptone starch agar medium (PSAM) containing 10 g/l soluble starch, 5 g/l peptone, 15 g/l agar and 0.05 g/l chloramphenicol with pH= 5.5. The agar medium was incubated at 30 0C. After 5 day of incubation, iodine solution (1% iodide and 2% potassium iodide) was flooded on the agar plate. The identification processes of the isolates were performed using mature cultures on standard potato dextrose agar (PDA) in order to ensure a good development of taxonomically relevant features, for following the identification keys [16,17].

The screening of isolated microorganisms is very critically important to obtain microorganisms with high ability, or to improve the productivity of some known microbial products such as enzyme. Although all of the three isolates showed the amylolytic activity, the isolate 13 caused clear zone with the widest diameter (78 mm) on agar medium. This isolate was identified as *P. chrysogenum.* Following this isolate, the isolate 19, which was identified as *P. griseofulvum*, showed high amylolytic activity. Considering these results, the following experiments for α-amylase production were performed with *P. chrysogenum* 13 . The results were shown in Table 1.

Table 1: Observation ofenzymatic ability of isolated fungi

Isolate code	Fungus species	Colony diameter (mm)	Clear zone diameter (mm)
13	*P. chrysogenum*	70	78
19	*p. griseofulvum*	58	66
65	*p. canansen*	55	62

The Effect of salt concenteration salinity on activity of enzymatic of isolated fungi was shown in visible 1



Visible 1: Effect of salt concenteration salinity (0, 5, 10, 15, 20, 25,30,35 % sea salt).

Identification of Fungi: Primers were designed for sequencing of isolated fungi afther DNA extraction and 28SrRNA was performed through pcr technique.So isolated fungi Identificated.

Fungus: *Penicillium chrysogenum* used in this study was isolated from soil samples collected from howz Soltan lake and identified by molecular technique it had 100% homology with *Penicillium chrysogenum* JCM 22826. Screening was carried on at first by plating method using Sabouraud Glucose Agar with an antibiotic (Chloromphenicol 0.1 g l^{-1}) and different salt density after all continue withe starch agar plate contain salt. It was found to be a good α-amylase producer and its enzyme property was investigated in our study .

Chemicals: All analytical reagents and media components were purchased from Hi-Media (Mumbai, India) and Sigma chemicals.

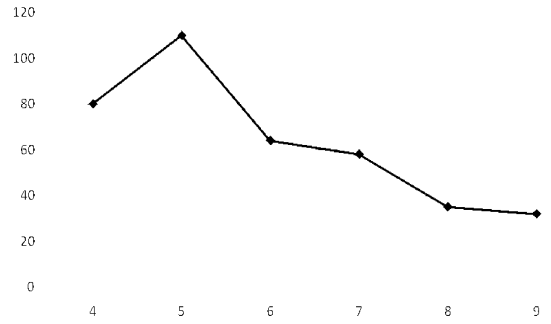
Inoculum Preparation: A volume of 7 mL of sterile distilled water was transferred to a sporulated (7-day-old) PDA slant culture. The spores were dislodged using the inoculation needle under aseptic conditions and the suspension, with appropriate dilution, was used as inoculum. A volume of 1 mL of spore suspension contained about 5.10^6 spores.

Solid-state Fermentation: Masses of 5 g of dry substrates were taken into 250-mL Erlenmeyer flasks. To adjust moisture levels (% by mass per volume), 0.1 M acetate buffer (pH=5.0) was added. The contents of the flasks were mixed thoroughly and autoclaved at 121°C for 20 min. The flasks were incubated at 28°C for 7 days.

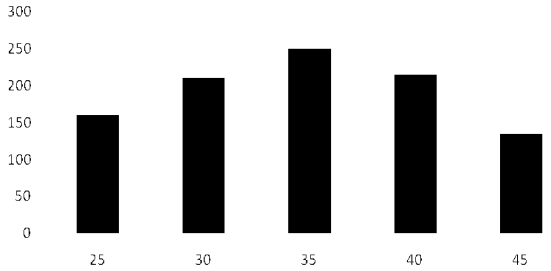
Enzyme Extraction: α-amylase enzyme was extracted in 50 ml of 0.1 M phosphate buffer (pH=7) on a rotary shaker at 200 rpm for 25 min. The content was filtered through muslin cloth, filtrate was centrifuged at 10,000 rpm for 10 min and supernatant was used as the enzyme source.

Enzyme Assay: α-amylase activity was determined by incubating a mixture of 0.5 ml of WB as enzyme source and 1 % soluble starch dissolved in 0.1 M phosphate buffer, pH=7, at 30 0C for 20 min [18]. The reaction was stopped by adding 1 ml of 3, 5-dinitrosalicylic acid and then followed by boiling for 10 min. The final volume was made up to 12 ml with distilled water and the reducing sugar released was measured at 540 nm [19]. One unit (U) of α-amylase activity was defined as the amount of enzyme that releases 1 micromole of reducing sugar as glucose per minute, under assay conditions and expressed as U/g of dry substrate.

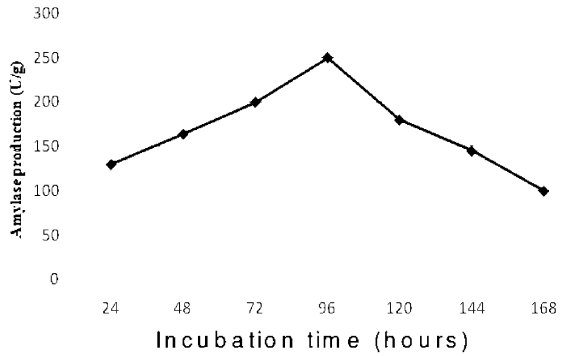
Impact of Process Parameters on A-amylase Production During Ssf with Comparison Submerged Cultivation: The impact of various process parameters influencing α-amylase synthesis by *P. chrysogenum* under solid state fermentation was studied. The strategy followed was to optimize each parameter, independent of the others and



Visible 2: The effect of initial pH on enzyme yield by fungus



Visible 3: The effect of incubation temperature on enzyme production by fungi



Visible 4: The effect of incubation time on enzyme production by the fungi

subsequently optimal conditions were employed in all experiments [20]. The effect of process parameters on enzyme production was determined by incubating at different pH (3-11), temperature (25-45°C), additional carbon source, nitrogen source, moisture content (20-80%), incubation time (24-168) h and surfactant.

Effect of Initial Ph of the Medium: The effect of initial pH on enzyme yield by fungus during solid state fermentation was studied by adjusting the pH of the mineral salt solution used to moisten the substrate to various pH levels (pH 4-9) using 1 N NaOH and 1 N HCl. The other conditions were kept constant. The result was shown in Visible 2.

Table 2:Effect of salinity on amylase production

Salinity (% of sea salt)	Amylase activity*(U/g)
0	380
5	365
10	260
15	105
20	40

Table 3: The effect of nitrogen source on enzyme production by isolated fungi

Nitrogen sources 0.2% (w/w)	Amylase activity (Ug)
Yeast extract	275
Meat extract	260
Beef extract	270
Peptone	320
Casein	260

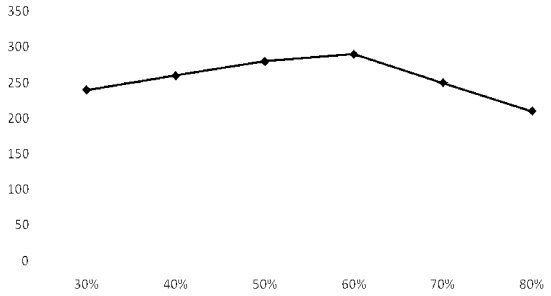
Effect of Incubation Temperature: The effect of incubation temperature on enzyme production by fungi during SSF was determined by incubating the flasks at different temperature (25-45°C) keeping other conditions constant. The result was shown in Visible3.

Effect of Incubation Time: The effect of incubation time on enzyme production by the fungi was studied by incubating the inoculated flask for a total period of 168 h and estimating the enzyme production at regular intervals of 24 h. The result was shown in Visible4.

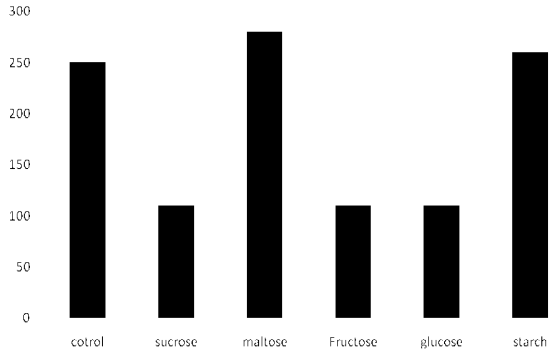
Effect of salinity on amylase activity by isolated fungi was shown in Table 2.

Effect of Nitrogen Source: The effect of nitrogen source on enzyme production by the fungi was studied by incorporating 0.2% (w/w) level of nitrogen in the SSF medium. The nitrogen sources tested include Yeast extract, Meat extract, Beef extract, Peptone, Casein. The effect of nitrogen source on enzyme production by the fungi was shown in Table 3.

Effect of Moisture Content: The results presented in visible5 indicate that an initial moisture content of 60% was optimum for maximum enzyme yield with wheat bran. Increase in moisture content resulted in clumping of the solid particles and consequent reduction in enzyme yield. Microbiological activity on a substrate will progressively decrease at lower water contents.Optimum yield was observed as 390 U/g at 60% (w/w) moisture content which decreased to 180 U/g at 80% (w/w) moisture content. Moisture causes swelling of substrate facilitating better utilization of the substrate. Increase in moisture content leads to reduction in product yield, during SSF,



Visible 5: Effect of moisture content on enzyme activity



Visible 6: Effect of additional carbon source on enzymatic activity

is due to reduction in interparticle spaces, decreased substrate degradation and impaired oxygen transfer [21, 22].

Effect of Additional Carbon Source: The results presented in visible6 indicate that maltose enhanced amylase production (380 U/gds) when compared to other carbon sources. Identical observations were earlier reported by [23, 24]. α-amylases is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product, maltose. another additional carbon sorce Fructose, sucrose and glucose inhibited growth and amylase production. Identical observations were earlier recorded in A. oryzae [25] α-amylases production is also subjected to catabolite repression by glucose and other sugars, like most other inducible enzymes [26, 27].

CONCLUSION AND DISCUSSION

The results have shown that the amylolitic enzyme produced by the isolated halotelorant *Penicillium* sp. α-amylase production will increase to tenfold when is used ssf in comparison smf .In the present study indicating its potential for industrial scale application in the future. Among chemical parameters, pH of the culture medium plays an important role on morphological changes in microbes and enzyme secretion. Also the pH change observed during the growth of microbes, effects on stability in the medium [28]. However, *Aspergillus oryzae* released amylase only in alkaline pH above 7.2 [29]. But found *Penicillium* sp. capable to produce α-amylase stable at alkalin is notable.Temperature optimum for amylase was found to be in a range between 25 and 37°C for the mesophilic fungi and the present study recorded 35°C as optimal,which agrees with earlier findings [28,29]. The influence of temperature on amylase production is related to the growth of microbes. The incubation period varies with enzyme productions Short incubation period offers potential for economical production of enzymes. In the present study the amylase activity increased steadily and reached maximum at 96 h of incubation (Visible 4). Although the fungal strain was isolated from coastal soil, it produced low concentration of amylase when production medium prepared with high salt concentration (Visible1). Hence, it can be a halotolerant terrestrial species.The nature of culture conditions and composition of media for optimal production of α-amylase by *P. chrysogenum* has been developed in this study.

REFERENCES

1. Sudo, S., T. Ishikawa, K. Sato and T. Oba, 1994. Comparison of acid stable -amylase production by Aspergillus kawachii in solid-state and submerged cultures. J. Ferment. Bioeng., 77: 483-489.
2. Pandey, A., C.R. Soccol, P. Nigam, D. Brand, R. Mohan and S. Roussos, 2000. Biotechnological potential of coffee pulp and coffee husk for bioprocesses. Biochem. Eng. J., 6: 153-162.
3. Kunamneni, A., K. Permaul and S. Singh, 2005. Amylase production in solid state fermentation by the thermophilic fungus *Thermomyces lanuginosus.* J. Bioscience Bioeng., 100: 168-171.
4. Wijbenga, D.J., G. Beldman, A. Veen and D.J. Binnema, 1991. Production of native starch degrading enzymes by a *Bacillus firmus*/lentus strain. Appl. Microbiol. Biotechnol., 35: 180-184.
5. Chessa, J.P., G. Feller and C. Gerday, 1999. Purification and characterization of the heat-labile α-amylase secreted by the psychrophilic bacterium TAC 240B. Can. J. Microbiol., 45: 452-457.
6. Rajagopalan, G. and C. Krishnan, 2008. α-Amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. Bioresour. Technol., 99: 3044-3050.

7. Pandey, A., C. Soccol and R.D. Mitchell, 2000. New developments in solid state fermentation: Bioprocesses and products. Process Biochem., 35: 1153-1169.
8. Sun, H., D. Zhao, B. Zhang and Y. SHI, 2008. Production of α-amylase by *Aspergillus oryzae* As 3951 in solid state fermentation using spent brewing grains as substrate. J. Sci. Food Agric., 88: 529-535.
9. Stredansky, M., E. Conti, L. Navarini and C. Bertocchi, 1999. Production of bacterial exopolysaccharides by solid substrate fermentation. Process Biochem., 34: 11-16.
10. Raimbault, M., 1998. General and microbiological aspects of solid substrate fermentation, Electron. J. Biotechnol., pp: 1.
11. Sun, X., Liu, Z. Qu and X. Li, 2007. The Effects of Wheat Bran Composition on the Production of Biomass-Hydrolyzing Enzymes by *Penicillium decumbens*. Appl. Biochem. Biotech., 146: 119-128.
12. Ellaiah, P., K. Adinarayana, Y. Bhavani, P. Padmaja and B. Srinivasulu, 2002. Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergilus* species, Process Biochem., 38: 615-620.
13. Butinar, L., S. Sonjak, P. Zalar, A. Plemenitas and N. Gunde-Cimerman, 2005. Melanized halophilic fungi are eukaryotic members of microbial communities in hypersaline waters of solar salterns. Bot. Mar., 48: 73-79.
14. Marbaniang, T. and S. Nazareth, 2007. Isolation of halotolerant *Penicillium* species from mangroves and salterns and their resistance to heavy metals. Current Sci., 92: 895-897.
15. Zalarl, P., M.A. Kocuvan, A. Plemenitas and N. Gunde-Cimerman, 2005. Halophilic black yeasts colonize wood immersed in hypersaline water. Bot. Mar., 48: 323-326.
16. Vonarx, J.A., 1981. Key to the Orders of Fungi, The Genera of Fungi Sporulating in Pure Culture. Cramer Hirschberg Germany.
17. Domsch, K.H., W. Gams and T.H. Anderson, 1993. Key to the Genera, Compendium of Soil Fungi. IHW, Eching, Germany.
18. Bernfield, P. 1955. Amylases, α and β. In: Methods in Enzymology, Vol. 1, Academic Press, New York, USA., pp: 149-158.
19. Balkan, B. and F. Ertan, 2007. Production of α-amylase from *Penicillium chrysogenum* under solid state fermentation by using some agricultural by products, Food Technol. Biotechnol., 45: 439-442.
20. Uyar, F. and Z. Baysal, 2004. Production and optimization of process parameters for alkaline protease production by a newly isolated *Bacillus* sp. under solid state fermentation. Proc. Biochem., 39: 1893-1898.
21. Ramesh, M.V. and BK. Lonsane, 1990. Critical importance of moisturecontent of the medium in α-amylase production by Bacillus licheniformis M 27 in a solid state fermentation system. Appl. Microbiol. Biotechnol., 33: 501-505.
22. Sandhya, X. and BK. Lonsane, 1994. Factors influencing fungal degradation of total soluble carbohydrates in sugar cane pressmud under solid state fermentation. Proc. Biochem., 29: 295-301.
23. Lachmund, A., U. Urmann, K. Minol, S. Wirsel and E. Ruttkowski, 1993. Regulation of Amylase formation in *Aspergillus oryzae* and *Aspergillus nidulans* transformants. Curr. Microbiol., 26: 47-51.
24. Morkeberg, R., M. Carlsen and J. Nielsen, 1995. Induction and repression of α-amylase production in batch and continuous cultures of *Aspergillus oryzae*. Microbiol., 141: 2449-2454.
25. Yabuki, M., N. Ono, K. Hoshino and S. Fukui, 1997. Rapid induction of alphaamylase by non-growing mycelia of *Aspergillus oryzae*. Appl. Environ. Microbiol., 34: 1-6.
26. Bhella, R.S. and I. Altosaar, 2004. Role of CAMP in the mediation of glucose catabolite repression of glucoamylase synthesis in *Aspergillus awamori*. Curr. Genet., 14: 247-252.
27. Gupta, R., P. Gigras, H. Mohapatra, V.K. Goswami and B. Chauhan, 2003. Microbial amylases: a biotechnological perspective. Process Biochem, 38: 1599-1616.
28. Kundu, A.K., S. Das and T.K. Gupta, 1973. Influence of culture and nutritional conditions on the production of amylase by the submerged culture of *Aspergillus oryzae*. J. Ferment. Technol., 51: 142-150.
29. Ueno, S., M. Miyama, Y. Ohashi, M. Izumiya, and I. Kusaka, 1987. Secretoryenzyme production and conidiation of *Aspergillus oryzae* in submerged liquid culture. Appl. Microbiol. Biotechnol., 26: 273-276.