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Optimization of Amylase Production by *Aspergillus Oryzae* Isolated from Various Sources

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ABSTRACT

Background & Objectives: *Amylases are a group of hydrolases that can specifically cleave the glycosidic linkage in starch. Fungal and bacterial amylases also have importantapplications in industry. The major advantage of using fungi for the production of amylases is economical bulk production capacity and ease of manipulation. The aim of present study was the isolation fungi on different sources and optimization of amylase production by Aspergillus oryzae.*
Materials and methods: *Sampling were performed from different sources such as moldy bread, mucky soil, and soil mucky containing potatoes waste in order to obtain the Aspergillus oryzae. Molecular identification and screening of amylase producing by different isolates of A. oryzae have been done by using submerged fermentation in plate culture on specific medium. The specificity of amylase enzymes in all A. oryzae strains were evaluated in different temperatures (15-90°C), buffers (sodium acetate, citrate-phosphate, Tris-HCL and Naoh-glycine) and ph (3, 4 and 5). In our study, the quality control strain was A. oryzae PTCC5164.*
Results: *All fungi strain were confirmed as A. oryzae by phenotypic and molecular tests. In addition to, all strains were positive for amylase production in submerged fermantation. Our results indicated that amylase production by A. oryzae isolated from soil mucky was more than to the other strains. As well, the best condition for amylase production by these strain was in Tm=45°C, glycine buffer and pH=5.*
Conclusions: *Amylases are among mostly used enzymes with applications in various industries from bakeries and sugar processing to detergents manufacturing. Efforts to find novel enzymes are still underway because these enzymes are interesting not only from industrial but also from enzymology point of views. The production of amylase with increasing of temprature in all strains of A. oryzae was declined. The best pH for amylase production was equal to 5 and glycine as the best buffer for the production of this enzyme.*

Key words*: Amylase production, Aspergillus oryzae, Submerged fermentation, pH, Temperature, Buffer*

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INTRODUCTION

At the moment, enzymes such as amylase, protease, catalase, isomer, penicillin- Asylase are produced industrially to be used in different industries. Amylase is and enzyme that hydrolyzes starch. Starch grains are insoluble in cold water, and are resistant to chemical substances or enzymes more than anything else. Amylase is the most important enzyme in technology biology and the significance of amylase lies in its broad application produced by microbial and fungus species. Amylase has a wide application in different industries like paper- making, detergents, bakery, food industry including producing fructose and glucose syrups and fruit juice, Although most of plants, animals and microorganisms are able to produce amylase, the enzyme can be used in the industry by microbial origin. Meanwhile, Aspergillus oryzae species produce a wide spectrum of amylase, being singnificant in industry. Amylase production depends on factors like strains, medium composition, culture approach, cell growth, and incubation. Alpha- amylase traits should be fit with resistance to heat and PH and application of amylase; therefore, the wide application of amylase made some researches to be continued to make the enzyme traits clear. Rate of starch hydrolysis by alpha- amylase is dependent on conditions such as temperature, substrate origin, substrate density, enzyme density, presence or absence of calcium ions. Alpha-amylase is produced by a large number of fungi and bacteria, the most important bacteria is Bacillus subtilis, Bacillus polymyxa, Bacillus caldolyticus, and its species like micrococcus, pseudomonas, Escherichia, proteus, termomonospora, aspergillus, Trichoderma, and penicillium that are able to produce alpha- amylase resistant to heat and ∝-amylase that is active in the presence of calcium and cobalt.
A.oryzae is a filamentous fungus growing mostly in china and Japan soil. The fungus has been used in Asia for more than 2000 years in the production of fermented food products such as soya

sauce, Miso, and rice wine. At the beginning of the twentieth century, A.oryzae was applied as a source for alpha- amylase, the first enzyme used in food production in an industrial scale. Since then, A. oryzae has been used in production of various native and heterologous enzymes in food industry. Diversity and microbial population evaluation in starch waste, and next. Evaluation on special abilities of microorganisms in amylase production and introduction of new and native strains with specific abilities are useful to be applied in different industries. The study aimed to examine thermal resistance and PH in amylase from A. oryzae isolates in different environmental sources.

Material and Method:

Sampling:

This cross- sectional study was conducted in 2015 for 6 months in Microbial research laboratory of Islamic Azad university of Damghan Branch. The samples were collected from moldy bread, mucky soil and mucky soil containing potato waste in order for isolating A. oryzae. The collected samples were incubated at 37°C for 5 days in potato dextrose agar (PDA).

The strain A. oryzae PTCC 5164 was used as test controlling strain.

Identification of fungus isolates:

The phenotypic study on fungal colonies grown in PDA, has been done in terms of colony's form and sporulation structure in coloring by Lacto phenol cotton blue. In order to molecularly identify fungal isolates, at first, DNA was boiled according to Millar et.al (2000). Polymerase chain reaction was conducted by designed primer from 16sr RNA, Bact_63f (5'-CAGGCCTAACACATGCAAGTC-'3) and Bact_1389r (5'-ACGGGCGGTGTGTACAAG- 3'), based on a protocol defined by Dantas et.al (2008). In additions sequencing of fungi was done, using forward primer Bact_63f. Aspergillus oryzae PTCC 5164 was purchased from industrial fungi and Bacteria's collection in Iran and was applied as test controlling strain.

Inoculation suspension:

Fungal spore suspension was prepared using Nebular slide in a number of 1×10^6 cell/ mL in physiology serum and tween 80 (0.8%).

Amylase production using immersion method:

The amount of $(NH_4)_2$ SO_4 0.14%, K_2 HPO_4, KH_2PO_4 0.20%, Mg SO_4. $7H_2O$ 0.01%, yeast extract 0.50%, peptone 0.20%, beef extract 0.20% (PH- 5) spore suspension 10^6 CFU/ mL were added to a flask of 25 mL containing medium of 1% carbon source to be inoculated, each of the amounts were chosen from each of A. oryzae isolates in different sources. The fermentation product was incubated at 37°C for 96 hours in shaker by 100 rpm. Biomass was separated for 20 minutes in 10000 gr, and supernatant is used to examine amylase activity. Fermentation medium had been in shaker incubator of 160 rpm for 5 days at 30°C. Biomass was separated in 10000 rpm for 20 minutes and supernatant was used to examine amylase activity.

Enzyme extraction:

The enzyme solution was extracted by adding up 100 mL of buffer acetate (PH- 4.8 by concentration of 50 m M) to culture samples and shaking them in 150 $rev.min^{-1}$ for 1 hour at 30 °C. The extract was isolated using what man filter paper (No.1) from medium waste. Suspended particles were sediment by centrifuge in 6000 rpm for 15 minutes, and the resulting supernatant was used in determining amylase activity.

Enzyme activity measurement:

The enzyme activity was measured using Bemfeld method. At first, 0.5 mL of crude enzyme solution was boiled in testing tubes. The solution containg starch for 1% (Merck Company) was used in buffer acetate as a substrate at 40°C in a shaking water bath for 5 minutes. The enzyme was measured by DNS (Di- nitro salicylic acid). The resulting supernatant from extraction and centrifuge is so dense to examine amylase, that's why, it was subtilized in rate of one percent by buffer acetate to measure its amyl lytic power. The reduced sugar from the above tests was measured using method DNS. 1 unit of enzyme activity is an amount of activity that can produce 1 mM of reducing sugar per minute based on equivalent.

Study on the effect of temperature and PH:

Activity of the resulting enzyme solution was measured at 15 - 90°C and its figure has been drawn. In addition, enzyme activity in various PH (3, 4, 5) and different buffer (Tris, sodium acetate, Citrate phosphate, and Glycine) was determined and the graph has been drawn.

Results:

The results of culture and direct test indicated A. oryzae isolation. Furthermore, the analysis results confirmed the fungus isolates of A. oryzae. Aspergillus oryzae isolates from mucky soil, bread, mucky soil containing potato were positive in terms of alpha- amylase production through immersion method. The analysis results of temperature resistance for different isolates showed that the most enzyme activity was observed in A. oryzae isolates from mucky soil at 45 °C and the least activity was shown in A. oryzae isolates from moldy bread at 90 °C. The most enzyme production of A. oryzae isolates were seen in mucky soil at different temperatures and the most enzyme activity if for all A. oryzae isolates at 45 °C in which an increase in temperature caused a decrease in activity in all of isolates (figure 1).

The results from PH resistance and buffer for A. oryzae isolates from mucky soil indicated that the maximum activity was seen in PH- 5 and moreover, the maximum activity was seen in buffer Glycine (Figure 2). The maximum enzyme activity for isolates from moldy bread was observed in PH- 5 and the minimum in PH- 4. In addition, the maximum activity was seen in buffer Tris and the least in citrate phosphate (Figure 3). The maximum and minimum activity of enzyme for A. oryzae isolates from mucky soil containing potato is in PH- 6 and PH- 5, respectively. In addition, in buffer analysis, the most activity of the strain was in buffer Glycine and the least in sodium acetate (Figure 4).

For standard isolate of Aspergillus oryzae PTCC 5164, the maximus enzyme activity was in PH- 5 and the least in PH- 6.

Also, the most activity in buffer analysis for standard isolate of A. oryzae PTCC 5164 was seen in Glycine and the least in citrate phosphate (Figure 5).

Discussion:

Despite amylase production in different creatures, microbial sources that's to say bacterial and fungus amylase are mostly used in industrial production because of benefits including less cost, more resistance, saving the intended time and place for production and simple process of improvement and optimization. In the present study, the A. oryzae strain produced from fungus and bacteria collection was used as standard strain and also the A. oryzae isolates from mucky soil, moldy bread and mucky soil containing potato was used to be analyzed in terms of amylase resistance in themal spectrum of 15 - 90°C and in different PH by sodium acetate, citrate phosphate, Tris and Glycine. The results indicated that the maximum thermal resistance is concerned with A. oryzae isolates from mucky soil and the minimum belongs to A. oryzae isolates from moldy bread. Also, the results indicated that an increase in temperature caused a decrease in enzyme activity of amylase in all of isolates. The effect of temperature on enzyme production is related to microorganism growth, the fact is clearly observed in the results, and by an increase or decrease of growth in different temperatures, alpha- amylase production is in accordance with growth changes in various temperatures. The broad temperature range is reported to be 35 to 80 for optimized growth and production of alpha- amylase in fungi. The existing differences in the optimized temperature in different species may be because of temperature effect in alpha-amylase production. In other words, when incubation temperature increases, along with an increase in stabilized enzyme production into cell, the released alpha- amylase from the cell decreases by A. oryzae. Also, the results of present study showed that the best PH for amylase high activity in A. oryzae isolates from mucky soil, moldy bread, mucky soil containing potato and standard isolate were PH- 5, PH- 5, PH- 6 and PH- 5, respectively. The significance of PH in alpha- amylase activity refers back to the enzyme application in industry. There are different alpha-amylase by resistance range against acidic or alkaline PH, each of which are used in different industries based on need. For instance, the enzymes should be resistant to alkaline PH in washing industry. Those groups of alpha- amylase that are highly resistant to acidity are also produced by fungi, including different species of Aspergillus. The intended alpha-amylase is optimized in neutral PH and can be applied in melting of starch. In addition, the best buffer for high activity amylase in isolates under the study, was mucky soil (Glycine), moldy bread (Tris), mucky soil containing potato (Glycine) and standard isolate (Glycine). In this study, A. oryzae isolate from mucky soil showed the best enzyme activity compared with the standard strain. The results of the study show that fungi use Glycine as a source of nitrogen for their growth in the presence of different buffers, and this fact makes high activity of amylase easier using the fungi. Moravej et.al (2010) reported the optimized condition (moisture of 55%, PH- 615, at 25°C, the fermentation rate of 10^6 and nitrogen source of 363- 8 U/g (NH_4) 2 SO_4) for amylase production in Trichoderma longibrachiatum. In this study, the maximum activity of ∝ - amylase after 7 days of immersion fermentation was reported in PH = 7 at 28°C of room temperature 450 U/mg for A. oryzae. Deljou et.al (2015) indicated that the resulting enzyme from bacillus licheniformis, has the maximum activity at 80°C and buffer Tris- HCL with PH-7. Saleem et.al (2014) isolated 46 species of fungi belonging to 26 genus out of 125 samples of beans seeds in agar dextrose of potato at 28°C. Samples were collected from different places in Medina, Saudi Arabia. The most common species were Aspergillus flavus, A. Niger, A. ochraceus, A. Terreus, America nidulans, penicillium chrysogenome, stolonifera rhizopus. Among 46. Species of fungi that are analyzed to produce amylase, 8 isolates (17/4% of the total isolates) had the most activity, 27 species (58.7%) had the average amylase activity, and 11 species (23.9%) had the least activity in amylase production. The maximum amylase was produced after 6 days of incubation at 30°C by the primary PH of 6 and through combination of starch and phosphate ammonium that are sources of Nitrogen and carbon. The results of this study is somehow accordant with the recent one. Amylase is one of the most important enzymes possessing a significant role in industrial biotechnology. Therefore, using amylase from Aspergillus oryzae is suggested in processing the fermented starch and producing carbohydrate products.

Conclusion:

The amount of amylase production was decreased in all A. oryzae strains by an increase in temperature. The best suitable PH to produce amylase equals to 5 and buffer Glycine was proved to be the best source of amylase production. A.oryzae strains can be used in food industry for a broader production of enzyme, due to the capability of being cultured in starch substrate and its easy recovery from medium.

References

1. Souza PMd. Application of microbial α-amylase in industry-A review. Brazilian Journal of Microbiology. 2010;41(4):850-61.
2. Omemu A, Bankole M, Teniola O. Hydrolysis of raw tuber starches by amylase of Aspergillus niger AM07 isolated from the soil. African journal of Biotechnology. 2005;4(1):19.
3. Deb P, Talukdar SA, Mohsina K, Sarker PK, Sayem SA. Production and partial characterization of extracellular amylase enzyme from Bacillus amyloliquefaciens P-001. SpringerPlus. 2013;2(1):1.
4. Eggleston G ,Montes B, Monge A, Guidry D, editors. Optimization of α-amylase application in raw sugar manufacture. Proceedings of the Sugar Processing Research Conference; 2006.
5. Francis F, Sabu A, Nampoothiri KM, Ramachandran S, Ghosh S, Szakacs G, et al. Use of response surface methodology for optimizing process parameters for the production of α-amylase by Aspergillus oryzae. Biochemical Engineering Journal. 2003;15(2):107-15.
6. Carlsen M, Nielsen J, Villadsen J. Growth and α-amylase production by Aspergillus oryzae during continuous cultivations. Journal of Biotechnology. 1996;45(1):81-93.
7. Carlsen M, Spohr AB, Nielsen J, Villadsen J. Morphology and physiology of an α-amylase producing strain of Aspergillus oryzae during batch cultivations. Biotechnology and Bioengineering. 1996;49(3):266-76.
8. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B. Microbial α-amylases: a biotechnological perspective. Process Biochemistry. 2003;38(11):1599-616.
9. Soni SK, Kaur A, Gupta JK. A solid state fermentation based bacterial α-amylase and fungal glucoamylase system and its suitability for the hydrolysis of wheat starch. Process Biochemistry. 2003;39(2):185-92.
10. Kammoun R, Naili B, Bejar S. Application of a statistical design to the optimization of parameters and culture medium for α-amylase production by Aspergillus oryzae CBS 819.72 grown on gruel (wheat grinding by-product). Bioresource Technology. 2008;99(13):5602-9.
11. Pedersen H, Nielsen J. The influence of nitrogen sources on the α-amylase productivity of Aspergillus oryzae in continuous cultures. Applied microbiology and biotechnology. 2000;53(3):278-81.
12. Millar BC, Jiru X, Moore JE, Earle JA. A simple and sensitive method to extract bacterial, yeast and fungal DNA from blood culture material. Journal of microbiological methods. 2000;42(2):139-47.
13. Dantas G, Sommer MO, Oluwasegun RD, Church GM. Bacteria subsisting on antibiotics. Science. 2008;320(5872):100-3.
14. Sandhya C, Sumantha A, Szakacs G, Pandey A. Comparative evaluation of neutral protease production by Aspergillus oryzae in submerged and solid-state fermentation. Process Biochemistry. 2005;40(8):2689-94.
15. Kundu A, Das S. Production of amylase in liquid culture by a strain of Aspergillus oryzae. Applied microbiology. 1970;19(4):598-603.
16. Gupta A, Gupta V, Modi D, Yadava L. Production and characterization of α-amylase from Aspergillus niger. Biotechnology. 2008;7(3):551-6.
17. Chang M-Y, Juang R-S. Activities, stabilities, and reaction kinetics of three free and chitosan–clay composite immobilized enzymes. Enzyme and microbial technology. 2005;36(1):75-82.
18. Malhotra R, Noorwez S, Satyanarayana T. Production and partial characterization of thermostable and calcium-independent α-amylase of an extreme thermophile Bacillus thermooleovorans NP54. Letters in applied microbiology. 2000;31(5):378-84.
19. Patel AK, Nampoothiri KM, Ramachandran S, Szakacs G, Pandey A. Partial purification and characterization of alpha-

amylase produced by Aspergillus oryzae using spent-brewing grains. Indian Journal of Biotechnology. 2005;4(4):336-41.

20. Djekrif-Dakhmouche S, Gheribi-Aoulmi Z, Meraihi Z, Bennamoun L. Application of a statistical design to the optimization of culture medium for α-amylase production by Aspergillus niger ATCC 16404 grown on orange waste powder. Journal of Food Engineering. 2006;73(2):190-7.
21. Bhella RS, Altosaar I. Purification and some properties of the extracellular α-amylase from Aspergillus awamori. Canadian journal of microbiology. 1985;31(2):149-53.
22. Moravej R, Azin M .Production of amylase by T. longibrachiatum on solid-state fermentation:optimization of culture by Taguchi method.iranian journal of biology.2013;25(4),501-508. .
23. Deljou A, Arzi I. Alpha amylase production by a native thermophilic strain Bacillus licheniformis-AZ2 isolated from Qinarje Hot spring in Ardebil Province. Journal of Agricultural Biotechnology. 2015;7(1):75-92.
24. Saleem A, Ebrahim MK. Production of amylase by fungi isolated from legume seeds collected in Almadinah Almunawwarah, Saudi Arabia. Journal of Taibah University for Science. 2014;8(2):90-7.

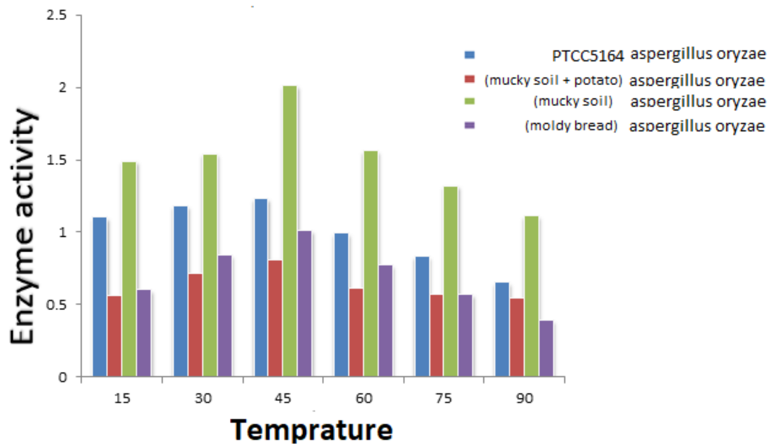

Figure 1) Amylase activity in different isolates of Aspergillus oryzae affected by different temperatures.

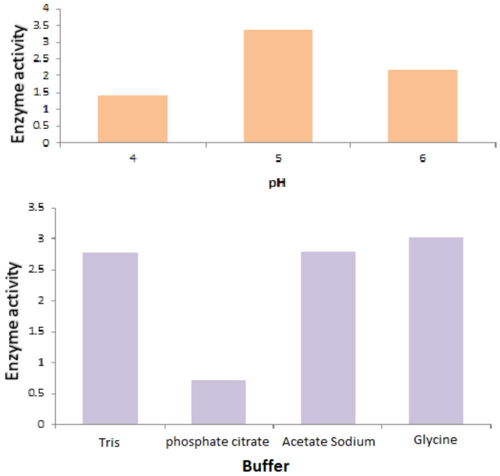

Figure 2) Amylase activity in A. oryzae isolates from mucky soil in different buffers and PH.

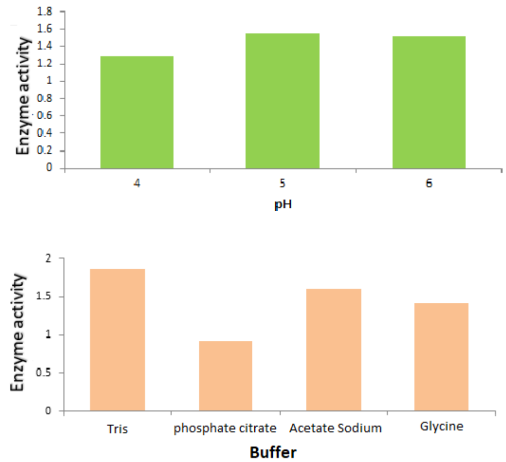

Figure 3) Amylase activity in A. oryzae isolates from moldy bread in different PH and buffers.

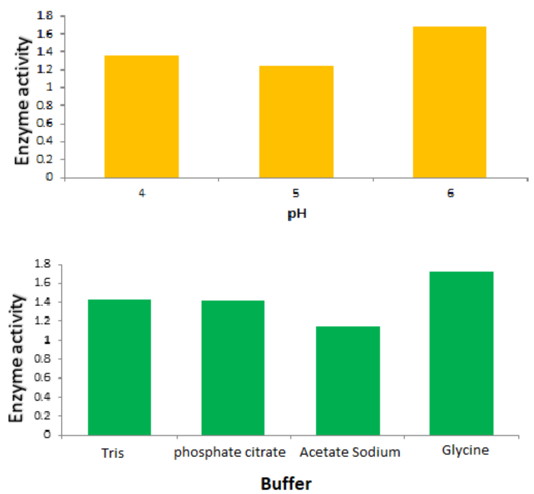

Figure 4) Amylase activity in A. oryzae isolates from mucky soil and potato in different buffers and PH.

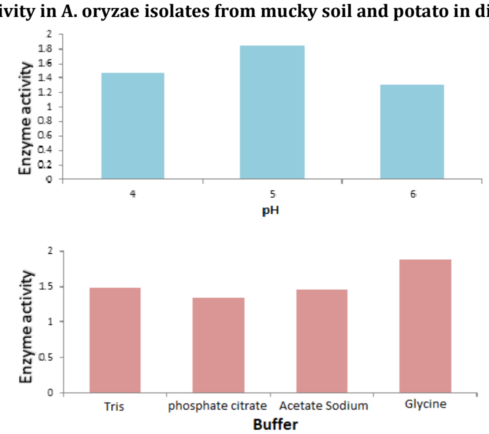

Figure 5) Amylase activity in standard isolates of A. oryzae PTCC 5164 in different buffers and PH.