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Full Length Research Paper

Solid state fermentation using agro industrial residues for alpha amylase production by an actinomycete isolate from red sea soil

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During the screening process to obtain the most potent α -amylase actinomycete producer from soil in red sea region, *Streptomyces* sp.HA2 was isolated and identified as working strain in solid state fermentation using agro industrial residues. Investigation of four agricultural residues such as banana peel, sugarcane bagasse, wheat bran and rice bran to select the best substrate for production of α -amylase. Selection of banana peel as substrate improved the activity. Various process parameters were optimized for maximum amylase production resulted in incubation period of (96 h), pH (8.5), inoculum size (8%), and moisture content (40%). Also, production of 1500 u/gds was achieved by supplementing the media with 1% starch and 1% peptone on 10g banana peel. The partially purified enzyme by ammonium sulphate was found stable at temperature up to 50°C for 120 minute. By using these optimized cultural conditions, this α -amylase may be utilized in wide spread applications like detergent, leather, and agriculture, scarification, pharmaceutical industry as well as molecular biology techniques.

Keywords: alpha amylase , Solid state fermentation, *Streptomyces* sp, 16S rDNA, agro industrial residues

INTRODUCTION

Amylases are extracellular endo enzymes ranks first in terms of commercial exploitation and considered as one of the most important enzymes used in industry. Such enzymes degrade the starch molecules progressively into smaller polymers composed of glucose units. Spectrum of applications of α -amylase has widened in many sectors such as textiles, food, fermentation, and paper, fine chemical and pharmaceutical industries (de Souza et al., 2010). Amylases have been derived from several sources, including plants, animals, several fungi, yeast, bacteria and actinomycetes. However, due to effective production

management strategies, the amylases of microorganisms have a broad spectrum in industrial applications expanded into many other fields such as clinical, medicinal and analytical chemistry (Pandey et al., 2000). Microbial amylases have successfully replaced chemical hydrolysis of starch in starch processing industries. Among the major technical advantages of using microorganisms for production of amylases is the higher yields obtained with in the shortest fermentation time, economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics. Among

microorganism actinomycetes produces different industrially important enzymes. Various reports on amylase production by actinomycetes such as *Streptomyces clavifer* and *Streptomyces spp.* have been confirmed (Ragunathan and Padmadas 2013). The production of α -amylases has usually processed through liquid-based medium using submerged fermentation (SmF), but in solid state fermentation (SSF), the provided medium is on moist substrate act as water-insoluble solid with or without soluble nutrients (Anaegbu et al., 2017). The cost of enzyme production in submerged fermentation is high, which necessitates reduction in production cost by alternative methods. Compared to submerged fermentation the (SSF) process shows high volumetric productivity with lower capital cost and energy requirements followed by increase concentration of product, very less effluent and water output and very handful fermentation equipment with easier product recovery (Renge et al., 2012). The optimization of fermentation conditions, particularly physical and chemical parameters, is important for maximum product concentration development in the fermentation processes (Francis et al., 2003). The major factors that affect microbial synthesis of enzymes in a SSF system include selection of a suitable substrate and microorganism, particle size of the substrate, inoculum concentration and moisture level of the substrate. Thus it involves the screening of a number of agro-industrial materials for microbial growth and product formation (Sodhi et al., 2005). The most promising residues for SSF include agricultural by products which are generally considered as the best substrates makes SSF an attractive alternative method. Suitable low cost fermentation medium for production of α -amylase using agricultural by-products has been reported. Paddy husk, wheat bran, rice processing waste and other starch containing wastes have gained importance as supports for microbial growth during enzyme production. Also, utilizing coconut oil cake as substrate for production of α -amylase under solid-state was carried out (Ramachandran et al., 2004). Supplementation of the substrates with different *nitrogen* sources and sugars influence on enzyme productivity as it induces enzyme-synthesis, provides balanced growth conditions for mycelial-colonization and biomass formation. Moreover, for maximum production of α -amylase, microorganisms principally required optimum cultural conditions (Ul-Haq et al., 2012). Hence, the present study was aimed to accomplish the objective of α -amylase enzyme production from various agricultural by-products by a potent actinomycete strain using solid state fermentation technique and investigation of its process optimization.

MATERIALS AND METHODS

Screening and isolation of amylolytic actinomycetes strain

Actinomycetes Isolation Agar (AIA) supplemented with glycerol (5ml/l) and nystatin (50 μ g/ml) as anti-fungal antibiotic to avoid fungal contamination was utilized for isolation by serial dilution and pour plate technique. The plates were incubated at 32 \pm 2 $^{\circ}$ C for 7 days. Actinomycetes colonies were picked and purified by streak plate technique on starch agar consisted of following (g/l): starch 20g; beef extract 5g; peptone 3g and agar 24 g adjusted at pH7. Production of amylase was detected by flooding plate with gram's iodine solution. Efficiency of starch hydrolysis was determined on the basis of decolorization of zone diameter against the blue colour background (Vidhale and Pundkar 2016). The isolate showing maximum zone of hydrolysis was maintained on yeast extract-malt extract agar (ISP2) and were preserved in slants at 4 \pm 2 $^{\circ}$ C for further identification (Saravana et al., 2012).

Sequencing of 16S rRNA and analysis of sequence data

The chromosomal DNA of the selected strain was isolated and the 16S rRNA gene was amplified with forward primer 27f (5'-AGAGTTTGATCMTGCCTCAG-3') and reversed 16S rRNA primer 1492r (5'-TACGGYTACCTTGTTACGACTT-3') and water was added up to 100 μ l. The amplified DNA fragment was separated on 1% agarose gel, eluted from the gel and purified using a QIA quick gel extraction kit (Qiagen). The purified PCR product was sequenced with two primers, 518F; 5'-CCA GCA GCC GCG GTA ATA CG-3' and 800R; 5'-TAC CAG GGT ATC TAA TCC-3' using a Terminator Cycle Sequencing kit (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA). The 16S rRNA gene sequence was determined by the dideoxy chain-termination method with the Big-Dye terminator kit using an ABI 310 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequence of strain generated in this work (1200 bases) was aligned with 16S rRNA gene sequences of other members of the genus *Streptomyces*. A sequence similarity search was done using GenBank BLASTN (Singh et al., 2012).

Inoculum and fermentation medium

Agro-industrial wastes such as banana peel, sugarcane bagasse, wheat bran and rice bran were taken for

production of α -amylase without any pretreatment. All agro-industrial waste was collected from local area. Sugarcane bagasse was powdered in a grinder to get 4-5 mm particle size while wheat bran and rice bran were used at mesh size 2-4 mm. In solid state fermentation 10 g of each agro industrial waste was weighed and hydrated with basal salt solution at pH 7 using 1 N HCl or 1 N NaOH containing: $(\text{NH}_4)_2\text{SO}_4$ 2g/l ; KH_2PO_4 1g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5g/l in 250 mL Erlenmeyer flask (Chakraborty et al., 2009), and adjusted with moisture content at 20%. After sterilization in autoclave, flasks were inoculated with one ml of spore suspension (1×10^7 spores/ml) from a 7 days old culture of the selected actinomycete isolate and incubated under static condition at $32 \pm 2^\circ\text{C}$ for 144 h. Triplicate flasks were maintained for each treatment. The contents in the flasks were periodically mixed using a sterilized stirring bar. At interval of 24h, the enzyme was extracted with 10 ml of distilled water. The pooled enzyme extract was centrifuged at 5000 rpm for 20 min in a refrigerated centrifuge and the clear supernatant was used for enzyme assay.

Enzymes Assay

α -amylase activity was measure by spectrophotometric assay. The activity of α - amylase was assayed by 0.5 ml crude enzyme with 0.5 ml soluble starch (1%w/v) in phosphate buffer at pH 7. Reaction mixture was incubated at 37°C for 30min. After incubation, the reaction was stopped by placing the reaction mixture in boiling water bath (Mohapatra et al., 2003). The level of liberated glucose was estimated by dinitrosalicylic acid method (Miller 1959) and expressed as one unit (u) defined as the amount of enzyme which releases $1 \mu\text{mole}$ of reducing end groups of glucose per minute in 0.02M sodium phosphate buffer (pH, 7) with 1% soluble starch as substrate at 37°C . In SSF, unit of enzyme activity is calculated as units per gram of dry solid (u/gds). All experiments were carried out in triplicate and average values were given in presented data. Standard deviations in all the assays were below 5%.

Optimization of fermentation process under SSF

The optimization of medium components and fermentation process for the production of extracellular amylase occurred through varying process conditions like incubation time (24, 48, 72, 96, 120 and 144), initial pH (5, 6, 7, 8, 9 and 10), inoculum size (2–10 %), initial moisture content (10, 20, 30, 40, 50, and 60 %), while nutrient supplementation with carbon sources 1 % (w/v) such as starch, sucrose, glucose, maltose, fructose and lactose, and organic nitrogen sources such as peptone, beef extract, casein and yeast extract supplemented independently with 1 % (w/v).

α - amylase partial purification

The various steps of enzyme purification were carried out at 4°C unless otherwise mentioned. The crude enzyme was treated with solid ammonium sulphate with continuous overnight stirring and separation into the following saturation ranges: 0–20%, 20–40%, 40–60%, and 60–80%. The precipitates collected by centrifugation were dissolved in 0.1 M phosphate buffer, pH 7.0. The enzyme solution was dialyzed against the same buffer for 12 h with several changes to remove the salt and assayed by the method described by Roe (Roe 2001).

Temperature stability of α - amylase

Determination of temperature stability of the partially purified enzyme was studied by incubating the enzyme for 120 minutes at various temperatures ranging from 30°C to 70°C in phosphate buffer at pH 8.5 and suitable aliquot was removed and residual activity was determined.

RESULTS AND DISCUSSION

Isolation and identification of the selected actinomycete

The enzymatic hydrolysis of starch is gradually replacing the traditional acid hydrolysis process for many years in industrial scale (Saxena and Singh 2011). In the present study, screening of α -amylase producing actinomycetes was carried out on starch plate assay method. The most potent amylase producing strain was selected and identified by morphological and colony according to Bergey's Manual of Determinative Bacteriology (Karanja et al., 2010). The observation showed that they are Gram positive and produced aerial mycelium abundantly bearing short and spiral spore chain on aerial mycelium. Mesodiaminopimelic acid (meso-DAP), arabinose and galactose were found to be major constituents of cell wall. The electron micrograph picture of the culture grown on starch agar medium are shown in Figure1.

Molecular identification

Genomic DNA of the selected strain was isolated and 16S rRNA gene was PCR amplified with specific forward and reverse primers. The BLAST search analysis revealed 98 % homology with the genus *Streptomyces* sp. and designated as *Streptomyces* sp. HA2. A neighbor-joining tree based on 16S rRNA gene sequence revealed that the strain belongs to a new species as it occupies a distinct phylogenetic position and different from other strains of *Streptomyces* (Figure. 2). *Streptomyces* are an important source of enzyme and bioactive products. They are also



Figure 1. Electron micrograph (X-1000) of actinomycete selected isolate showed spiral spore chain.

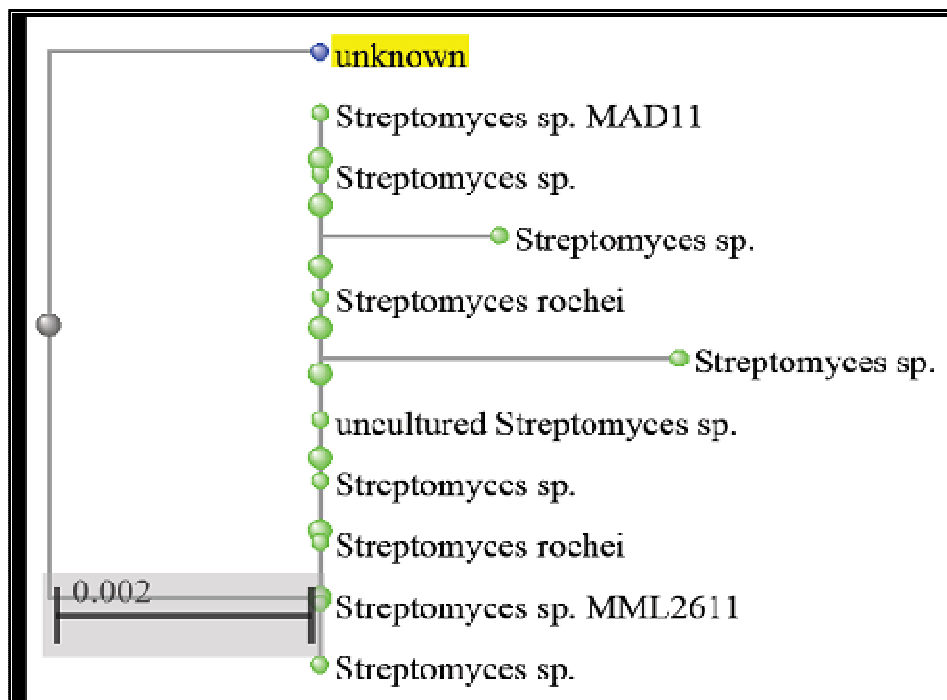


Figure 2. Neighbour-joining phylogenetic tree of the *Streptomyces* sp. HA2 (the unknown) based on 16S rRNA gene sequences showing HA2 strain and the nearest related taxa.

major producers of industrially relevant enzymes (Dharmaraj 2010).

Screening of agro-industrial residues as substrates for SSF

In SSF, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials as substrate for microbial growth and enzyme production (Akcan et al., 2012). Evaluation of different substrates i.e., banana peel, sugarcane bagasse, wheat bran and rice bran for the production of α -amylase was represented in

Figure 4. All the substrates supported enzyme formation by the culture, while banana peel proved superior to the other substrates. The maximum production of enzyme (430 u/g ds) at 96h was observed when banana peel was used as growth substrate. It is because it contains about 31.70 % of total mass from fiber with carbohydrates content of 59 %. Present study on utilization of agro-industrial wastes for the production of α -amylase by the selected actinomycete showed that the production is directly related to the raw starch content in agro-industrial wastes (Singh et al., 2012). Therefore, the order of substrate suitability was banana peel > rice bran > wheat bran > sugarcane bagasse. The selection of a substrate for enzyme

Sequences producing significant alignments:

Select: [All](#) [None](#) Selected 0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces rochei strain KTS-05 16S ribosomal RNA gene, partial sequence	1260	1260	99%	0.0	99%	KT927093.1
Streptomyces sp. MML2611 16S ribosomal RNA gene, partial sequence	1260	1260	99%	0.0	99%	KF734436.1
Streptomyces sp. strain AC384_FSS937 16S ribosomal RNA gene, partial sequence	1258	1258	99%	0.0	99%	KJ928426.1
Streptomyces sp. strain 13-123 16S ribosomal RNA gene, partial sequence	1258	1258	98%	0.0	99%	KJ508025.1
Streptomyces rochei strain OFT MFB 147341/RL24 16S ribosomal RNA gene, partial sequence	1258	1258	98%	0.0	99%	KF241001.1
Streptomyces sp. MAD11 16S ribosomal RNA gene, partial sequence	1258	1258	99%	0.0	99%	GQ249760.1
Streptomyces sp. strain AC383_FSS800 16S ribosomal RNA gene, partial sequence	1254	1254	99%	0.0	99%	KJ928436.1
Streptomyces sp. strain AC380_FSS933 16S ribosomal RNA gene, partial sequence	1254	1254	99%	0.0	99%	KJ928422.1
Streptomyces sp. strain Al-Chabi-97 16S ribosomal RNA gene, partial sequence	1253	1253	98%	0.0	99%	MH201350.1
Uncultured Streptomyces sp. clone ADCLB 16S ribosomal RNA gene, partial sequence	1253	1253	98%	0.0	99%	MH142930.1

Figure 3. Similarity of 16S rRNA gene sequences in the GenBank

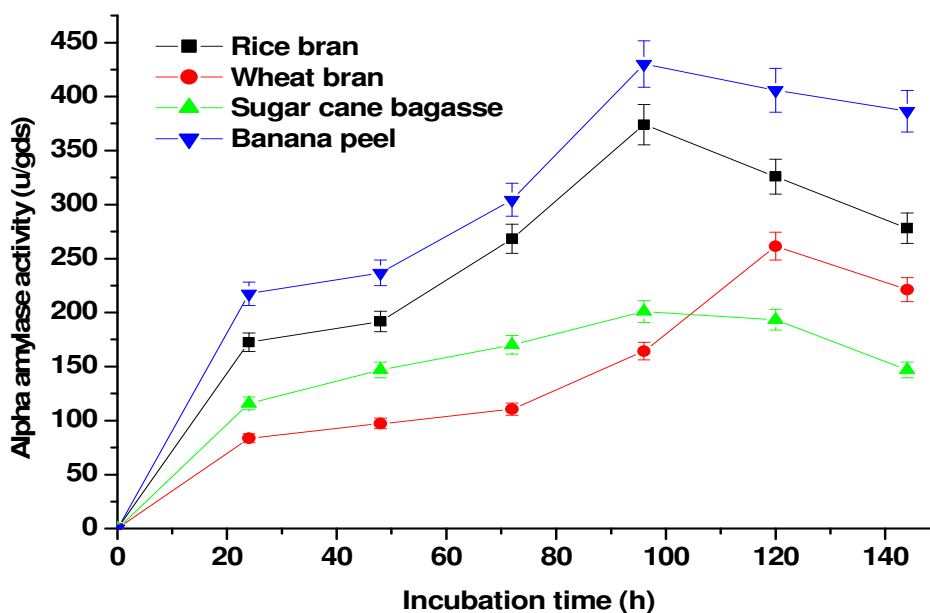


Figure 4. Effect of different agro-industrial substrates on α -amylase production by the selected strain *Streptomyces* sp. HA2. The bars indicate the standard deviation of three analyzed replicates

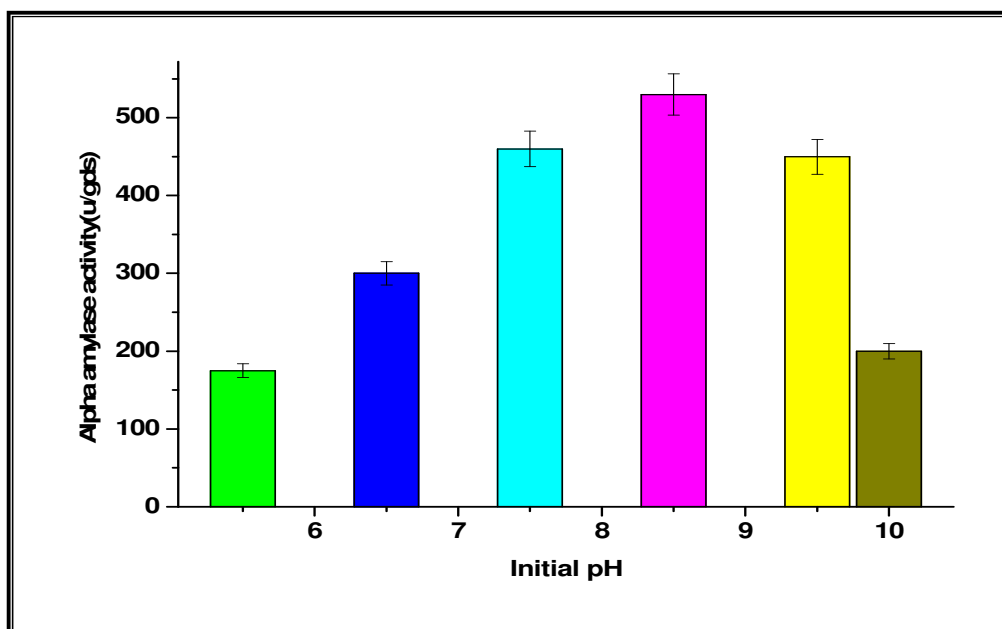


Figure 5. Effect of different initial pH on α -amylase production by the selected strain *Streptomyces* sp.HA2. The bars indicate the standard deviation of three analyzed replicates

production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro-industrial residues (Kapilan 2015). Incubation period varies with enzyme productions. It governed by characteristics of culture and also based on growth rate and enzyme production (Krishnakumar et al., 2015). It was detected that the production of α -amylase by the selected isolate *Streptomyces* sp. HA2 in the fermented substrates began from 24h increased gradually and reached maximum level at 96 h after inoculation. Further increase in incubation period decreased the enzyme production. It might be due to the reason that the isolated strain *Streptomyces* sp. HA2 entered the stationary phase after 96 h as maximum α -amylase accumulation takes place at the end of the logarithmic phase or during early stationary phase. Rapid decrease in α -amylase production after 96 h may be due to the accumulation of toxic byproducts, release of high levels of intracellular proteases activity concomitant with the spore formation. Similar reports on α -amylase were reported on *Streptomyces albidoflavus* and *S. erumpens* MTCC 7317(Singh et al., 2011). Hence, incubation period of 96 h was optimized and banana peel was used as the substrate for the production of α -amylase in the further experimental work. It is evident from this study that the agro-industrial waste can be a good source of amylase if utilized by actinomycetes under solid state fermentation. It is an environmentally friendly method of waste management which can minimized environmental impact (Saxena and Singh 2011).

Effect of initial pH

Among the physicochemical parameters, the pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme secretion. The production of α - amylase is very responsive to initial pH of the fermentation medium describes that pH played a sensitive role in enzyme production and growth of the selected isolate *Streptomyces* sp.HA2. Maximum amylase activity was recorded when initial medium pH was 8.5, which yielded 530 u/gds (Figure. 5). Further increase in pH resulted in decrease of α -amylase production. These data clearly indicate the alkaline nature of the enzyme. These enzymes are used in detergents for laundry and automatic dishwashing to degrade the starch from cloth and porcelain. This growing new area of application of α -amylases optimally working at moderate temperatures and alkaline pH can help to improve the washing performance of modern detergents at low temperatures. Since the working pH range for detergent compatibility lies between 8 and 11, therefore, demand for alkaline α -amylases is growing day by day (Chakraborty et al., 2015). Similarly, earlier reports also found that the activity of α - amylase was maximum at pH near 9.0 in *Aspergillus awamori*. Other researchers reported that pH of 6.0 for *Streptomyces clavifer* and the pH of 7.0 for *Streptomyces* sp. SLBA-08 were optimum for amylase production (Ragunathan and Padmadas 2013). Therefore, in the subsequent experiments, the initial pH of the fermentation medium was adjusted to pH 8.5.

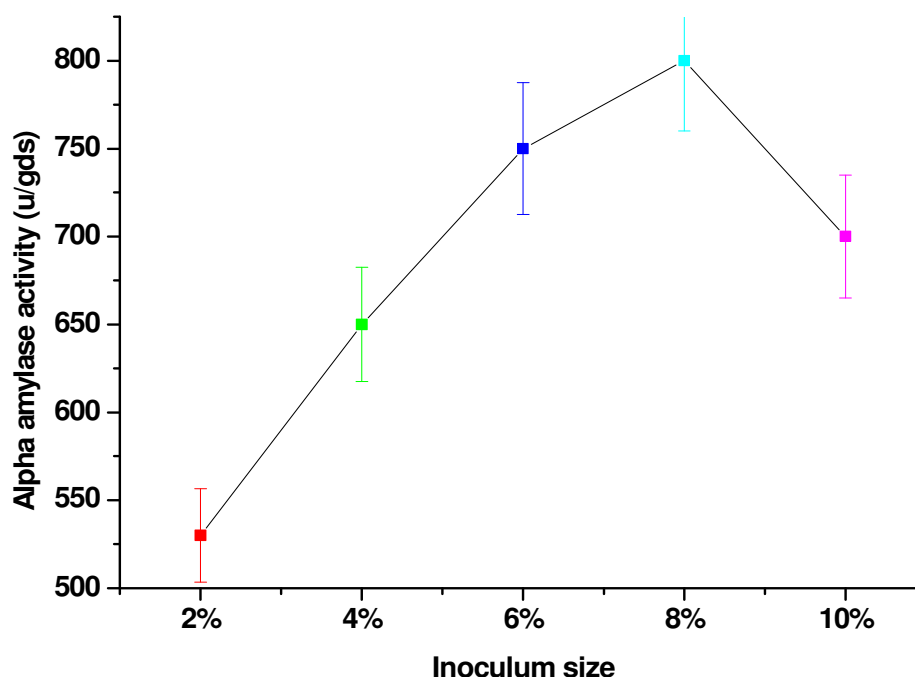


Figure 6. Effect of inoculum size on α -amylase production by the selected strain *Streptomyces* sp.HA2. The bars indicate the standard deviation of three analyzed replicates

Effect of inoculum size

The effect of size of inoculum was also an important factor for the production of α -amylase. The highest enzyme production 800 u/g ds was obtained at an inoculum level of 8% of spore suspension (1×10^7 spores/ml) as shown in Figure. 6. The enzyme production with all the other inoculum levels was rather low. The less enzyme production at lower inoculum level might be because of the less number of viable cells in the production medium require a longer time to grow to an optimum number to utilize the nutrients in substrate for enzyme production. However, less enzyme production at higher inoculum level may be due to limiting nutrient availability for the large number of viable cells, or rapid accumulation of toxic metabolites (Ul-Haq et al., 2012). Similarly, it was reported that inoculums volume per gram of substrate was among the most effective parameters in stimulating amylase production by *Gibberella fujikuroi*. Also, the size of inoculum was recognized as being the most controlling parameters on the production of α -amylase by *A.oryzae*. On the other hand it was demonstrated that the tested range of inoculum did not result in any significant variation in α -amylase production by *Bacillus amyloliquefaciens*. (Mouna and Mahmoud 2015).

Effect of initial moisture content of substrate

The initial moisture content of the substrates is known to have a profound influence on the microbial growth and enzyme production in SSF. The optimal moisture level of substrate for enzyme production was found to be 40 %, with 860 u/gds when compared to 10%, 20% and 30%. With further increase in moisture content up to 40 %, a decrease in alpha amylase activity was detected (Figure.7). These results are in good agreement with those indicated that moisture levels in SSF processes vary between 30 and 85 % (Sivaramakrishnan et al., 2006). At low moisture content, there is high water tension and low solubility of the nutrients which causes the low yield of enzyme. On the other hand, a reduction in enzyme production at high initial moisture content leads to suboptimal enzyme production may be due to changes in the structure of substrate particles, a reduction in substrate porosity and reduction of gas diffusion and volume results in impaired oxygen transfer (Sivaramakrishnan et al., 2006). In subsequent experiments, therefore, 40 % moisture content of substrate was adjusted for the optimal amylase production.

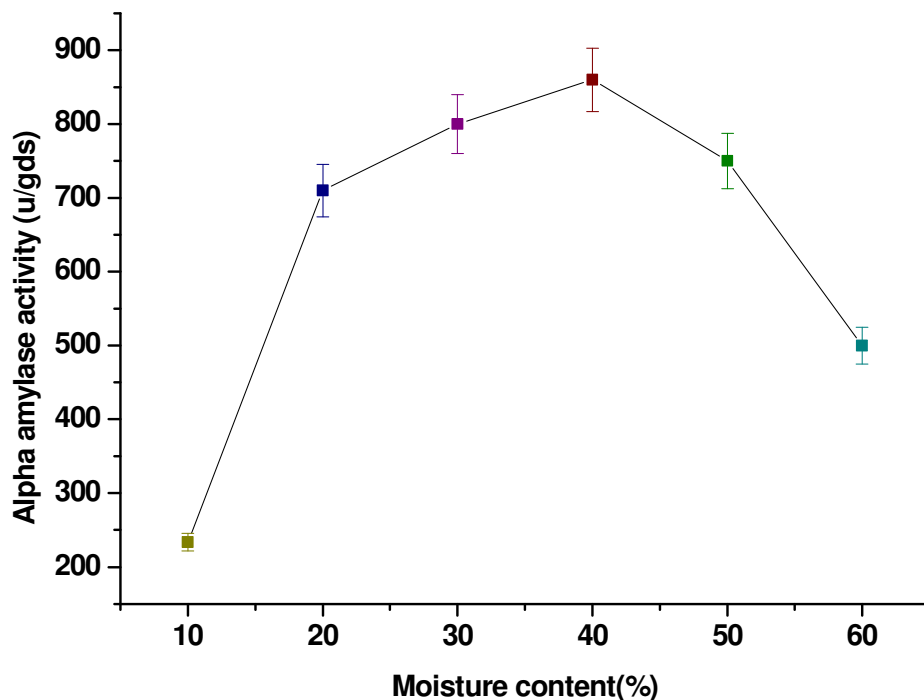


Figure 7. Effect of moisture content on α -amylase production by the selected strain *Streptomyces* sp.HA2. The bars indicate the standard deviation of three analyzed replicates

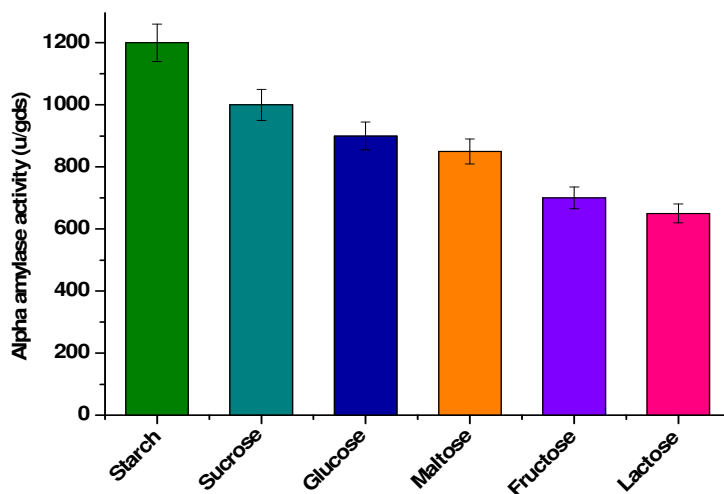


Figure 8. Effect of different carbon sources on α -amylase production by the selected strain *Streptomyces* sp.HA2. The bars indicate the standard deviation of three analyzed replicates

Effect of carbon source supplementation on α -amylase production

The supplementation of banana peel with different carbon sources such as starch, sucrose, glucose, maltose,

fructose and lactose in the production of α -amylase by the selected isolate *Streptomyces* sp.HA2 was investigated in order to obtain a suitable medium. As shown in Figure.8, of the carbon sources tested, starch increased α -amylase production the most (1200 u/gds) followed by sucrose ,

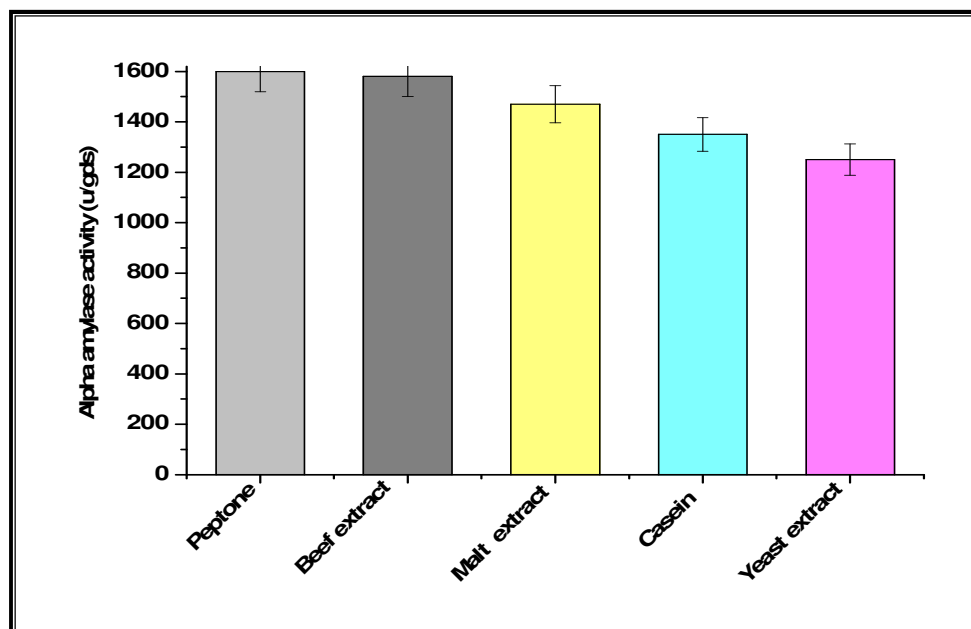


Figure 9. Effect of different nitrogen sources on α -amylase production by the selected strain *Streptomyces* sp.HA2. The bars indicate the standard deviation of three analyzed replicates

glucose, maltose, fructose and lactose which was similar to the findings of Kunamneni *et al.* Starch was also known to increase enzyme production in TA1 strain of *Aspergillus nidulans*. Furthermore, it was observed that starch was the best inducer for α -amylase production in *Bacillus* sp. PS-7, *Bacillus* sp. I-3, *B. subtilis* IMG22 and *Bacillus subtilis* CBTK 106 (Özdemir *et al.*, 2009).

Effect of nitrogen source supplementation

The nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth and the enzyme production. Addition of organic nitrogen sources such as peptone, beef extract, casein, yeast extract and malt extract to the medium showed various effects on enzyme production. As shown in Figure.9, the highest level of α -amylase production by the selected strain *Streptomyces* sp.HA2 was observed in presence of peptone, reached 1500 u/gds which proved to be the best among all the organic nitrogen sources followed by beef extract, malt extract, casein and yeast extract. A similar result was reported by Ramachandran *et al.* who observed that peptone gave an increase in enzyme yield in SSF using coconut oil cake as substrate. Thippeswamy *et al.* reported that the addition of peptone increases amylase production by *Bacillus* sp.

Partial purification of α -amylase by ammonium sulphate precipitation

Several industrial processes are carried out using whole cells as the source of enzymes but the efficiency can be improved using isolated and purified enzymes. Partial purification of amylase from crude enzyme extracts obtained from solid state fermentation of banana peel was achieved by various steps using $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by dialysis was carried out. In the present study, the ammonium sulphate precipitation (60% saturation) followed by dialysis of crude α -amylase yielded 50% of the enzyme with 3-fold purification are represented in Figure.10. It was reported before that α -amylase was recovered from *Thermobifida fusca* NTU22 and *Geobacillus* LH8 strain fermentation cultures with 1.3-fold purification by ammonium sulphate precipitation. Similar to the present study, 34.29% yield of α -amylase produced by *Bacillus amyloliquifaciens* TSWK1-1 with 4.29-fold purification was reported (Singh and Kumar Kapoor 2014). In the present work, a good yield and purification of α -amylase was achieved compared to previous reports.

Stability of α -amylase at different temperatures

Temperatures influences the bioactivity of enzymes therefore, thermal stability of the selected isolate *Streptomyces* sp.HA2 α -amylase between 30°C and 70°C

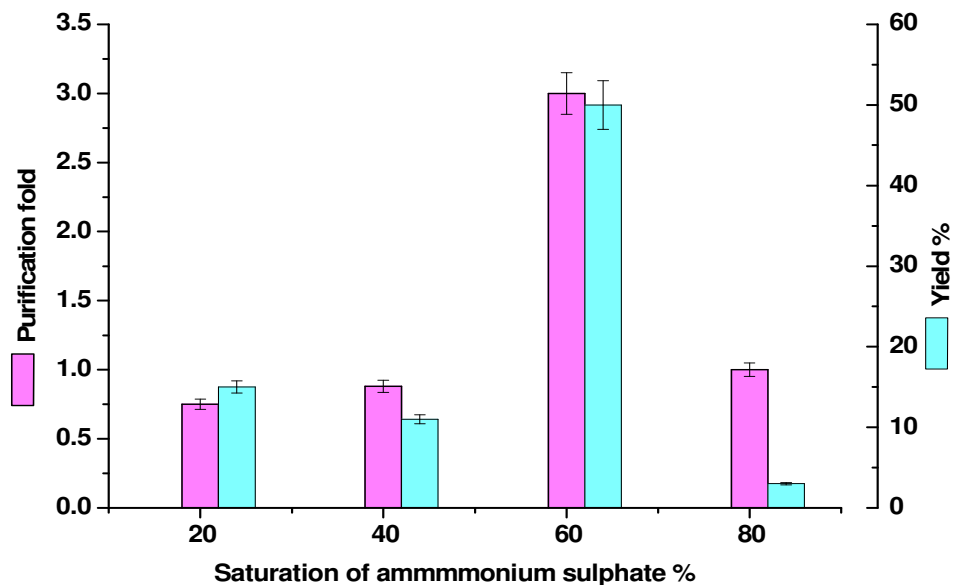


Figure 10. Partial purification of α -amylase by ammonium sulphate precipitation. The bars indicate the standard deviation of three analyzed replicates

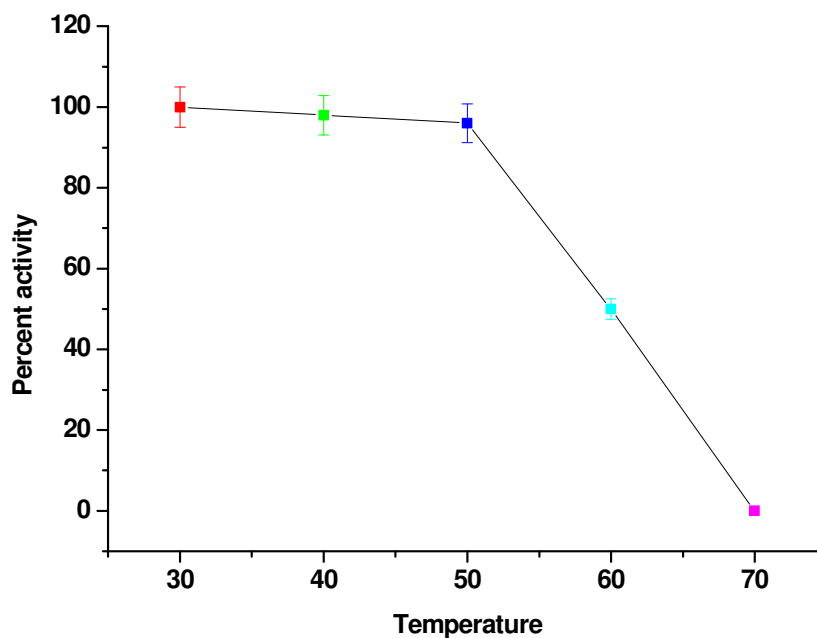


Figure 11. Effect of temperature on the stability of α -amylase.

at pH 8.5 using phosphate buffer was studied and results are presented in Figure.11. The enzyme retained almost its full activity up to 50°C for 120 min. Further increase in temperature, enzyme activity diminished gradually until lost at 70°C.

CONCLUSIONS

Based on the present findings, it is concluded that the soil is a potential source for amylase producing microorganisms, which could be exploited for the

production of important industrial amylase. In the present study, the use of different solid agro industrial materials as substrates like banana peel, sugarcane bagasse, wheat bran and rice bran can be a good source of amylase if utilized by the selected isolate *Streptomyces* sp.HA2 under solid state fermentation, which can minimized environmental impact and serve the purpose of waste management. Among various substrates used, banana peel was found to be the best substrate for the production of α -amylase. Also, the effect of various fermentation parameters such as incubation time, pH, inoculum size, initial moisture content, carbon sources and nitrogen sources for the production of α -amylase by the selected strain were tested. In addition, supplementation with 1 % starch and 1 peptone positively enhanced the enzyme synthesis. However, the present study has to be further improved for a large-scale SSF.

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