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Isolation, Optimization and Production of amylase from Microorganisms isolated from kitchen waste contaminated soil samples

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ABSTRACT

Microbes are the most ideal sources of enzymes due to their extensive biochemical diversity. The production of amylase is essential for conversion of starches into oligosaccharides. A large number of amylase has its application in different industrial sectors such as food, textile, paper and detergent industries. Therefore the present study is focused to improve an amylolytic activities of the bacterial and fungal isolates obtained from the soil sample dumped with kitchen waste. In the present investigation, 5 different soil samples were collected and the microbes were isolated and optimized for amylase production. Among the 7 bacterial isolates predominant was *Bacillus subtilis* and among the 2 fungal isolates the leading one was *Aspergillus niger*. The enzyme was produced and was assayed by Bernfeld method using 3, 5-dinitrosalicylic acid. The production of amylase enzyme was optimized with different parameters such as use of various carbon sources, nitrogen sources, pH, temperature and incubation time which enhance the enzyme production. Maximum growth was obtained at 1% concentration of sugars for both bacteria and fungi. The optimum temperature for the bacterial amylase production was 37°C and for fungi 27°C whereas the optimum pH was found to be 7 for bacteria and 6 for fungi. The most favourable incubation time was 24 hrs for bacteria and 96 hrs for fungi. The result showed that the amylase production from *Bacillus* spp was higher than fungi and it can be further used for large scale industrial production.

Keywords: Amylase, Screening, Optimization, *Bacillus subtilis, Aspergillus niger*

1. Introduction

Microorganisms are the most significant sources for enzyme production. To produce enzymes in higher yield which is used for industrial use, isolation and characterization of new proficient strains using cheap carbon and nitrogen source is a continuous process. Microorganisms have become increasingly important as a producer of industrial enzymes (Pandey *et al.,* 2000 & Ashwini *et al.,* 2011). Due to the immense diversity and ease of production of enzymes by microorganisms they are now replacing many conventional techniques. Amylases are starch degrading enzymes and captured approximately 25-30% of enzyme market (Mishra *et al.,* 2014). Degrading amylolytic enzymes has the importance in the industries with huge applications in food, fermentation, textile and paper industries (Pandey *et al.,* 2000 & Ashwini *et al.,* 2011). In addition to the conventional application in food and fermentation industries, microbial enzymes have attained significant role in biotransformation involving organic solvent media, mainly for bioactive compounds. Current developments in biotechnology are yielding new applications for enzymes. The major amylase producing bacteria includes *Bacillus subtilis, B. cereus, B. amyloloquefaciens* and *B. megaterium, P. aeruginosa, E. coli* and fungi includes *A. Niger, Penicillum, Cephalosporium, Neurospora* and *Rhizopus* (Raju and Divakar, 2013).

There are about 3000 enzymes known today and only few are industrially exploited. Many research works has been carried out to produce amylase enzyme using *Bacillus* spp. The microbial source of amylase is preferred to other sources because of its plasticity and vast availability. Microbial amylase has almost surpassed the synthetic sources in different industries. Amylolytic enzymes are widely distributed in bacteria and fungi. They are categorized into exo-acting, endo-acting and debranching enzymes. Unusual bacterial amylases are found in acidophilic, alkalophilic and thermo acidophilic bacteria (Sethi *et al.,* 2013).

The production of microbial amylases from bacteria is depends on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, incubation period, pH, temperature, metal ions and thermostability. In fact, such industrially important microorganisms found within the genus *Bacillus*, can be exploited commercially due to their rapid growth rate leading to short fermentation cycles, capacity to secrete proteins into the

extracellular medium and safe handling. *Bacillus* is endowed to produce thermostable α-amylase and also large quantities of other enzymes (Vijayalakshmi *et al.*, 2012).

Among different types of enzymes obtained from microbial sources, amylases are the one which is used widely in industries, pharmaceutical and biotechnogical aspects. Considering the importance of amylase for above purpose, the present investigation deals with the isolation, optimization and production of amylase from bacteria and fungi isolated from soil samples and screened for the production of amylase. Finally it was optimized with several parameters to enhance the amylase production.

2. Materials and Methods

2.1 Sample collection

Kitchen waste contaminated soil samples were collected from different environments using aseptic polyethylene bags and transferred to labeled screw-capped bottles.

2.2 Isolation of Bacteria and Fungi

Serial dilution was made from 10^{-1} to10^{-7} range. Higher dilutions were plated on nutrient agar and lower dilutions were plated on Potato Dextrose Agar (PDA) by spreading 0.1ml of the diluted sample. Then nutrient agar plates were incubated at 37°C for 24 hours and PDA plates were kept in room temperature for 3-4 days. The isolates were purified and identified by standard procedures for bacteria and fungi.

2.3 Morphological and Biochemical Characteristics:

Bacteria were identified morphologically by Gram staining, Motility and biochemically by Indole production test, Methyl red, Vogues-Proskauer test, Citrate utilization test, Triple sugar iron test, Nitrate reduction test, Catalase, Oxidase, Gelatin liquefaction, Urease, H_2S production, Hydrolysis of casein and starch hydrolysis test.

The fungal isolates were morphologically identified by using Lacto Phenol Cotton Blue wet mount technique.

2.4 Screening of amylase producing bacteria and fungi (Starch Iodine test)

The bacterial and fungal isolates were tested for their amylolytic activity using starch agar media (Sethi *et al.,* 2013). Isolated colonies were picked up from pure culture plate and streaked in straight lines on starch agar plates with starch as the only carbon source. For bacteria, the plates were incubated at 37ºC for 24-48 hrs and for fungi; it was incubated at room

temperature for 3-4 days. After incubation the plates were flooded with Gram's iodine and results were recorded as amylase producers and non amylase producers. The colonies which were showing zone of clearance in starch agar plates were maintained on nutrient agar slants.

2.5 Production of amylase in the production medium (Sethi *et al.*, 2013)

10 ml of production medium ((g/l) Trypticase-10 gm, peptone-5 gm, $(NH_4)_2$ SO_4 3 gm, K_2HPO_4 2 gm, L-cysteine HCl-0.5 gm & $MgSO_4$ 0.2 gm) was taken and sterilized in autoclave at 121°C for 15 min. After cooling, the flask was inoculated with overnight grown bacterial and fungal culture. The inoculated medium was incubated at 37°C in shaker incubator for 24 hrs. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

2.6 Assay of enzyme by Bernfeld method (Bernfeld, 1955)

Amylase activity was measured by the release of glucose from starch by DNS (3, 5-Di Nitro Salicylic acid) reagent using glucose as standard. For determining amylase activity, 1ml of crude enzyme was taken and was added in a mixture of 1ml of standard 1% starch solution. This mixture was vortexes and kept in a water bath at 60°C for 60 minutes. After incubation the stand was removed and reaction was stopped by keeping the reaction tubes in boiling water bath at 100°C for 2 minutes. The mixture was brought to the room temperature and 3ml of DNS reagent was added to it and the mixture was vortexed, capped and kept in a pre-heated water bath at 90°C for 15 minutes. This mixture was cooled to room temperature and absorbance of solution was measured with respect to blank (Glucose solution) at 540nm.

2.7 Optimization for amylase production

2.7.1 Carbon Source

The effect of various carbon sources such as glucose, sucrose, maltose, lactose and fructose were tested at the concentration range of 1 to 5% in production medium were used for bacteria and fungi (Mahendran *et al.*, 2010)

2.7.2 Organic and Inorganic Nitrogen Sources

The amylase production by the selected bacterial and fungal isolates were also optimized by supplementing different organic nitrogen sources (casein, malt extract, peptone, urea, gelatin and yeast extract) and inorganic nitrogen sources (potassium nitrate, ammonium sulphate, sodium nitrate, ammonium nitrate) individually at the concentration of 0.5%. (Shankar *et al.*, 2011).

2.7.3 Effect of pH

The effect of pH for amylase production was determined by culturing the bacteria and fungi in the production media with different pH. The experiment was carried out individually at various pH 5, 6, 7, 8 and 9 (Shankar & Isaiarasu, 2011).

2.7.4 Effect of Temperature

Temperature is an important role for the production of amylase. The effect of temperature on amylase production was studied by incubating the culture media at various temperatures such as 35, 45, 55 and 60°C (Sathees *et al.*, 2011).

2.7.5 Effect of Incubation period

The amylase production by the selected experimental microorganisms was determined by optimizing the production media by bacterial and fungal culture. The experiment was carried out individually at various incubation periods such as 24, 48, 72 and 96 hours for bacteria and 4, 5, 6 and 7 days for fungi (Kanmani *et al.*, 2011).

3. Results & Discussion

3.1 Strain selection

A total of seven bacterial isolates and two fungal isolates were obtained from the soil samples dumped with kitchen waste (Table-1). Among the nine isolates, prevailing amylase producing bacteria (*B. subtilis*) and fungi *(A. niger*) were used for further study. The bacteria and the fungi were identified morphologically and biochemically.

TABLE-1: Isolation of bacteria and fungi from soil samples

S. No	Type of Sample	Type of organisms	Name of the isolates
1	Kitchen waste contaminated soil sample	Bacteria (07)	*Pseudomonas aeruginosa*
			Pseudomonas fluorescens
			Bacillus subtilis
			Bacillus cereus
			E. coli
			Klebsiella pneumoniae
			Proteus spp
		Fungi (02)	*Aspergillus niger*

			Aspergillus flavus

3.2 Screening of amylase production

Among the nine isolates, *B. subtilis and A. niger* was found to be the best amylase producers and were used for further study based on the highest zone of clearance obtained in the screening test carried out in starch supplemented medium (Table -2). The Optimal Density (OD) values were taken for those 2 strains (Table-3). The OD value for *B. subtilis* was noticed as 00.522 and for *A. niger* 00.375. The result was supported by the study carried out by Alkando A Ibrahim, 2011 and constrastly, the study conducted by Adeniran and Abiose, 2009 explained that the amylase production was higher in fungal isolates.

TABLE-2: Screening of amylase producing bacteria and fungi

S. No	Type of Organisms	Name of the isolates	Range of Amylase activity
1	Bacteria	*E. coli*	++
		K. pneumoniae	+
		P. aeruginosa	++
		P. fluorescence	++
		B. subtilis	+++
		B. cereus	++
		Proteus vulgaris	-
2	Fungi	*A. niger*	+++
		A. flavus	++

(- -Negative, + - Weak production, ++- Medium production, +++ - Strong production)

TABLE-3: Assay of amylase production

S. No	Name of the isolates	OD value

1	*B. subtilis*	00.522
2	*A. niger*	00.375

3.3 Effect of carbon sources

Various carbon sources such as glucose, fructose, lactose, maltose and sucrose were used and its OD values were assessed for both bacteria & fungi (Table- 4 & 5). Results showed that the amylase production has found to be higher when glucose was used as carbon source compared to others at 24 hrs of incubation for bacteria and 3 to 4 days incubation at room temperature for fungi. This result was similar to the study conducted by Sethi *et al.*, 2013. They reported that, glucose brought the highest amylase production compared to other sources. Rao and Sathyanarayana, 2003 reported that the different carbon sources have varied influence on the production of extracellular enzymes especially amylase strains. These results are similar to the findings of Heseltine *et al.*, 1996.

TABLE-4: Effect of various carbon sources on amylase production by *B. subtilis*

Sugars	Concentration of Sugars				
	1%	2%	3%	4%	5%
Glucose	03.604	00.761	00.163	00.075	00.055
Fructose	03.145	00.503	00.215	00.076	00.022
Lactose	03.004	00.256	00.173	00.015	00.003
Maltose	03.001	00.095	00.173	00.005	00.002
Sucrose	00.304	00.156	00.027	00.017	00.002

TABLE-5: Effect of various carbon sources on amylase production by *A. niger*

Sugars	Concentration of Sugars

	1%	2%	3%	4%	5%
Glucose	03.472	03.965	00.472	00.341	00.020
Fructose	02.496	00.375	00.273	00.123	00.075
Lactose	02.301	00.305	00.173	00.075	00.023
Maltose	03.004	00.762	00.472	00.321	0.031
Sucrose	00.396	00.253	00.172	00.075	00.025

3.4 Effect of organic & inorganic nitrogen sources

Four different inorganic and six different organic sources were used for both bacteria & fungi and the result was evaluated in table 6. It was found that the better amylase production was reported when ammonium sulphate and peptone has been used for both bacteria & fungi. But the study of Okolo *et al.*, 1996 reported that tryptone was found to be the best nitrogen source for amylase production. In a study conducted by Deb *et al.*, 2013, they demonstrated yeast extract, Casein and beef extract also showed stimulating effects on amylase production. They also added that the enzyme production was increased when ammonium nitrate was used as inorganic nitrogen source in the culture media. According to Coleman and Elliott, 1962, ammonium salts were stimulators of *B. subtilis* amylase production. It has also been reported that, ammonium nitrate and sodium nitrate were the best nitrogen sources for maximum production in a study carried out by Mahmood and Rahman, 2008.

TABLE-6: Effect of Inorganic & Organic nitrogen sources on amylase production

Nitrogen Source					
Inorganic	**OD value**		**Organic**	**OD value**	
	Bactetria	**Fungi**		**Bacteria**	**Fungi**
NH_4NO_3	00.283	00.020	Casein	00.020	00.093
NH_4SO_4	00.546	00.281	Gelatin	00.020	00.302
$NaNo_3$	00.039	00.250	Yeast extract	00.139	00.118
KNO_3	00.076	00.118	Malt extract	00.321	00.345

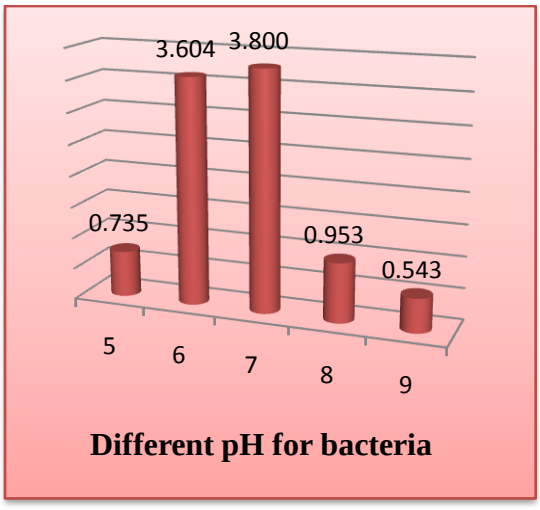
-	-	-	Peptone	01.004	01.118
-	-	-	Urea	00.158	00.027

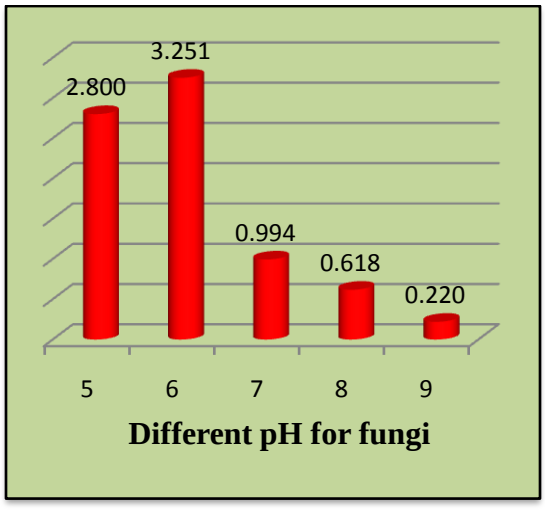
(**NH_4NO_3-** Ammonium nitrate; **NH_4SO_4-** Ammonium sulphate; **$NaNo_3$-** Sodium Nitrate; **KNO_3-** Potassium Nitrate)

3.5 Effect of pH on amylase production

The pH plays an important role in morphological changes of microorganisms and in enzyme secretion than other physical parameters. The bacterial isolates grown in media with different pH ranging from 5-9. Maximum enzyme activity was observed in medium possess pH 7. The fungal isolates were grown in different pH and the maximum amylase enzyme activity observed in media with pH 6 (Fig-1). Demirkan, 2010 in his studies with B. *subtilis* and its mutant derivatives stated that the optimum temperature and pH of both α-amylases were found to be 45^0C and 6.0 respectively. The work carried out by Suganyadevi *et al.*, 2012 in amylase production by *A. niger* revealed that the maximum yield of amylase was in pH-7 and the amylase production was 450 U/mg in *Ipomoea batatas.* The study of Behal *et al.*, 2006 explained that the thermostable amylase producing *Bacillus* sp showed an optimum enzyme activity at pH 8.0 whereas in other species, the optimum activity was at pH 7.0 (Sumrin *et al.*, 2011). Most of the earlier studies supported that the optimum pH for the bacterial growth and enzyme production range from 6 to 9 (Gupta *et al.*, 2003, Kundu *et al.*, 1970, Castro *et al.*, 1992).

Figure-1: Effect of pH for bacteria & fungi on amylase production

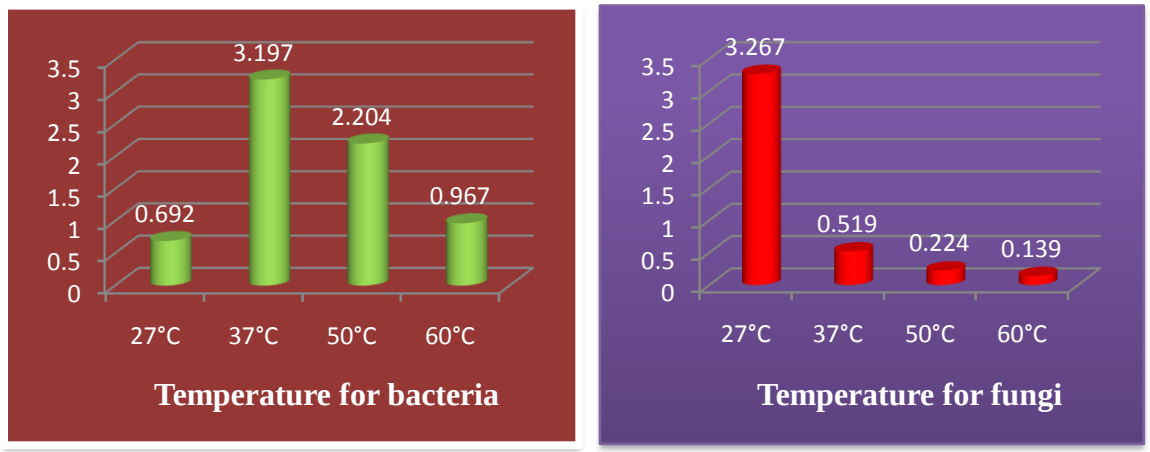




3.6 Effect of temperature on amylase production

Temperature is a vital environmental factor for microbial growth and production of metabolites by microorganisms which is usually varied from one organism to another **(**Banerjee and Bhattacharyya, 1992). The maximum bacterial amylase production obtained in incubation at 37°C and fungal amylase activity was observed in 27°C of incubation (Fig-2). Vidyalakshmi *et al.*, 2009 showed the effect of different incubation temperature on the production of amylase by *Bacillus* spp. They revealed that the maximum production of amylase was obtained at 35°C. The optimum temperature observed for the production of amylase from Banana stalk using *B. subtilis* was also 35°C as reported by Krishna and Chandrasekaran**,** 1996 Increase in incubation temperature, decreased the production of enzyme. The similar results were obtained by Suganyadevi *et al.*, 2012. They reported that the maximum activity of amylase was noted in the enzyme extracts incubated at room temperature (28°C) with pH-7 in both submerged fermentation and solid state fermentation. Mishra and Behera, 2008 studied the optimum temperature for amylase activity as 37°C for bacteria from kitchen wastes.

Figure-2: Effect of temperature for bacteria & fungi on amylase production

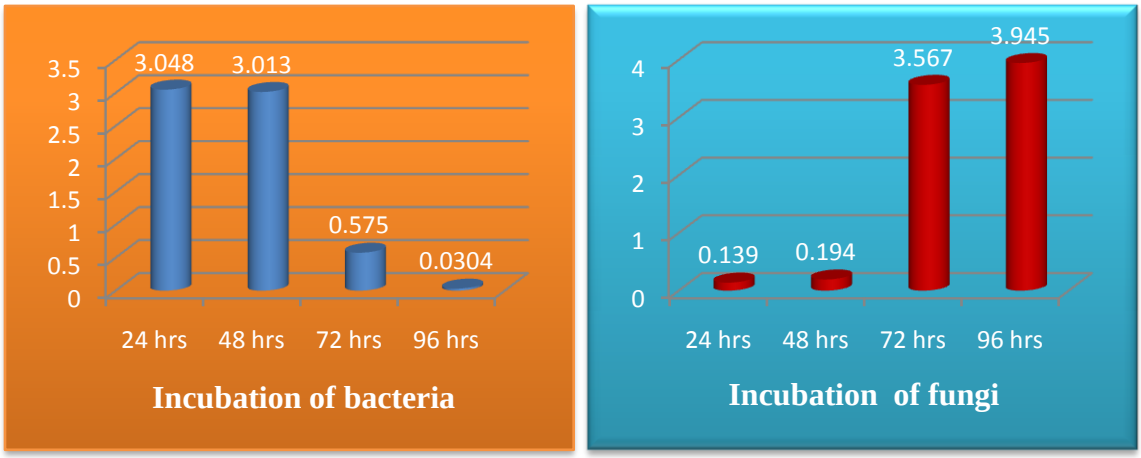


3.7 Effect of various incubation periods on amylase production

The effect of various incubation periods on amylase production reported that the maximum production has attained with 24 hrs incubation in bacteria. The fungal amylase production was achieved at 96 hrs of incubation (Fig-3). But in a study conducted by Vijayalakshmi *et al.*, 2012 the optimum incubation period for α-amylase production was 48 h (22.92 U/ml). They concluded that incubation beyond the optimum time course was generally

accompanied by a decrease in the growth rate and enzyme productivity, which gradually declined to 5.48 U/ml after 96 h of incubation. Vidyalakshmi *et al.*, 2009 concluded that incubation period did not show any significant increase in enzyme production rather it was decreased. Prabakaran and Hewitt, 2009 demonstrated that the optimum incubation period for *B. subtilis* amylase production was found to be 48-72 hrs and reached maximum activity at 48 hrs. Kathiresan and Manivannan, 2006 reported that the maximum activity was detected in 96 hrs (136U/ml) by *Penicillium fellutanum* under submerged fermentation but in case of bacteria it was noted as 24 hrs.

Figure-3: Effect of incubation period for bacteria & fungi on amylase production



Conclusion

Among different types of microbial enzymes obtained, amylases are one of the most widely used in industries, pharmaceutical and biotechnogical aspects and also in the preparation of fermented foods. Apart from those applications, they are also used in various other industries such as paper and pulp, textile, etc., From the present study, it can be concluded that the bacterial isolate produces amylase in higher level and different factors will be useful for large scale production. Among the 9 isolates, amylase production was highly obtained from *B. subtilis* followed by *A. niger*. Due to the importance of these findings, further studies need to be carried out in order to enhance the amylase production in large scale.

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