საქართველოს მეცნიერებათა ეროვნული აკადემიის მოამბე, ტ. 14, №2, 2020
BULLETIN OF THE GEORGIAN NATIONAL ACADEMY OF SCIENCES, vol. 14, no. 2, 2020

Microbiology

Amylase Production by Microscopic Fungi Isolated from South Caucasus

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(Presented by Academy Member Georgi Kvesitadze)

Starch degrading enzymes like amylase received a great deal of attention because of their technological significance and economic benefits. As a result of screening of Durmishidze Institute of Biochemistry and Biotechnology (DIBB) collection of mycelial fungi accounting 2500 individual strains are isolated from South Caucasus, 39 strains poducing amylases with different degree of α- and glucoamylase activities were revealed. In order to increase amylase biosynthesis of most active microscopic fungi cultures, optimal conditions for their depth cultivation were estimated. Influence of various physical and chemical factors such as pH, temperature, carbon and nitrogen sources on amylase production in liquid media were studied. As a result of optimization amylolytic activities were increased by 20-46%. *© 2020 Bull. Georg. Natl. Acad. Sci.*

Microscopic fungi, α-amylase, glucoamylase, optimization

Microbial enzymes are known to play a crucial role as metabolic catalysts, leading to their use in various industries and applications. Amylases are among the most important enzymes and are of great significance for biotechnology and different branches of industry [1,2].

Amylases are glycosidase which catalyze the hydrolysis of glycosidic linkage in starch to generate smaller sugars useful to bio industry. Two main enzymes are involved in starch hydrolysis – α-amylase and glucoamylase. α-amylase (E.C. 3.2.1.1) hydrolyses α-l.4- glycosidic bonds randomly in amylose, amylopectin and glycogen in an endofashion. α-amylases bypass α-1.6 glycosidic bonds and do not cleave them. Glucoamylase is an exo-acting enzyme (EC 3.2.1.3) hydrolyzing both 1.4-. α- and 1.6-. α-glucosidic linkages in polysaccharides yielding glucose. Amylases can be obtained from plants, animals and microorganisms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors as they are more stable than prepared with plant and animal amylases. A large number of microbial amylases are available commercially and they have broad spectrum of industrial applications.

Nowadays microbial amylases almost completely replaced chemical hydrolysis of starch in starch processing industry [3,4]. The major advantage of using microorganisms for the production of amylases is the economical bulk

production capacity and the fact that microbes are easy to manipulate in order to obtain enzymes of desired characteristics.

Traditionally, amylases of fungal and bacterial origin were used in different branches of food industry for a long time. These are: application of α-amylase and glucoamylase in starch hydrolysis to fermenting sugars. Obtained as a result of starch saccharification process fermentable sugars could further be fermented by yeast to produce alcohol. In addition to starch hydrolysis, amylases have a number of other industrial applications. The α-amylases were widely used in the bakery industry. These enzymes can be added to the dough of bread to degrade the starch in the flour into smaller dextrins, which are subsequently fermented by the yeast; the addition of α-amylase to the dough enhances the rate of fermentation and reduces the viscosity of dough, resulting in improvements in the volume and texture of the product. Amylases are used for the clarification of beer or fruit juices, or for the pretreatment of animal feed to improve the digestibility of fiber. 90% of all liquid detergents contain amylases. These enzymes are used in detergents for laundry and automatic dishwashing to degrade the residues of starchy foods such as potatoes, gravies, custard chocolate, etc. Amylases are used in textile industry for desizing process. Sizing agents like starch are applied to yarn before fabric production to ensure a fast and secure weaving process. The use of α-amylases in the pulp and paper industry is for the modification of starch of coated paper, i.e. for the production of low-viscosity, high molecular weight starch [5-7].

Isolation and selection of suitable organisms are very essential for the production of extracellular amylases. Selection of microorganisms with best amylase activity could contribute a lot for the discovery of novel potential amylases appropriate for different industrial and biotechnological applications [8,9].

Materials and Methods

The search for new microorganisms that can be used for amylase production is a continuous process. The selection of microscopic fungi was carried out among the diverse mycelial fungi strains collection of DIBB, isolated from different soil-climatic zones of south Caucasus, including extreme habitats.

The action of different growth parameters like incubation period (0-72 hrs), pH in the range from 2.0 to 10.0, temperature range of 15-55°C, on the growth and development of strains was studied. In order to increase the biosynthesis of most active producers optimal composition of nutrient medium was selected. Different carbon (starch 6%, 3%, disaccharides and some small molecular weight saccharides- maltose, glicerine, lactose, xylose, galactose, ramose and cellobiose – 0,8% with respect to carbon), nitrogen (peptone, $NaNO_3$, $KN0_3$, NH_4N0_3, NH_4CL beef extract and yeast extract) and phosphorus sources (NaH_2PO_4; $(NH_4)_2HPO_4$; KH_2PO_4; $KHPO_4$) were used in our studies.

Initially strains were grown on solid agar medium containing beer wort. Consistence of the medium per litre: 0.5 l beer wort 7°B, 0.5l running water, 20.0 g agar (pH 5.5 – 6.0). Nutrient medium was adjusted to required pH by addition of 0.1 M NaOH or 0.1 M HCl. The media in flasks were sterilized at 0,7 atm. for 40 min. Conidial suspensions of 10-day cultures, grown in test-tubes were used as inoculum (the test-tubes were placed in thermostat, at corresponding optimum growth temperature for each particular strain). While screening the amylase producers (α-amylase, glucoamylase) microscopic fungi cultures grew on a solid modified Czapeck's:

Medium #1, in %: starch – 2.0; $NaNO_3$ – 0.91; KH_2PO_4 – 0.1; $MgSO_4 \times 7H_2O$ – 0.05; KCl – 0.05; $FeSO_4 \times H_2O$ – 0.0002, agar-agar – 0.2.

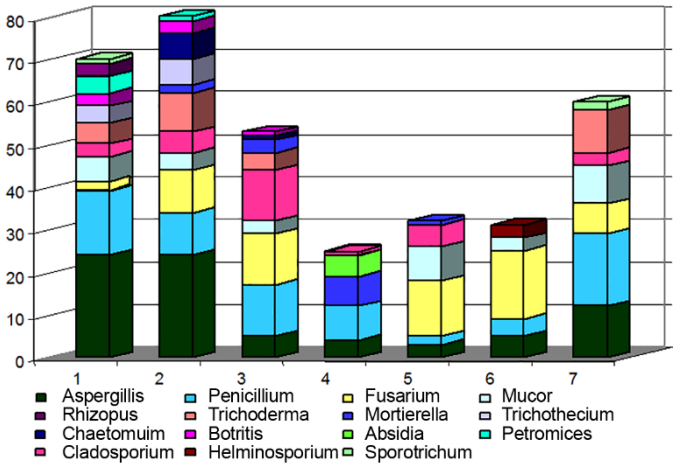


Fig. 1. Amylase producers of different genera.

In case of microscopic fungi, the cultures sowing material was 10-day old culture conidia suspension; submerged cultivation was carried out in 250 ml Erlenmeyer flasks, on temperature-controlled rotary shaker (180-200 rpm), at 30-45°C. In order to reveal amylases producers cultivation was conducted during 72 hours. The amount of nutrient media equalled to 50 ml. Composition of liquid medium for production of amylases:

Medium #2, in %: starch – 6.0; $NaNO_3$ – 0.91; KH_2PO_4 – 0.1; $MgSO_4 \times 7H_2O$ 0.05; KCl – 0.05; $FeSO_4 \times H_2O$ – 0.0002; malt extract 1.5 per 50 ml.

Determination of a-amylase activity in the cultural liquids was based on decreased staining value of blue starch-iodine complex [10]. Glucoamylaze activity was determined with respect of glucose [11]. The concentration of free glucose was assayed, using a glucooxidase reagent after 10 min hydrolysis.

Results and Discussion

307 strains of the DIBB culture collection, representatives of different genera of microscopic fungi Aspegillus, Absidia Actinomucor, Absidia, Chaetomium, Cladosporium, Mucor, Mortirella Myrothecium, Penicillium, Rhizopus, Sporothrichum were applied in the study. According to literary data and our experience these strains are known to be active producers of amylases [12,13].

As a result of screening under submerged cultivation 39 strains were selected with amylase activity. Most of them belong to Aspergillus genera (Fig. 1.). Tree from them Aspergillus niger Sh 42, Cladosporium sp. Hh 3, Mortierella sp. Sy 38 producing comparatively large quantity of enzymes and being thermopiles and thermotolerant were selected for further studies (Table 1).

Ambient factors significantly influence on the nature of metabolism in microorganisms. Consequently, selection of cultivation conditions is one of the ways to regulate biosynthesis activity of organisms without impact on genetic apparatus.

Microorganism vitality and potential to produce enzymes intensively greatly depends upon the selection of the appropriate nutrient medium in particular, the carbon, nitrogen and phosphorus sources; However, the carbon source plays a special role in biosynthetic processes in microorganisms including regulation of the synthesis of enzymes through induction and repression [14-15].

Starch and wheat bran served as a carbon source for the amylase producers, disaccharides and some small molecular weight saccharides, maltose, lactose and cellobiose – 0,8% with respect to carbon source were used. High amylase activity was observed with starch and maltose as a carbon source. Further the optimal concentration of starch

Table 1. Amylolytic activities in cultural filtrates of mycelial fungi strains (U/ml)

#	Strains	α-Amylase activity, U/ml	Glucoamylase activity, U/ml	Characterization
1.	*Absidia sp. Ts 45*	0.5	-	Thermophile
2.	*Aspergillus niger Ef 1*	1.2	4.2	Mesophile
3.	*Aspergillus niger Ag 4*	1.3	4.0	Mesophile
4.	*Aspergillus niger My 2*	0.9	3.1	Mesophile
5.	*Aspergillus niger Sk 1*	1.1	3.5	Mesophile
6.	*Aspergillus niger Ts 7*	2.1	5.0	Thermotolerant
7.	*Aspergillus niger Ts 11*	3.2	11.0	Thermotolerant
8.	*Aspergillus niger Sh. 3*	1.7	6.0	Thermophile
9.	*Aspergillus niger Sh 42*	5.9	15.0	Thermophile
10.	*Aspergillus niger Ch 17*	1.7	7.8	Thermotolerant
11.	*Aspergillus niger Ch. 25*	1.5	8.0	Thermotolerant
12.	*Aspergillus oryzae Sk 9*	1.2	6.5	Mesophile
13.	*Aspergillus fumigatus Sk 13*	0.9	-	Thermotolerant
14.	*Aspergillus nidulans Ts 10*	0.9	3.5	Thermotolerant
15.	*Cladosporium sp. Hh 3*	4.2	12.5	Thermophile
16.	*Chaetomium sp.Sy 12*	0.8	3.8	Thermotolerant
17.	*Chaetomium sp.Sh 15*	0.5	2.5	Thermotolerant
18.	*Mucor sp. Sy 21*	0.3	1.1	Mesophile
21.	*Mucor sp. Ar 14*	0.8	4.1	Mesophile
22.	*Mortierella sp. Sy 38*	2.8	7.0	Thermotolerant
23.	*Mortierella sp. Rm 4*	1.2	5.0	Mesophile
24.	*Myrothecium sp. My 21*	0.7	2.6	Mesophile
26.	*Rhizopus sp.Sy 20*	2.1	3.3	Mesophile
27.	*Rhizopus sp. Rk 6*	2.5	3.7	Mesophile
28.	*Penicillium sp.Sy 41*	3.1	3.9	Mesophile
29.	*Penicillium sp.Sh 32*	2.8	5.0	Mesophile
32.	*Penicillium sp. Mm 11*	1.9	4.2	Mesophile
33.	*Paecilomyces sp.Sh 50*	0.4	-	Thermophile
34.	*Verticillium sp. Ag 3*	0.7	1.4	Thermotolerant
35.	*Verticillium sp. Ar 1*	0.6	1.1	Mesophile
36.	*Aspergillus niger A 1-1*	3.2	7.0	Thermophile
38.	*Aspergillus niger A 6-3*	trace	1.3	Mesophile
39.	*Cladosporium sp.T48*	0.7	-	Thermotolerant
40.	*Cladosporium P36*	5.1.	9.1	Thermophile
41.	*Mucor sp. R33*	trace	3.3	Thermotolerant
42.	*Mucor sp.K22*	0.7	-	Thermophile
43.	*Penicillium sp. T57*	1.5	3.8	Mesophile
45.	*Rhizopus sp.R21*	-	0.9	Mesophile

Table 2. Effects of carbon source on biosynthesis of amylolitic enzymes

Carbon source	Strain					
	Aspergillus niger Sh 42		*Cladosporium sp. Hh 3*		*Mortierella sp. Sy 38*	
	α–amylase un/ml	Glucoanylase un/ml	α–amylase un/ml	Glucoanylase un/ml	α–amylase un/ml	Glucoanylase un/ml
starch 6%)	5.5	15.5	4.0	12.5	2.6	7.0
starch 9%)	5.9	12	4.2	12.0	2.8	7.0
cellobiose	3.2	8.5	3.2	7.8	1.1	4.2
glicerine	2.1	5.2	1.5	4.5	1.0	2.5
maltose	4.8	8.5	3.8	9.5	1.4	6.2
lactose	2.8	4.6	2.2	2.8	1.05	1.3
xylose	1.5	3.0	2.5	1.3	1.2	1.1
galactose	2.3	4.1	3.0	3.2	1.7	2.1
ramose	1.5	1.8	2.1	1.3	1.2	1.2

Table 3. Effects of nitrogen source on biosynthesis of amylolitic enzymes

Nitrogen source	Strain					
	Aspergillus niger Sh 42		*Cladosporium sp. Hh 3*		*Mortierella sp. Sy 38*	
	α–amylase un/ml	Glucoanylase un/ml	α–amylase un/ml	Glucoanylase un/ml	α–amylase un/ml	Glucoanylase un/ml
$NaN0_3$	6.0	15.0	4.8	12.5	3.8	7.8
$KN0_3$	5.8	14.5	4.2	10.0	3.6	7.1
NH_4N0_3	4.1	12.0	4.2	9.0	3.0	6.8
NH_4CL	1.15	5.1	1.1	3.5	2.8	3.5
urea	4.3	7.5	3.2	7.5	2.4	4.2
malt extract 0,5%	3.8	6.8	2.2	7.8	2.1	4.0
malt extract 1.0%	4.0	7.0	3.1	7.8	2.8	4.5
malt extract 3.0%	5.1	7.5	3.8	8.0	3.0	6.2

Table 4. Effects of phosphorus source on biosynthesis of amylolitic enzymes

Nitrogen source	Strain					
	Aspergillus niger Sh 42		*Cladosporium sp. Hh 3*		*Mortierella sp. Sy 38*	
	α–amylase un/ml	Glucoanylase un/ml	α–amylase un/ml	Glucoanylase un/ml	α–amylase un/ml	Glucoanylase un/ml
KH_2P0_4	6.2	15.0	8.5	10.5	3.8	8.0
K_2HP0_4	5.8	14.5	7.0	9.2	3.2	7.8
$NaHP0_4$	4.8	12.1	5.5	8.0	2.6	6.2
$(NH_4)_2$ $HP0_4$	3.5	7.8	2.1	4.5	1.8	4.5

Table 5. Amylases activities of selected mycelial fungi strains under optimum condidions of cultivation

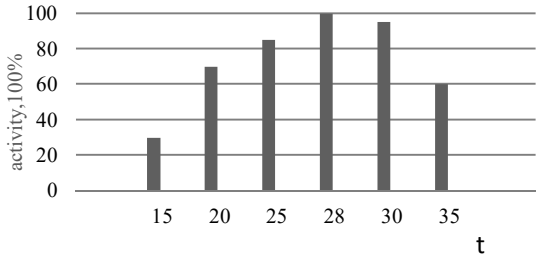
Culture	α-Amylase activity, U/ml			Glucoamylase activity, U/ml		
	Initial	After optimization	Increased activity in%	Initial	After optimization	Increased activity in %
Aspergillus niger Sh 42	5.9	7.08	20	15.0	22.0	46
Cladosporium sp. Hh 3	4.2	6.0	42	12.5	18.0	44
Mortierella sp. Sy 38	2.8	3.5	25	7.0	9.0	28

as a selected carbon source was determined. The α-amylase producers revealed high enzyme activity, with 9% starch; the glucoamylase gave the elevated amount of the enzyme growing on – 6% of starch (Table 2). Nitrogen is another important component of the microorganisms cultivation medium. Microorganisms uptake nitrogen of either organic or inorganic forms. Following nitrogenous salts were applied as inorganic nitrogen sources in nutrient media: $NaNO_3$, KNO_3; $(NH_4)_2SO_4$; NH_4NO_3 and $(NH_4)_2HPO_4$; Peptone, urea and yeast extract at different concentrations were applied as the nitrogen organic sources. For all the studied producers optimum source of nitrogen for amylase biosynthesis appeared $NaNO_3$, $KN0_3$ and malt extract (Table 3). The optimal phosphorus source for these cultures was also determined while applying NaH_2PO_4; $(NH_4)_2HPO_4$; KH_2PO_4; K_2HPO_4. K_2HPO_4 and KH_2PO_4 were most effective for synthesis of amylolitic enzymes (Table 4).

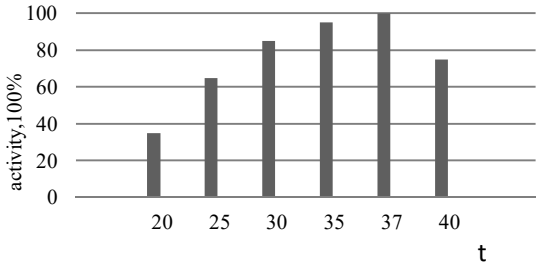
As far as, the temperature is one of the most important factors in the regulation of growth and physiological activity of mycelial fungi cultures, first of all, effect of the temperature on culture growth and development and enzyme synthesis by selected strains were established. Submerged fermentation (SF) of selected producers was carried out in the above described conditions at different temperatures (15°C to 55°C). Optimum tempe-

ratures, at which the maximum amount of enzymes is produced during submerged fermentation of strains, are represented in Fig. 2. According to the data, during submerged fermentation, optimum temperature for mesophilic strain Aspergillus niger Sk 1 fluctuates between 27-30°C, for thermotolerant Mortierella sp. Sy 38 between 35-37°C, and for thermophiles Aspergillus niger Sh 42, Cladosporium sp. Hh 3 between 42-45°C.

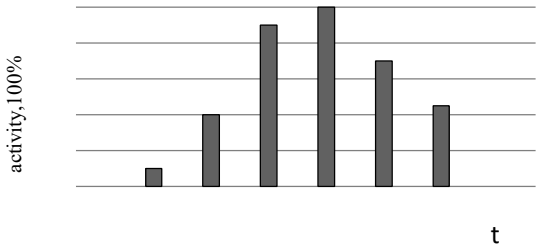
To investigate the pH optimum of enzyme production, cultivation of strains was carried out in diapason of pH 2.5-9.0, at 0.5 pH intervals under the above described conditions of cultivation. According to the search results, during submerged fermentation, optimum pH for majority strains fluctuates between 5.5-6.5 (Fig. 3). As a result of performed experiments amylolytic activities was increased by 20-46% (Table 5).



Strain Aspergillus niger Sk 1- mezophile

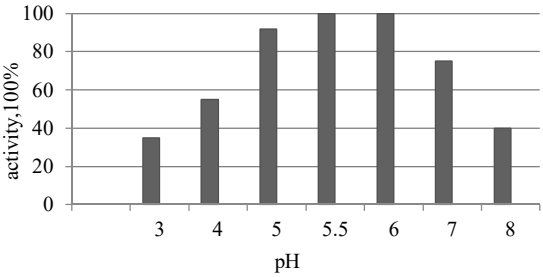


Strain *Mortierella* sp. Sy 38 – thermotolerant

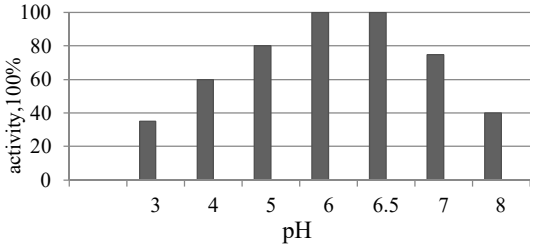


Strain *Aspergillus niger* Sh 42 thermophil

Fig. 2. Influence of cultivation temperature on amylases biosynthesis.



Strain *Aspergillus niger* Sh 42



Strain *Aspergillus niger* Ts 11

Fig. 3. Effect of cultivation pH on amylase biosynthesis by the selected enzyme producer.

Conclusions

– As a result of screening 39 strains, out of which there are 18 mesophilic and 21 extremophilic strains, were revealed among 307 strains from the DIBB microscopic fungi collection;
– Representatives of the genera Aspergillus, Cladosporium, Penicillium and Trichoderma are distinguished by comparatively high activity;
– Composition of nutrient medium for deep cultivation of strains, active producers of amylases were established;
– Optimal growth conditions (temperature, pH and duration of cultivation) for strains, active producers of amylases were established;
– As a result of optimization of nutrient media and cultivation conditions activities of enzymes produced by the strains were increased by 20-46%.

The work was funded by the Shota Rustaveli National Science Foundation of Georgia (SRNSF), project #2017-59.

მიკრობიოლოგია

სამხრეთ კავკასიიდან გამოყოფილი ამილაზების პროდუცენტი მიკროსკოპული სოკოები

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ამილაზები, როგორც სახამებლის დამშლელი ფერმენტები, მათი ტექნოლოგიური მნიშვნელობიდან გამომდინარე, ფართოდ გამოიყენება ეკონომიკის სხვადასხვა დარგში. საქართველოს აგრარული უნივერსიტეტის დურმიშიძის ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტის მიკროსკოპული სოკოების საკოლექციო შტამებს შორის ჩატარებული სკრინინგის შედეგად გამოვლენილია ამილაზური ფერმენტების პროდუცენტი 39 შტამი. ამილაზური ფერმენტების ბიოსინთეზის გაზრდის მიზნით სიღრმული კულტივაციისას შერჩეულია განსაკუთრებით აქტიური და სტაბილური ფერმენტების პროდუცენტი შტამების ოპტიმალური საკვები არეები და კულტივირების პირობები. შესწავლილია ნახშირბადის, აზოტის, ფოსფორის სხვადასხვა წყაროს და აგრეთვე კულტივირების ტემპერატურისა და pH-ის გავლენა ამილაზების სინთეზის ინტენსივობაზე. ოპტიმიზაციის შედეგად ამილაზური ფერმენტების სინთეზი გაიზარდა საშუალოდ 20-46%-ით.

REFERENCES

1. Poonam Singh Nigam (2013) Microbial enzymes with special characteristics for biotechnological applications. *Biomolecules,* **3**(3): 597-611.
2. Kandra L. (2003) α-Amylases of medical and industrial importance. *Journal of Molecular Structure (Theochem),* 666-667; 487-498.
3. Pandey A., Nigam P., Soccol C.R., Soccol V.T., Singh D., Mohan R. (2000) Advances in microbial amylases. *Biotechnol. Appl. Biochem.,* **31**(Pt 2): 135-152.
4. Ritu Saini, Harnek Singh Saini and Anjali Dahiya (2017) Amylases: characteristics and industrial applications. *Journal of pharmacognosy and phytochemistry,* **6**(4): 1865-1871.
5. Lundkvist H., Olsen HS. (2007) Enzyme applications in bread making. *Biozoom* no. 4. Novozymes.
6. Noomen H., Ali N., El-H., Haddar A., Kanoun S., Alya SK et al. (2009) Alkaline proteases and thermostable α-amylase coproduced by Bacillus licheniformis NH1: Characterization and potential application as detergent additive. *Biochemical Engineering Journal*, 47: 71-79.
7. Vander MJEC, Maarel B., VanderVeen JCM, Uitdehaag HLM, Dijkhuizen L. (2002) Properties and application of starchconverting enzymes of the alpha amylase family, *J. Biotechnol.,* 94: 137-155.
8. Tiwari SP, Srivastava R., Singh CS, et al. (2015) Amylases: an overviev with special reference to alfa amylase. *Journal of Biosciences,* ISSN 2320-1355, **4**(1): 1886-1901.
9. Rukhlyadea A.P., Goryacheva M.T (1961) Determination of enzyme amylolytic activity. *Enzyme and Alcohol Industry*, 1: 9.
10. Dahlguist A. J. (1961) *Biochem.,* **80**: 547.
11. Khokhar I., Mukhtar I., Mushtaq S. (2006) Isolation and screening of amylolytic filamentous fungi. *J. Appl. Sci. Environ. Manage,* **15**(1): 203-206.
12. Luis Henrique S., Guimarãesl et al. (2006) Screening of filamentous fungi for production of enzymes of biotechnological interest. *Brazilian Journal of Microbiology,* 37: 474-480.
13. Umar D.M. (2008) Effect of alternative carbon and nitrogen sources on production of alpha-amylase by Bacillus megaterium. *J. Biotechnol.,* 136: 293-302.
14. Francis F., Sabu A. et al. (2002) Synthesis of alpha amylase by oryzae in solid state fermentation. *J. Basic Microbiol.,* 42: 320-326.
15. Shah I.J., Gami P.N. et al. (2014) Optimization for α-amylase production by Aspergillus oryzae using submerged fermentation technology. *Basic Research Journal of Microbiology,* ISSN 2354- 4082, **1**(4): 01-10.

Received February, 2020