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Isolation, Characterization and Optimization of Bacteria producing Amylase

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Abstract

The present work comprises the amylase enzyme production by isolated amylase producing microorganism. To isolate amylase producing strain, soil samples were collected from different sources. All isolates were screened for amylolytic activity by starch agar plate method. A total of 20 strains of bacteria were isolated which showed amylolytic activity. Out of these, isolate 2a was selected on the basis of maximum hydrolysis for furtherstudy.2a culture was identified as *Pseudomonas mendocina*on the basis of morphology and biochemical tests. Effects of temperature, pH, different carbon and nitrogen sources, metal salts and different substrate concentrations of the medium using SmF were optimized. The maximum enzyme production was found after 72 h (1.688 U/min) of incubation at temperature 40°C and pH 7. Among various C and N sources,1% glucose and 1 % Tryptone was found to be best for amylase production. It was found that 0.1% of Mg^{2+} increased enzyme production whereas other metal ions exhibited inhibitory effects. The enzyme production was maximum at 5% substrate (starch) concentration which shows inducing effect of substrate. These characteristics of *Pseudomonas mendocina* suggest that this is a promising isolate which merits further investigations for potential applications in various biotechnological processes.

Keywords: Amylase, Starch degradation, DNS method, *Pseudomonas mendocina*, SmF.

Introduction

Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper (Pandey *et al*., 2000). Amylases can be obtained from several sources such as plant, animal and microbes (Kathiresan and Manivannan, 2006). Amylases contribute as a class of industrial enzymes constituting approximately 25% of the enzyme market (Sindhu *et al*., 1997). Among the amylases, β-amylase is exo-acting whereas α-amylase is endo-acting enzyme as well as hydrolytic enzymes (Oseni and Ekperigin, 2013).

Microbial amylases have a broad spectrum of industrial applications as they are more stable with great genetic diversity, high enzymatic activity in a wide range of conditions (extreme pH, temperature, osmolarity, pressure etc.), simple and cost effective production and easy manipulation to obtain enzymes of desired characteristics (Tanyildizi *et al.,* 2005. &Vidyalakshmi *et al.*,2009).

Many microorganism used in α- amylase and β-amylase production including *Bacillus subtilis, B. cereus, B. polmyxa, B. amyloliquefaciens, B. coagulans, Lactobacillus, Escherichia, proteus, B. lincheniformis, B. steriothermophilus B. megaterium, Strepotmyces sp., Pseudomonas sp.* etc.. α- amylase are used in bread and chapatti industry for the improvement of the quality, taste, aroma and porosity

of the bread. It is also used in the textile industries for increasing the stiffness of the finished products and desizing agent for removing starch from the grey cloths before its further processing, sugar and glucose industries alcohol industries for the production of glucose from the starch, paper industry for the hydrolyzing of the raw starch that is used for sizing and coating the paper, detergent, building product and feed industries for improvement of detergency of laundry bleach composition and bleaching without color darkening(Singh and Rani, 2014).

The objective of this work was to isolate and identify a bacterial strain, which can produce amylase with desired characters that can be used in industrial sectors.

Materials and Methods

Sample Collection:

Samples were collected in containers under sterile conditions from soil contaminated with decaying materials i.e. soil receiving kitchen waste, bakery waste, garden soil and compost.

Isolation of Bacteria:

1.0g of the freshly collected sample was mixed with 9.0 ml of sterile distilled water in sterile test tube, serial dilutions were followed. 0.5ml of 10^{-5} dilution was pipetted into a sterile petri dish and overlaid with 20ml of nutrient agar. This was incubated at 37^{o}C for 24 hours. Many colonies were observed and each sub-cultured until a pure isolate was obtained. Pure isolates were maintained on nutrient agar slant and stored at 4^{o}C for further studies

Screening of Amylase Producing Microorganisms:

Spot inoculation of pure cultures was carried out on starch agar plate. Bacterial cultures were incubated at 37^{o}C for 24hrs. After incubation the plates were checked for amylase production by addition of gram's iodine solution. The production of amylase was indicated by the clear zone of starch hydrolysis surrounding the colony.

Identification of Cultures:

The cultures were identified by the morphological and cultural characteristics which include growth pattern on plate.

The Bacterial culture was identified by the Gram's staining and Biochemical kit of gram positive and gram negative. The various tests e.g. Citrate utilization, Lysate utilization, Ornithine utilization, Urease, Phenylalanine deamination, Nitrate Reduction, H_2S production, as well as sugar fermentation of Glucose, Adonitol, Lactose, Arabinose and sorbitol were carried out to identify the culture.

Amylase Production using SmF:

The amylase production was enhanced using starch as inducer. 2% of Bacterial culture (inoculum) was inoculated into the production media containing Starch (1%), Bacteriological peptone (0.6%), $MgSO4.7H_2O$ (0.05 %) and KCl (0.05%). Fermentation flasks were incubated at 37^0 C at 100 rpm. Fermentation broth was harvested after every 24 hrs and enzyme was extracted using filtration. Filtrate was collected and used as amylase source. Amylase activity was carried out using spectrophotometric method.

Determination of Amylase Activity:

Amylase activity was carried out by measuring the amount of sugar released using DNSA method. Amylase activity was determined by incubating a mixture of 1 ml of each enzyme source and 1% soluble starch dissolved in 0.1 M phosphate buffer, at pH 7, at 55° C for 15 min. The reaction was stopped by adding 1 ml of 3, 5 Dinitro salicylic 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. One unit of amylase activity was defined as the amount of enzyme that releasing 1µmole glucose equivalent per minute under the standard assay conditions. Reducing sugar (Glucose or maltose) concentration was determined from a standard curve under same condition using glucose (Senthilkumar *et al.*, 2012).

Calculation of Enzyme Activity: U/ml = µg of glucose/ml of enzyme x incubation time.

Optimization of Amylase Production: (Senthilkumar *et al.*, 2012; Ajayi and Fagade, 2006)

Effect of pH and Temperature:

The effect of varying pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) and temperature (20, 30, 40 and 50°C) on α-amylase production medium was investigated.

Effect of Carbon and Nitrogen Sources

The effect of carbon (glucose, maltose, sucrose and starch) and nitrogen sources (organic nitrogen-peptone, tryptone and inorganic nitrogen-urea and ammonium sulphate) each at 1% concentration was investigated on amylase production.

Effect of Metal Ions

The effect of metal salts on α- amylase production was studied by adding different metal salts like Cu as $CuSO_4$, Mg as $MgSO_4$, Mn as $MnSO_4$, Zn as $ZnSO_4$ and Pb as $PbSO_4$ in the medium at 0.1%concentration.

Effect of Substrate Concentration

Effect of substrate concentration was measured at different concentrations of starch (1.0, 2.0, 3.0, 4.0 and 5.0 %) in the production medium.

Results and Discussion

Isolation and Characterization of Amylase Producing Bacteria:

Different cultures were screened for amylase production on starch agar medium. 20 different bacterial strains were shown amylase production. Similar method has been used by Clark, *et al.*, 1958.Observation of starch hydrolysis zone on starch agar plate by different cultures was shown in fig.1.1. From 20 cultures, 1 best culture 2a was selected based on starch hydrolyzing zone shown in table 1.1.Vaseekaran *et al.* was also used halo diameters for selection of efficient amylase producer.

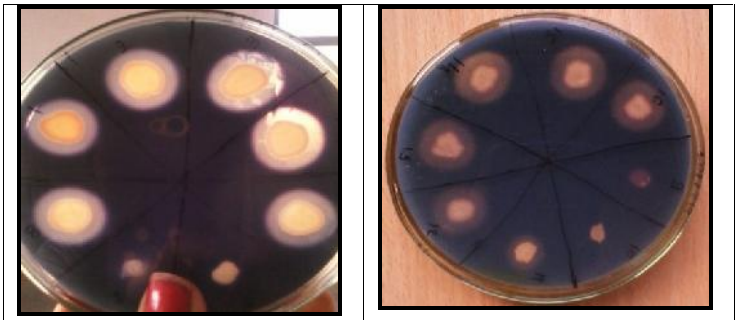

Fig: 1.1: Screening of Amylase producing microorganism on Starch agar plate.

Table 1.1.Starch hydrolyzing zone on Starch Agar Plate:-

Sr. No.	Name of culture	Starch hydrolysis zone in mm	Sr. No.	Name of culture	Starch hydrolysis zone in mm
1	1a	1.5	11	**2a**	**1.9**
2	1b	1.2	12	2b	1.0
3	1c	1.4	13	2d	1.1
4	1d	1.3	14	2f	0.7
5	1e	1.5	15	2g	1.2
6	1f	1.8	16	2h	0.8
7	1h	1.7	17	2i	1.5
8	1j	1.1	18	2j	1.5
9	1k	1.6	19	2k	1.0
10	1l	1.2	20	2m	0.9

Identification of the Amylase producing culture 2a:

Various biochemical tests were conducted on Himedia identification kit for isolate 2a. Results of Biochemical test was shown in table 1.2

Culture 2a was identified as *Pseudomonas mendocina* using morphological characteristics and biochemical characterization

Table 1.2. Observation of Morphological and biochemical characteristics of culture 2a:-

Morphological characteristics	Culture 2a	Biochemical Test	Culture 2a
Size	Medium	**Citrate utilization**	Positive
Shape	Round	**Lysine utilization**	Negative
Margine	Entire	**Ornithine utilization**	Negative
Elevation	Convex	**Urease**	Negative
Surface	Smooth	**Phenylealanine Deamination**	Negative
Cosistency	Moisty	**Nitrate Reaction**	Positive
Opacity	Opaque	**H_2S production**	Negative
Pigmentation	Cream white	**Sugar Fermentation**	
Gram's reaction	Gram negative	**Glucose**	Positive
		Adonitol	Negative
		Lactose	Negative
		Arabinose	Positive
		Sorbitol	Positive

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Optimization of Amylase production using SmF:

Effect of Temperature and pH on amylase production:

Temperature is one of the important physical factors influencing the enzyme production by *Pseudomonas mendocina*. Among the selected four temperature, maximum activity (1.688 U) of amylase was determined at 40^0C after 72 hrs. of incubation, shown in fig.1.2. Increase in incubation temperature, decreased the production of enzyme. The results showed a positive correlation between the growth/enzyme production and the incubation temperature up to 40°C, followed by a gradual decrease and this was also observed during study of Pandey*et al.*, 2000. At higher temperature the bacterial growth gets suppressed and consequently enzyme formation also gets inhibited

Earlier reports on amylase production indicates greater influence by pH. At pH 7, optimum amount (1.303 U) of amylase was produced by *Pseudomonas mendocina* and below and above this pH, the production gets either decreased or denatured shown in fig.1.3. Different organisms have different pH optima, and decrease or increase in pH, on either side of the optimum value results in poor microbial growth. These results suggest that there is a stimulation of enzyme synthesis at neutral pH and that the higher enzyme production at this pH was a result of increased cell growth. Neutral pH was found to be optimal for amylase production as reported in *B. thermooleovorans* NP54 by Malhotra *et al.*, 2000.

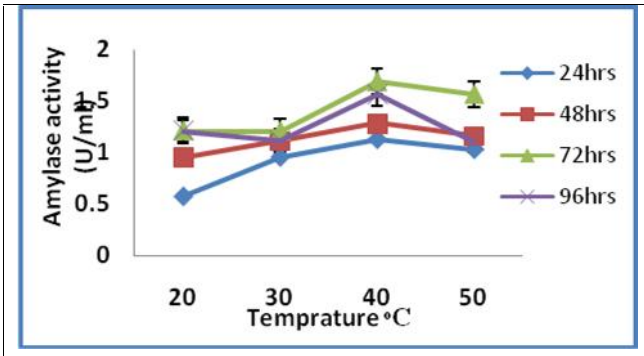

Fig.1.2. Effect of temperature on amylase Production

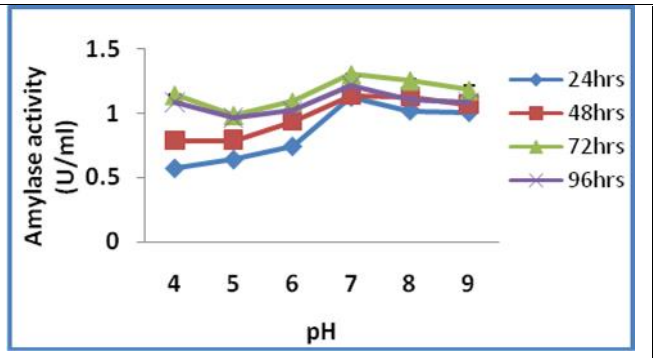

Fig. 1.3. Effect of pH on amylase Production

Effect of Different Carbon Source and Nitrogen Source on Amylase Production:

Among the different Incubation time tested, maximum production of amylase was noted at 72hrs. However the highest production of amylase by *Pseudomonas mendocina* bacterium after 72hrs was found. Among the four carbon sources1% glucose was found to be best and 1.490 U. maximum activity of amylase was determined, shown fig.1.4.

Effect of different organic and inorganic nitrogen sources were tested on amylase production. Among the organic nitrogen sources Tryptone was found to be best and gave 0.882 U/ml/min amylase activities. Among inorganic nitrogen sources ammonium sulphate was found to be best. Amylase activity in Ammonium sulphate was found to be 0.470 U. shown in fig.1.5.

Among the organic nitrogen sources, tryptone proved to be the most suitable as compared to the inorganic N2. It has been found in *Bacillus subtilis* RSKK96 that organic nitrogen sources like tryptone, peptone and yeast extract usually have stimulating effects (Akcan, *et al.*,2011) and our findings are similar to them. Various other organic nitrogen sources have also beenreported to support maximum α-amylase production by various *Bacillus* species (Bozic *et al.*, 2011 and Rasooli *et al.*,2008).

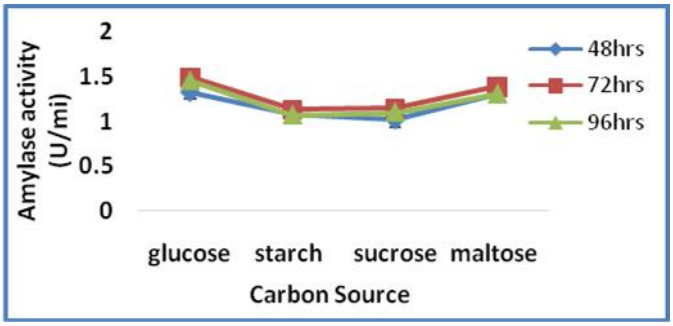

Fig. 1.4. Effect of Carbon Source on Amylase Production

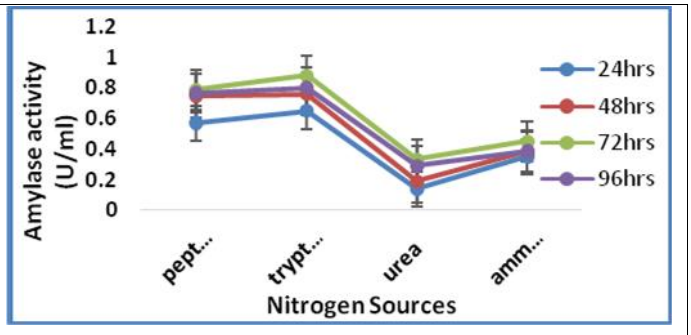

Fig. 1.5.Effect of Nitrogen source on Amylase Production

Effect of Substrate Concentration on Amylase

As starch is primarily considered as substrate for amylases. Concentrations of starch were varied in growth medium from 1 to 5%. 5% starch concentration was best for the amylase production. Even at this concentration *Pseudomonas mendocina* was able to exploit its amylase produce machinery at highest efficiency as indicated by maximum enzyme activity which was determined 1.386 U., shown in fig.1.6. Maximum enzyme activity was found with 2% starch as the substrate when the crude enzyme of *Bacillus subtilis* KC3 was allowed to react with different substrate concentrations.(Vijayalakshmi *et al.*, 2012).

Effect of metal ions on amylase production:

Most of the α-amylases are known to be metallo enzymes. Supplementation of salts of certain metal ions provided good growth of bacteria and thereby better enzyme production.

Different metal ions (Cu, Mn, Mg, Pb and Zn) have been tested for amylase production using *Pseudomonas mendocina.* Maximum activity of 0.995 U. of amylase was determined in presence of Mg ion after 72 hrs. and was shown in fig. 1.7. The production of α-amylase by *B. subtilis* KC3 was increased in the presence of 0.1% $CaCl_2$, which is similar to *Bacillus sp*. TSCVKK and *Bacillus sp.* 64. So Mg^{+2} and Ca^{2+} had significant effects on the metabolism and physiology of bacteria and that was also found to be effective on enzyme activity. (Deshpande and Cheryan, 1984.)

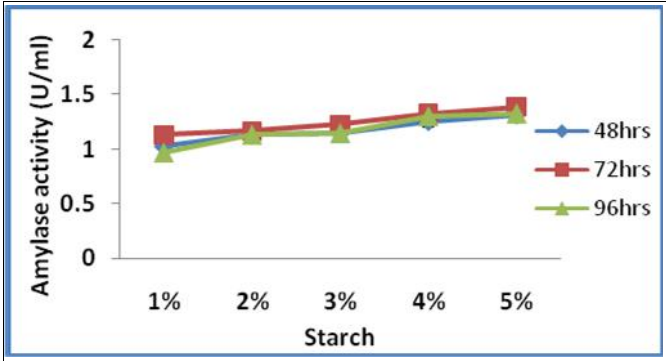

Fig. 1.6. Effect of Starch Conc. on Amylase Production

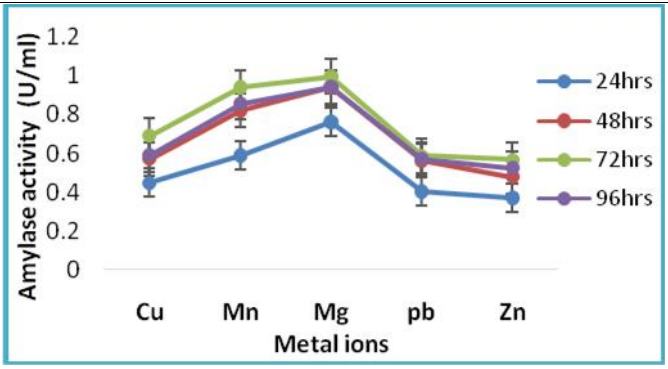

Fig. 1.7. Effect of Metal ions on Amylase Production

Conclusion

Total of 20 bacterial strains which produced clear halos in the starch agar medium were isolated and purified. Among the 20 bacterial strains, one strain was selected as best amylase producer and identified as *Pseudomonas mendocina.* The optimum temperature and pH for the activity of the amylase obtained from this strain were 40°C and 7.0, respectively. Tryptone as an organic nitrogen source and addition of Magnesium sulphate as metal ion resulted in enhance the enzyme activity. Among various carbon sources 1% starch gave maximum production of amylase and starch act as an inducer for amylase production. This study has shown that these Gram negative, rod shaped, bacteria are able to synthesize amylase that is evidenced in the hydrolysis of starch which is very important in biotechnology.

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