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ISOLATION AND CHARACTERIZATION OF AMYLASE PRODUCERS AND OPTIMIZATION OF ENZYME PRODUCTION

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ABSTRACT

The soil sample was screened for isolating amylase producing soil bacteria. The isolates were observed for microscopic and selected biochemical tests for characterization. The results of gram staining reveal that the strains VO1, VO2 and VO3 were gram positive rods. The initial amylase production was confirmed by inoculating the isolates in Berg's broth. The organisms were acclimatized to higher temperatures to evolve thermal stable enzyme by inoculating g the colonies at 40^0, 45^0 and 50^0C.

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INTRODUCTION

Microorganisms are the most important sources of enzyme production. Selection of the right organisms plays a key role in high yield of desirable enzymes for the production of enzymes for industrial use. Soil microorganisms can be classified as bacteria, fungi, actinomycetes, algae and protozoa. Each of these groups has characteristics that define them and their function in soil. Bacteria and Archea are the most abundant microorganisms in the soil. Enzymes are macromolecular biological catalysis. The molecules at the beginning of the process are called substrates and the enzyme converts these into different molecules called products (Berg et al., 2006). All enzymes are proteins but all proteins are not enzymes. Amylase enzyme is one of the most widely used enzymes in the industry. The history of the industrial production of enzymes dates back to the time when Dr. Jhokichi take amino began the production of digestive enzyme preparation by dextrose powder and dextrose crystals from starch using a amylase and glucoamylase began in 1959.

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Amylases are starch degrading enzymes. They are widely distributed in microbial, plant and animal kingdoms. The degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. Initially the term amylase, amylopectin, glycogen and their degradation products (Boyer and Ingle, 1972). Since then, amylases are being used for various purposes. Conversion of starch into sugar, syrups and dextrin forms the major part of starch industries (Marshall, 1975). The hydrolysates are used as carbon sources in fermentation as well as source of sweetness in a range of manufactured food products and beverages. Hydrolysis of starch products containing glucose and maltose in brought about by controlled degradation (Norman, 1982). In hydrolyses starch and used commercially for the production of sugar syrups from starch which consist of glucose, maltose and higher oligosaccharides (Hagihara et al.,2001). Amylases are of great significance in biotechnological applications ranging from food, fermentation, detergent, pharmaceutical, brewing and textile to paper industries (Miller, 1959). To meet the higher demands of these industries, low cost production of amylase is required (Kathiresan and Manivannan, 2006). However, due to efficient production strategies, microorganisms have substantial potential to contribute to a number of industrial applications (Sodhi et al., 2000).

Such industrially important microorganisms are found within the Bacillus species because of their rapid growth rates that lead to short fermentation cycles, their capacity to secrete proteins into extra cellular medium and general handling safety (Pandey et al., 2000). Production of these amylases has been investigated through submerged (SMF) and solid state fermentation (SSF) However, the contents of a synthetic medium are very expensive and uneconomical, so they need to be replaced with more economically available agricultural and industrial by products, as they are considered to be good substrates for SSF to Produce enzyme. Temperature and pH are known to be important parameters in the production of enzymes from bacteria (Guerra et al., 2003). In recent years the technique of solid state fermentation (SSF) process has been developed and used more extensively. It has advantages over SMF like simple technique, low capital investment, cheaper production of enzyme having better physiochemical properties, lower levels of catabolic repression and better product recovery (Baysal et al., 2003). The major factors that affect microbial synthesis of enzymes in a SSF system include selection of a suitable substrate and microorganisms, particle size of the substrate inoculums concentration and moisture level of the substrate. Thus it involves the screening of a number of agro industries materials for microbial growth and product formation (Sodhi et al., 2000). Some amylases, especially alkaline amylases are used in detergents. To some extent amylases are also used as digestive aids (Beazell, 1942). To supplement the diastolic activity of flour and to improve digestibility of some of the animal feed ingredients. Liquefaction is a process of dispersion of insoluble starch granules in aqueous solution followed by partial hydrolysis using thermo stable amylases. In industrial process, the starch suspension for liquefaction is generally in excess of 35 (w/v). Therefore the viscosity is extremely high following gelatinization. Thermo stable a-amylase is used as a thinning agent, which brings about reduction in viscosity and partial hydrolysis of starch. Retro gradation of starch is thus avoided during subsequent cooling. Amylase is used in many production processes in which cheap starch is converted and prepared for further use. An example is the production of citric acid. Biotechnologically produced amylase is used in food industry. Amylase is also used in clothing detergents to dissolve starches, which are long chains saccharoses from fabrics. Amylase plays an important role various purposes. Natural amylase in the mash and the addition of artificial a-amylase can increase fermentation performance.

METHODS

Collection of sample

Soil sample was collected from leaf litter field located in Viswanathapuram area, Madurai. The sample was collected in sterile screw cap bottles and brought to the laboratory. Isolation and primary screening of amylase producing bacteria: The collected soil samples were diluted by serial dilution technique. The diluted samples of 10^{-1} to 10^{-8} were spread with L-shaped glass rod by spread plate technique on the starch agar plates. After incubation at 37^0 C for 24 hrs, the plates were added with a pinch of iodine crystals. The colonies forming clear halo zones of hydrolysis were selected.

Characterization of the isolates

The isolates were subjected to Gram's staining procedure and various biochemical tests viz., indole test, methyl red test, voges-proskauer test, simmons citrate test, catalase test, oxidase test, urease test, nitrate reduction test, gelatin hydrolysis test, starch hydrolysis test and H_2S production test based on the standard protocols described by Cappuccino and Sherman (2005).

Confirmation of amylase production

Initially amylase production was tested using medium Berg's broth supplemented with 1% starch and incubated at 45^0 C. the composition of fermentation medium was 0.05g $MgSo_4$, 0.05g K_2HPO_4, 0.1g $FeSo_4$, 0.25g $CaCl_2$, 0.2g $MnSo_4$ and 1g starch.

Screening of thermo stable bacterial amylases

The isolated strains were further screened for their ability to produce thermo stable amylases, with assay temperatures such as 40^0, 45^0 and 50^0C. The selected strains were re-inoculated into starch solid media and incubated at various temperatures. The colonies showing highest zones of clearance with respective temperature were selected for further optimization process.

Fermentation process

Submerged and solid fermentation process were carried out by following the methodology defined by Varalakshmi et al., (2009) with slight modifications using subudana and rice husk as substrates respectively.

Impact of substrate concentration on amylase production under submerged fermentation

The impact of concentration of the substrate concentration on amylase production under submerged fermentation was carried out in 250ml conical flask containing 0.5g, 1g, 1.5g and 2f of Sabudana. Amylase was extracted and Enzyme assay was carried out periodically from 24 to 6 hours.

Impact of substrate concentration on amylase production under submerged fermentation

The impact of different concentrations of rice husk such us 5g, 7.5g, 10g, 12.5g and 15g was assessed. The fermentation medium was moistened with mineral salt medium and the enzyme assay was carried out after 96hours in order to optimize the best concentration for amylase production.

Enzyme assay

The amylase activity was assayed by measuring the reducing sugar liberated in the reaction mixture by the standard methodology described by Miller (1959). The reaction mixture consisted of 0.5ml of 1% (W/V) soluble starch and 0.5ml appropriately diluted enzyme source in 2ml of 0.1M phosphate buffer (pH 6 to 8). After incubation at (30^0, 40^0,45^0C) temperature for 20 minutes the reaction was stopped by addition of dinitro salicylic acid. Then the tubes were boiled in a water bath for 15min and thus reducing sugar released by enzymatic hydrolysis was determined. One unit of amylase activity was defined as the amount of enzyme that relates one micromole of reducing sugar as glucose per minute under assay conditions and enzyme activity is expressed as the specific activity represented as U/mg.

RESULTS

Isolation of amylase producers

The soil sample was screened for isolating amylase producing soil bacteria. The serially diluted soil sample was inoculated onto starch agar plates and observed for zone of clearance after the addition of iodine solution. Three bacterial strains showing zone of clearance were isolated, designed as VO1, VO2 and VO3 and considered for further analysis.

Characterization and identification of the isolates

The isolates were observed for microscopic and selected biochemical tests for characterization.

Microscopic characterization

The results of gram staining reveal that the strains VO1, VO2 and VO3 were gram positive rods.

Biochemical characterization

The results of the response of the isolates VO1, VO2 and VO3 to the selected biochemical tests are given in Table 1. Based on the above results, the three strains were provisionally identified as *Bacillus licheniformis, Bacillus firmis* and *Paenibacillus glucanolyticus* respectively.

Confirmation of amylase production

The initial amylase production was confirmed by inoculating the isolates in Berg's broth and the results are given in table 2. Thermal stability of bacterial amylase: The organisms were acclimatized to higher temperatures to evolve thermal stable enzyme by inoculating g the colonies at 40^0, 45^0 and 50^0C. Maximum zone of clearance was obtained with the organisms grown at 45^0 C.

Table 1. Biochemical characteristics of the amylase producing bacterial isolated form leaf litter field located in Viswanathapuram, Madurai

S.No.	Biochemical tests	VO1	VO2	VO3
01	Starch hydrolysis	+	+	+
02	Catalase test	+	+	+
03	Indole production	-	-	-
04	Methyl red test	+	+	+
05	Citrate utilization	+	-	-
06	Hydrogen sulphide	+	+	+
07	Motility	+	+	+
08	Voges-Proskauer test	-	-	-
09	Oxidase test	+	+	+

DISCUSSION

Amylases are a group of important enzymes which are mainly employed in the starch processing industries for the hydrolysis of polysaccharides like starch into simple sugars (Akpan et al., 2002). Many successful industrial fermentation processes heavily depend on enzymes from microorganisms. Amylases constitute one of the important groups of enzymes that are used in a wide range of starch industries and account for nearly 25% of the total sale of enzymes. Although there are many microbial sources available for producing amylases, only a few such as *Bacillus subtilis, Bacillus licheniformis* and *Bacillus amyloliquifaciens* are recognized as commercial producers. Amylases have wide variety of applications ranging from coversion of starch to sugar syrups to the production of cyclodextrins for the pharmaceutical industry. Amylases are also used in textile, detergent, paper and distilling industries (Elela et al., 2009). Amylases are used commercially for starch liquefaction, paper, desizing of textile fabrics, in preparing starch coatings of paints, in removing wall paper, food in the vrewing industry, sugar induction by production of sugar syrups from starch which consist of glucose, maltose and higher oligosaccharides, pharmaceutical and in preparing cold water dispersible laundry starches. To meet the demands of these industries low cost medium is required for the production of amylases (Balkan and Figen, 2007). Nowadays the potential of using microorganisms as a biological source of industrially economic enzymes has stimulated interest in the exploitation of extracellular enzymatic activity in several microorganisms. Amylases can be obtained from several sources such as plant, animal and microbes such as bacteria and fungi (Murakami et al., 2008).

The microbial source of amylases is preferred to other sources because of its plasticity and vast availability. Until now all commercial enzymes have been derived from cultivated bacteria or fungi. Bacterial amylases are generally preferred for starch processing. Among bacteria, Bacillus species such as *B. subtilis, B. stearothermophilus, B.macerans, B.megaterium and B. amyloliquefaciens* were the best producers of thermostable a-amylase using submerged fermentation and these have been widely used for commercial production of the enzyme for various applications (Enhasy, 2007). For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous precess. Microorganisms have become increasingly important as producer of industrial enzymes.

Table 2. Production of amylase enzyme in Berg's medium by the selecte3d isolates

S. No	Hours	Enzyme Activity (U/ml)		
		Bacillus licheniformis	Bacillus firmus	Paecibacillus glucanolyticus
01	24	1350	1350	1530
02	48	810	1080	990
03	72	990	1170	1440
04	96	810	810	900

Table 3. Activity of the enzyme produced in Rice Husk by the selected isolates under different incubation conditions

S.No	Temp pH	*Bacillus licheniformis*			*Bacillus firmus*			*Paenibacillus glucanolyticus*		
		30^0 C	40^0 C	45^0 C	30^0 C	40^0 C	45^0 C	30^0 C	40^0 C	45^0 C
01	6	630	1440	1800	810	1620	1620	1260	1620	1800
02	7	1440	1260	1350	1170	2340	2340	360	990	1890
03	8	720	1620	1890	1260	1800	1800	1890	810	2250

Due to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes. Hence in the present study an attempt has been made to isolate amylase producing microbes from soil sample. The attempt has resulted in three bacterial strains such as *Bacillus licheniformis, Bacillus firmis and Paenibacillus glucanolyticus* that are producing amylases. Extracellular enzymes produced by *Bacillus sp.* Are used in many industrial applications (Hiller et al., 1996). The enzymes obtained and isolated form bacteria are the numerous and have vast variety of applications. The organisms like *Bacillus liceniformis, Bacillus amylofaciens* and other species of Halobacillus are the main produces of bacterial enzymes. Also various acidophilic and thermo acidophilic bacteria produce enzymes like amylases. Thermo stable enzymes have increased use in industries as they are able to function even in harsh extreme temperature conditions (Gupta *et al.,* 2003).

Thermostability is a desired characteristic of most of the industyrial enzymes. Use of thermostable amylases from *Bacilli* for industrial application is significant. Thermostable amuylases have been reported from several bacterial strains and have been produced through the use of submerged fermentation as well as solid state fermentation. The temperature properties for enzyme activity and stability make the enzyme quite suitable for biotechnological applications, especially in starch processing, laundry and textile industry (Teodoro and Martins, 2000). Thermostable enzymes isolated form thermophilic organisms have a number of commercial and industrial applications because of their stability. Amylases working at high temperature are important for industrial application. This high temperature help to decreased viscosity of the medium, increase substratesolubility and reduce risk of microbial contamination (Kuchner and Arnold, 1997). The organisms isolated in the present study were acclimatized to survive up to 45^{o} C and their productivity was tested at 40^{o} C. There are two major methods for large scale production of amylase: (a) solid state fermentation (SSF) process and (b) submerged fermentation (SmF) process.

Submerged fermentation utilizes free flowing liquid substrates or broths efficiently to produce desired bioactive compounds. Normally, such bioactive compounds are secreted into the fermentation broth. This fermentation technique is generally best appropriate for bacteria that normally require high moisture content. An additional advantage of this technique is that recovery of products is relatively simple (Subramaniyan and Vimala, 2012). Another best advantage of submerged culture is the technique for sterilization and process control is easier to engineer in these systems (Vidyalakshmi *et al.,* 2009). Both solid state fermentation and Submerged state fermentation process has been carried out in the particular study. The nature and amount to carbon source in culture media is important for the growth and production of extracellular amylase in bacteria. Amylase is produced from many sources of carbon (Including fructose glucose, maltose, galactose, sucrose, lactose, dextrose), industrial waste (like date syrup and molasses), agricultural waste involving sugarcane bagasse and rice husk (Bhutto and Umar, 2011). The use of agricultural wastes makes solid state fermentation (SSF) an attractive alternative method (Ellaiah *et al.,* 2002). Baysal *et al.* (2003) have reported a amylase production in solid-state fermentation with wheat bran and rice husk as substrates. Biosynthesis of amylases was performed on agro-industrial wastes and by-products such as starchy materials to solve pollution problems and obtain a low cost medium (Haq *et al.,* 2005). Rice husk, whgeat bran and potato starchy wastes were used as a low cost carbon substrate for amylase activity by *B. subtils* (Asghar *et al.,* 200). Similarly in the present analysis, agricultural products such as rice husk and sabudana were used as media for fermentaton. Stomford *et al.,* (2001) have optimized various parameters and the growth was found to be maximum at the following conditions with starch as a substrate (1%), pH 7.5. temperature 40^{o} C, 0.3% salt concentration, 1% of nitrogen source and 0.3% of ionic concentration k_2HpO_4, where as enzyme production was maximum at 35^{o}C, 1% starch as a substrate, pH 7.5, 0.5% salt concentration, 1% of nitrogen source and 0.3% of ionic concentration K_2HPO_4.

The growth was found to be minimum when maltose was used as a carbon source, temperature kept at 45^{o}C, pH 9.5 with 2.0% of nitrogen source 0.5% of salt concentration, wherweas the minimum of enzyme production was observed at 40^{o}C, pH 9.5 when fructose used as carbon source. In the present analysis various concentration of sabudana and rice husk were studied for the optimal amylase production. The result revealed the *Paenibacillus glucanolyticus* showed maximum enzyme production (13680 U/ml) with 2g of substrate under submerged fermentation. *Bacillus firmus* was able to the produce maximum yield (9810 U/ml) with 15g of rice husk and the same yield was observed in *Paenibacillus glucanolyticus* with 12.5f of substrate. Various physical and chemical factors have been known to affect the production of amylase such as temperature, pH, and period of incubation. pH is one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. Yasser *et al.*(2012) isolated *B.licheniformis* with optimal temperature and pH of 60-80^{o}C and 6-7.5. Kaur *et al.* (2003) isolated amylase producing bacteria strain from soil samples collected from potato field of north India.

The strain produced maximum enzyme activity at 37^{o}C to 50^{o}C with pH 6-8.the effect of pH on enzyme activity was analysed by varying pH ranging from 6 to 8. *Bacillus licheniformis* was more active in pH 8. The optimum pH *Bacillus firmis* was found to be pH 7 with the maximum yield of 2340 U/ml. both the species were found to be less active at pH 6. The enzyme secreted by *Paenibacillus glucanolyticus* was active at pH 8. Amylase have been isolated for a long time from such organisms as *B. amyloliquefaciens, B.licheniformis* and *B.subtilis.* The enzymes obtained from *B.licheniformis* generally were stable than those from other *bacillus* species (Fossi *et al.,* 2005). In such studies the optimum temperature was recorded at 50-60^{o}C (Asghar *et al.,* 2005;De-Souza and Martins, 2000) the effert of pH on enzyme stability and activity is also dependent on time and temperature. In general, enzymes are less stable at high temperatures over time at pH values near the limit of the optimum. For this reason, the optimum pH should be determined to be under conditions close to those encountered in specific industrial applications. In such cases, it is important to chose an enzyme with as broad a pH optimum as possible. Amylases are generally stable a pH ranges from 4 to 11 (Gimbi and Kitabatake, 2002; Gupta *et al.,* 2003; Omenue *et al.,* 2005). Mishra *et al.* (2014) have optimized medium for amylase producing bacterial strain isolated form potato field of Bhatinda, Punjab, India.

The maximum, enzyme activity was observed at 45°C. singh *et al.* (2010) have revealed that the highest enzyme activity was observed at 45°C. similarly in the present study in the present study *Bacillus licheniformis, Bacillus firmis* and *Paenibacillus glucanolyticus* were more active with the maximum yield of 2340U/ml at 45°C. the enzyme yield was observed to be low at 30°C. Thus the present investigation has resulted in indentifying enzymes which are active at 45°C and will have great application potential. Further studies on acclimatization and optimization may lead to develop strains of survival value and stability of the enzyme at still higher temperatures.

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