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PRODUCTION OF α-AMYLASE FROM *ASPERGILLUS* SP. CMST-04 ISOLATED FROM ESTUARINE SOIL BY SOLID STATE FERMENTATION

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ABSTRACT

Production and partial characterization of α-amylase under solid-state fermentation by *Aspergillus* sp. isolated from estuarine soil has been investigated. Different starch based agricultural wastes were used as substrates (blackgram bran, wheat bran**,** corn waste and rice bran) for the production of extracellular α-amylase**.** Growth on rice bran gave the highest amylase production (255 U/g) and productivity (3.54 U/g/h), corn waste and wheat bran showed low α-amylase activities (126-204 U/g) and productivities close to that obtained with rice bran (2.243 U/g/h). Higher production of α-amylase was obtained at pH 6.0 and the temperature was found to be 30^{o}C. Crude enzyme was purified using ammonium sulfate fractionation and Sephadex G 50 column chromatography. The overall yield of the purified enzyme was 25.32% and the purified enzyme gave single protein band on SDS-polyacrylamide gel electrophoresis (~50 kDa). This enzyme exhibited optimum activity at pH 6.5, performed stability over a broad range of pH 5.5–8.0, and was optimally active at 30-35 ^{o}C. Enzyme activity was enhanced in the presence of calcium whereas in the presence of EDTA gave reverse effect. Thus, the newly isolated *Aspergillus* sp. was found to be a promising strain for higher production of α-amylase.

Key words: *Aspergillus* sp. amylase, SSF, rice bran.

INTRODUCTION

Amylolytic enzymes are the most important industrial enzymes which can be used in a number of industrial processes including brewing, baking, textile and detergent [1,2]. The α-amylases with desirable properties like low pH stability, raw starch digestibility and utilization of high concentration of starch, can be very useful in related applications [3]. They are produced commercially in bulk from microorganisms (α-amylase, EC3.2.1.1 and β-amylases EC 3.2.1.2.) and represent about 25–33% of the world enzyme market. The global market for amylase enzymes is expected to increase by 3.3% annually [3].

Traditionally, α-amylases have been produced by submerged fermentation (SmF). The last few decades, the solid-state fermentation (SSF) processes have been increasingly applied for the production of α-amylases [4]. SSF compared to SmF is simpler, has superior productivity, absence of accurate control of fermentation parameters and requires low cost for downstream processing [5]. In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. Starch based agro-industrial residues are generally considered the best substrates for the SSF of α-amylases [6,7]. Amylase of fungal origin was found to be more stable than the bacterial enzymes on a commercial level. Several attempts have been made to optimize culture conditions and suitable strains of fungi [2,8].

The demand for amylase is increasing day by day, as the starch processing industry require more efficient strain for higher production of α-amylases [1]. Considering the industrial need, an urgent need is required to explore new efficient amylase-producing organisms from extreme sources. Wildly used amylases in industrial applications are produced from terrestrial or fresh water mesophilic and thermophilic fungal sources especially *Aspergillus* sp. Alva et al. [9] reported that *Aspergillus* sp. produces commercially valuable amylases. Salt tolerant estuarine isolates are usually considered to be good sources of novel enzymes having potential values. The production of amylolytic enzymes from the estuarine fungi is scanty. Thus, the present study was aimed to isolate amylase producing strains of *Aspergillus* sp. from estuarine soil sample and optimization of the enzyme production. Further, crude α-amylase was partially characterized.

MATERIALS AND METHODS

Fungal culture

A total of 11 fungal strains isolated from estuarine soil collected from Pantri estuary Rajakkamangalam, Kanyakumari District, Tamil Nadu, were screened for amylase production on starch agar plates containing (g/L): soluble starch 10, peptone 10, beef extract 10, NaCl 5, K_2HPO_4 3, and $MgSO_4$. $7H_2O$ 0.5, $CaCl_2$. $6H_2O$ 0.5, $FeSO_4$. $7H_2O$ 0.1, pH 5.5 at 30 ^{0}C for 48 h. They were identified on the basis of morphological characteristics [8,10]**.** Efficiency of individual isolates amylase activity was determined by the zone of clearance shown on starch–agar plates. *Aspergillus* sp. cmst-04 was selected for further studies. It was maintained at 4 ^{0}C on starch agar slants containing 1% starch (w/w).

SSF AND CULTURE CONDITIONS

The spore suspension of the isolate *Aspergillus* sp. cmst-04 was prepared from a 4 days old culture grown on PDA plates at 30 ± 2^{0}C by adding 10 ml of sterile distilled water containing 0.01% (v/v) Tween 80 and suspending the spores with a sterile loop [11]. The spore suspension containing about 5.67 x 10^8 ±0.77 spores/ml was used as inoculums for SSF study. Agricultural wastes such as wheat bran, corn waste, rice bran and maize waste were used as substrate for α-amylase production. The substrates were ground into coarse powder with a blender. SSF was carried out using all the substrates individually. Starch content of individual substrates was checked by Anthrone method [12]. For production of enzymes in SSF, the fungi were grown at 30ºC in 250 ml Erlenmeyer flasks containing 15 g of the coarsely ground substrate and moistened (80 %) with 25 ml of mineral solution (composed of (g/L); $(NH_4)_2SO_4$ 4, $MgSO_4$.$7H_2O$ 1, $FeSO_4$. $7H_2O$ 0.02, K_2HPO_4 1.4 and KH_2PO_4

0.6, at pH 5.5). The solid media were inoculated with 1 ml of inoculum and the production media were incubated under static conditions at 30 ± 2 ^{o}C for 8 days. Enzyme production was estimated at different incubation periods (2, 4, 6 and 8 days).

Optimization of culture conditions

Optimization of SSF was carried out in 250 ml Erlenmeyer flasks containing 15 g of the coarsely ground substrate (rice bran). Optimization experiment was carried out at different incubation temperatures (20, 25, 30, 35 and 40 ºC) and pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0; adjusted with 0.1 N HCl and 0.1N NaOH). Different carbon sources (2 % (w/w); maltose, glucose, lactose and starch) and nitrogen sources (0.05 % (w/w); yeast extract, peptone and yeast) were supplemented with the production media to determine their effect on amylase production.

Enzyme assays

Enzyme was extracted with 50 ml, 0.1 M phosphate buffer (pH 6.0). Extraction buffer mixed with fermented medium were shaken on a rotary shaker at 250 rpm for 30 min. The mixture was filtered through cheese cloth and centrifuged at 6000 rpm at 4ºC for 15 min and the supernatant was used as the crude enzyme source. α-Amylase activity was assayed by measuring the reducing sugar released with starch as substrate. The reaction mixture consisted of 1 ml of 1% (w/v) soluble starch (Hi-media, India) in 100 mM phosphate buffer, pH 6.0, and 0.1 ml of enzyme solution and incubated at 37 °C for 30 min. The reaction was terminated by adding 0.5 ml of 3,5-dinitrosalicylic acid, then followed by boiling for 10 min and the amount of reducing sugar released was measured [13] using glucose as the standard. One unit of amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar as glucose per min under the assay conditions. Enzyme activity is expressed as specific activity, which is represented as U/g. The protein concentration was determined by the Lowry's method [14] using bovine serum albumin as the standard.

CHARACTERIZATION OF α-AMYLASE

Effect of temperature and pH

To determine thermostability of the crude α-amylase enzyme, the crude enzyme was pre-incubated at various temperatures from 20 to 80°C for 60 min and then rapidly chilled on ice, after which the residual activity was determined under the standard assay conditions. The pH stability profile was determined by suspending crude enzyme in appropriate buffer (pH 3.0 – 5.0, acetate buffer; pH 6.0 -7.0, phosphate buffer; and pH 8.0-9.0, Tris buffer) and pre-incubated for 24 h at room temperature. The activity of the enzyme was determined under the standard assay conditions.

Effect of calcium and EDTA

Calcium and EDTA were added in assay buffer system at concentrations of 5 mM and 10 mM to check their effect on enzyme activity.

Purification and molecular size determination

The supernatant of 500 mL culture was brought to 65 % ammonium sulphate saturation in an ice bath. The precipitated protein was collected by centrifugation at 20000 × g at 4°C and dissolved in phosphate buffer (0.1 M, pH 6.0; two pellet volumes), and dialyzed against the same buffer (overnight at 4°C; cut-off 10 kDa membrane). Concentrated form of the enzyme was loaded onto Sephadex G-50 column (Sigma-Aldrich; packed in glass column 1.5 x 15 cm; flow rate 5 ml/h) pre-equilibrated with 0.2 M phosphate buffer (pH

6.2), and eluted with the same buffer containing 0.2 M NaCl according to the method of El-Safey and Ammar [15]. Active fractions were collected and the relative molecular mass of the native enzyme was measured by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (10% polyacrylamide) which was performed by the procedure of Laemmli [16].

Statistical analysis

All experiments were carried out in triplicates, and repeated three times. The samples collected from each replicate were tested for amylase production and activity. Means of amylase activity and production were calculated and significant differences were calculated by determining standard deviation.

RESULT AND DISCUSSION

Screening of α-amylase producer

Preliminary screening of the α-amylase producer was carried out by starch hydrolysis method. Among the eleven fungal isolates, *Aspergillus* sp. cmst-04 has showed higher starch hydrolytic zone of about 2.47 ±0.25 cm and the hydrolyzing index was 1.18. Other isolates has low hydrolytic zone with hydrolytic index (Table-1). The use of starch agar and iodine for screening amylase producing microorganisms has been reported by Forgarty and Kelly [17] and Iverson and Millis [18]. Alva et al. [9] primarily screened efficient amylase producing *Aspergillus* sp. by the similar starch clearance test.

Table-1: Primary screening of amylase producing fungal isolates by starch hydrolysis method. Fungal spore inoculated plates were incubated at 30 ^{o}C for 72 hours.

Isolate No	Colony Size (cm)	Zone of clearance (cm)	Index*
cmst-01	1.67 ± 0.06	1.73 ± 0.06	1.04
cmst-02	2.03 ± 0.12	2.2 ± 0.1	1.08
cmst-03	1.7 ± 0.2	1.7 ± 0.2	1.0
cmst-04	2.1 ± 0.15	2.47 ±0.25	1.18
cmst-05	2.17 ± 0.15	ND	ND
cmst-06	2.23 ± 0.12	2.3 ± 0.17	1.03
cmst-07	2.6 ± 0.26	ND	ND
cmst-08	1.63 ± 0.3	1.73 ± 0.4	1.06
cmst-09	2.03 ± 0.15	2.1 ± 0.17	1.03
cmst-10	1.6 ± 0.1	1.63 ± 0.12	1.02
cmst-11	1.63 ±0 .15	1.77 ± 0.15	1.09

* Index was calculated from the mean values; Zone of clearance (cm) / colony size (cm).
ND = not determined.

α-Amylase production using solid substrates

Wheat bran, rice bran, blackgram bran and corn waste are the byproducts of traditional agriculture in India and is used for preparing a variety of animal and aqua feeds, and also traditionally used as the substrate for SSF of variety of industrial enzymes [19]. Mulimani et al. [20] reported rice components and wheat barn as solid substrate for α-amylase production. In this study, different agricultural by products were used as substrates for α-amylase production by SSF, rice bran (255 ± 7.0 U/g) has showed maximum enzyme production than wheat bran (204 ± 6.0 U/g) and coan waste (156 ± 7.0 U/g) (Figure 1). Chemical analyses of the substrates have showed that rice bran has higher starch content (18.2 % (w/w) dry matter) than the other agricultural byproducts used (Table-2). Substrate which contains high concentration of starch gives maximum enzyme production. Cereal waste like rice bran contains 17 % of starch [21] and wheat bran contains about 15-30 % dry matter of starch [22]. Anto et al. [23] suggested that rice bran was the suitable substrate for fungal growth and production of glucoamylase. Ellaiah et al. [24] reported that wheat bran gave higher enzyme yield compared to rice

bran and other agro-wastes. Anto et al. [23] reported that wheat bran have higher starch and nutritive values than rice bran, but, Arasaratnam et al. [6] reported that rice bran and husk support the growth and mycelial development of fungi. The results of the present study clearly indicated that, rice bran has showed the potential of replacing food grade starch in amylase production.

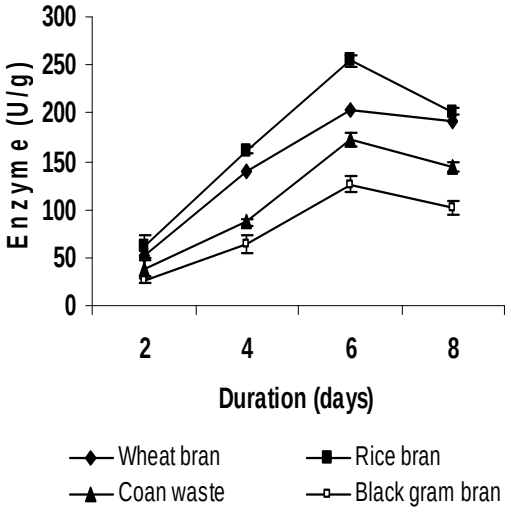

Figure – 1: Substrate selection for optimum a-amylase production by *Aspergillus* sp. cmst-04. Solid substrate media prepared with different raw materials (w/v, dry weight basis, substrate: basal salt solution, 15:10) and incubated at 30 ^{o}C.

Table-2: Starch content of agricultural byproducts used for amylase production.

Substrate	% of starch (dry matter)
Rice bran	18.2
Wheat bran	14.9
Black gram husk	2.8
Corn waste	12.7

Effect of temperature and pH on α-amylase production

Optimum temperature for maximum enzyme production was studied by the spore inoculated production media incubated at different temperature. It was observed that amylase production was high at 30 °C, followed by 25 and 35 °C of growth temperature. While highest amylase production at 30 °C occurred after 6 days of incubation, highest production at 35 °C was observed after 6 days. Maximum enzyme production could be obtained only after a certain incubation time which allows the culture to grow at a steady state. Increasing incubation temperature, decrease the growth and amylase production (Figure 2a). Similar observation was reported by Mukherjee and Majomdar [25] and Alva et al. [9]. Suganthi et al. [8] revealed that 45 ^{o}C was suitable for higher amylase production of *A. niger* in rice bran medium.

Growth and physiological activities of the fungi along with enzyme production is regulated by extracellular pH [3]. Different organisms have different optimum pH for growth and enzyme production [26]. In this study, maximum amylase production was observed at pH 5.0 on 6th day of incubation (Figure 2b) (289.67 ± 6.03 U/g). This result suggests that the enzyme production would be regulated by the extracellular media pH. Similar result was reported by Nahan and Waldermarin [27] and Chimata et al. [26]. But Varalakshmi et al. [28] reported the maximum enzyme activity at pH 9.5. Few reports suggested that different organisms are having various pH optima for amylase production [1, 2, 29].

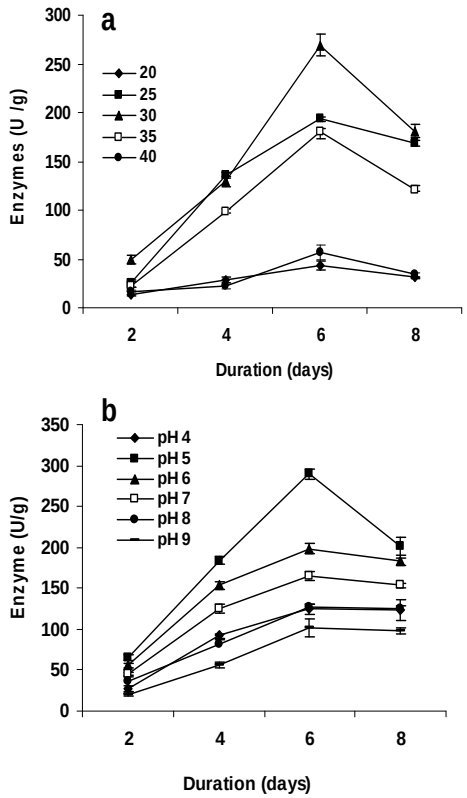

Figure – 2: Optimization of media for a-amylase production by *Aspergillus* sp. cmst-04. a) Different initial media pH on enzyme production (rice bran media (w/v) dry weight basis, substrate: basal salt solution 15:10, and incubated at 30 ^{o}C), b) effect of incubation temperature on a-amylase production (rice bran media (w/v) dry weight basis, substrate: basal salt solution 15:10, and adjusted the media pH 6.0).

Effect of additional carbon and nitrogen source on amylase production

The effect of supplementation of additional carbon (1% w/w) sources like glucose, starch, maltose and lactose on the enzyme production by SSF was tested. Maximum enzyme production was exhibited by starch supplementation (294.67 ± 7.63 U/g) followed by maltose (256.33 ± 5.51 U/g) and glucose (255 ± 4.58 U/g) on 6th day of incubation (Figure 3a). But lactose has shown low amylase production (Figure 3a). Many researchers have studied the effect of additional carbon supplementation on the substrates [23, 24]. Starch source and variation in molecular structure of starch greatly influence amylase induction in microorganisms [30]. Peixoto et al. [31] reported that, starch and its hydrolytic products induce amylase production compared to other carbon sources.

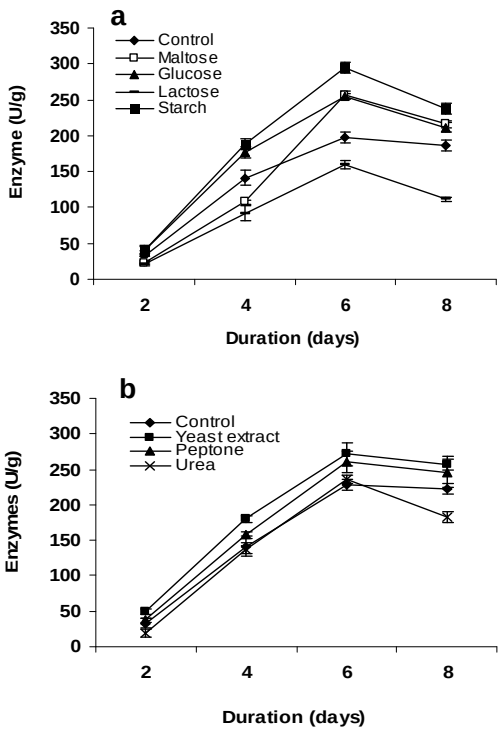

Figure -3: Optimization of media for a-amylase production by *Aspergillus* sp. cmst-04, media pH was maintained at 6.0 and incubated at 30 ^{o}C. a) Rice bran media additionally supplemented with different carbon sources (1 %, w/v on dry weight basis), b) Rice bran media additionally supplemented with different nitrogen sources (0.5 %, w/v on dry weight basis).

The effect of additional nitrogen sources like peptone, yeast extract and urea at 0.05 % (w/w) supplementation on the medium was tested. Among all the nitrogen sources tested, the maximum enzyme production was obtained with yeast extract (271 ± 15.82 U/g at 0.05 % w/w) (Figure 3b) followed by peptone (260 ± 15.0 U/g) and urea (235.67 ± 5.13 U/g). Peptone and urea also showed a

considerable amount of amylase production than the control. Frolova et al. [32], Hernandez et al. [29] and Anto et al. [23] reported maximum enzyme production on supplementation of yeast extract to the medium for fungal culture. Ellaiah et al. [24] obtained maximum enzyme production on supplementation with urea. Shaista et al. [33] reported maximum enzyme activity upon supplementation with 0.2 % peptone as a nitrogen source for *Bacillus* sp. The optimized media supplemented with 1 % (w/w) starch and 0.5 % (w/w) yeast extract with pH 5.0 and temperature 30 ^{o}C showed optimum enzyme production (323.33 ± 22.54 U/g) than the control medium (228 ± 7.55 U/g). That is 1.42 fold higher than control (Figure - 4).

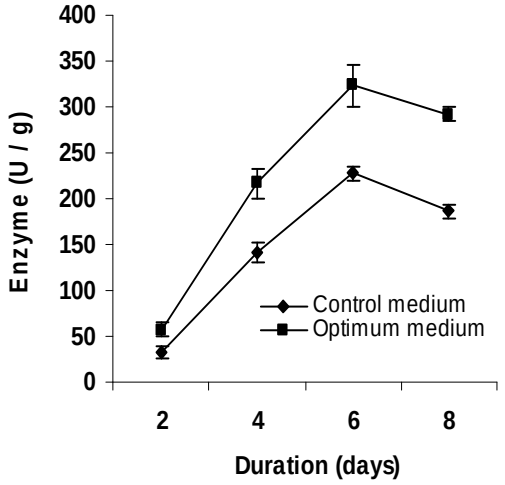

Figure - 4: Comparison of a-amylase production by using optimized media (rice bran media (w/v) supplemented with 1 % starch and 0.5 % yeast extract and maintained the moisture content of 80 %).

Crude enzyme characterization

Preliminary attempt has been made to characterize the α-amylase produced from the fungi *Aspergillus* sp. cmst-04 for its enzymatic activity at different pH, temperature and metal ions. The amylase exhibited a broad pH stability range, from pH 4 to pH 9 (Figure 5a). The crude enzyme has retained maximum activity at pH 6.0 (90.3 %). Most fungal amylases are unstable at low pH [34]. In this study newly isolated *Aspergillus* sp. cmst-04 produced amylase enzyme has shown higher activity at pH 6.0 (Figure 5a). Similar activity was also reported for *Scytalidium thermophilum* amylase enzyme [35]. The strain cmst-04 produced amylase has showed a high catalytic activity at 50°C when the enzymatic reaction was carried out at different temperatures, showing that the enzyme is low thermo active (Figure 5b). Aquino et al. [35] resulted *S. thermophilum* enzyme has optimum activity at temperature 60 ^{o}C. Sidkey et al. [33] reported that *A. falvus* α-amylase showed higher activity at 30 ^{o}C and pH 6.4.

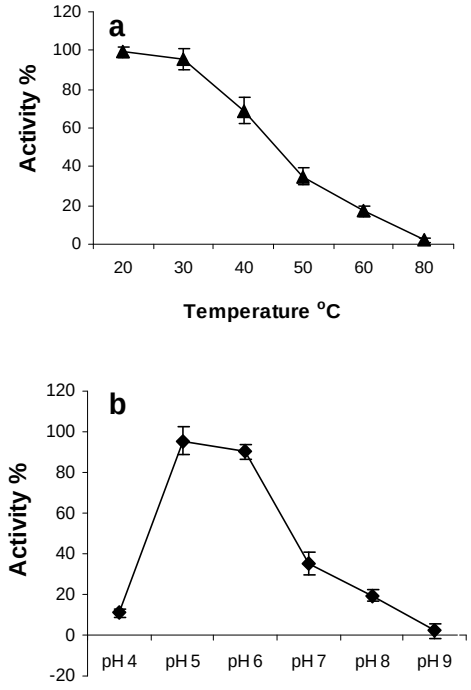

Figure -5: Characterization of the crude enzyme. a) Effect of pH on enzyme activity. For determination of optimum assay pH of the enzyme reaction, acetate buffer - pH 3 and 4;

sodium phosphate buffer - pH 5, 6 and 7; Tris buffer - 8 and 9 (100 mM) were used. The enzyme activity was measured and the results are presented on graph. b) Effect of temperature on enzyme activity. To study the effect of temperature on enzyme reaction activity and results are presented on graph. Bars represent means ± standard deviations for three replicates.

An increase in enzyme activity was seen in the presence of calcium ions (121.26 % at 5 mM and 118.16 % at 10 mM) and reversely of EDTA, which highly affected the normal enzyme activity (63.47 % at 5 mM and 19.03 % at 10 mM) (Figure 6). But, Asgher *et al.* [36] reported that Ca^{2+} had no effect on enzyme activity. Babu and Satyanarayana [37] revealed that 10 mM calcium inhibits the enzyme activity but EDTA has no effect. In the present study, results indicate that the α-amylase of *Aspergillus* sp. was stable in the presence of Ca^{2+} but not in EDTA.

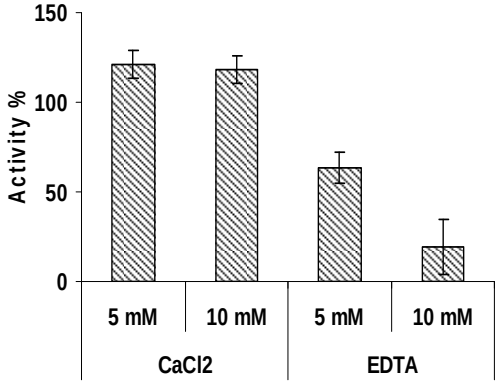

Figure -6: Effect of $CaCl_2$ and EDTA on enzyme activity. The enzyme assay was performed after pre-incubation of the enzyme with various concentrations of the salts. The results are presented on graph. Bars represent means ± standard deviations for three replicates.

The enzyme was partially purified by ammonium sulphate precipitation (65 % saturation level). The purity of the enzyme was 4.94 fold greater than the crude preparation. Sephadex G 50 purification has showed higher purity (25.32 fold) than the ammonium sulphate precipitation but the yield was reduced to 27.52% (Table -3). The partially purified enzyme preparation was subjected to SDS-PAGE for molecular weight determination (Figure 4). The enzyme was run on the protein gel and the molecular weight was determined (~50 kDa). Chakraborty et al. [38] noticed the purified α-amylase of *Bacillus* sp. as a monomeric protein with molecular mass of 64 kDa. Sidkey et al. [39] reported fungi *A. flavus* produces 56 kDa α-amylases. Varalakshmi *et al.* [28] reported that *A. niger* JG124 produces ~ 43 kDa α-amylase enzyme.

Table-3: Purification of α- amylase and its activity

	Total activity (U / mL)	Total Protein (mg / mL)	Specific Activity	Yield	Fold
Crude enzyme	356	69	5.16	100	1
Ammonium sulphate (65 % saturation level) precipitated	313	12.3	25.48	87.92	4.94
Sephadex G 50 purified	98	0.75	130.67	27.52	25.32

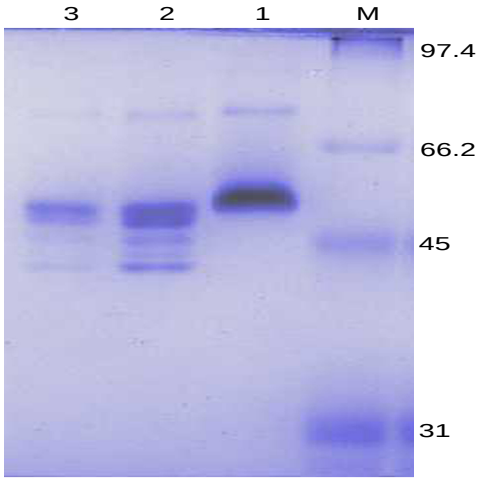

Figure -7: SDS-PAGE shows different steps of α-amylase purification. (1) Enzyme after Sephadex G-50 chromatography purification, (2) Crude

enzyme, (3) Enzyme after salting out, and (4) Protein marker (Bio-Rad Low range), stained with Coomassie brilliant blue R250.

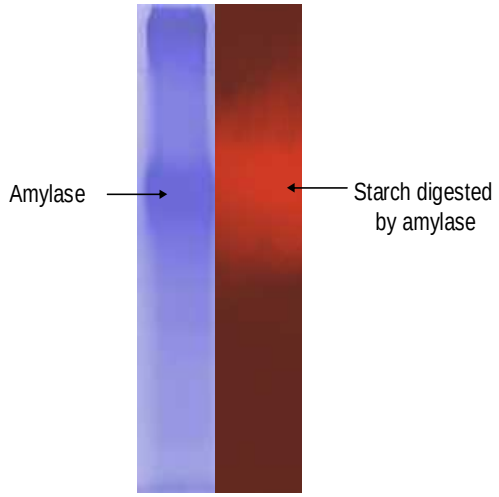

Figure -8: Zymogram analysis of α-amylase produced by *Aspergillus* sp. cmst-04.

CONCLUSION

The results obtained in the present study indicated that *Aspergillus* sp. cmst 04 could be a potential strain for α-amylase production by SSF with rice bran as the substrate. The enzyme production was highly influenced by pH and temperature. Among the various parameters of SSF pH, temperature, supplementation of carbon and nitrogen sources played an important role for α-amylase production. Further, optimized media showed improved production of α-amylases to a maximum of 323 ± 22.54 U/g. It was concluded that the enzymes produced from *Aspergillus* sp. cmst-04 have low temperature stability and are sensitive to EDTA, this would be a novel one which could be used for biotechnological applications.

REFERENCES

1. Gupta A, Gupta V K, Modi D R, & Yadava L P, Production and characterization of α-amylase from *Aspergillus niger*, Biotechnology, 7 (2008) 551-556.
2. Abu E A, Ado S A & James D B, Raw starch degrading amylase production by mixed culture of *Aspergillus niger* and *Saccharomyces cerevisae* grown on *Sorghum pomace*, Afr J Biotechnol, 4 (2005) 785- 790.
3. Sivaramakrishnan S, Gangadharan D, Madhavan K N & Pandey A, Solid Culturing of *Bacillus amyloliquefaciens* for alpha amylase production, Food Technol Biotechnol. 44 (2006) : 269-274.
4. Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P & Srinivasula B, Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergilu*s species, Process Biochem, 38 (2002) 615–620.
5. Baysal Z, Uyar F & Aytekin C, Solid state fermentation for production of a α-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water, Process Biochem, 38(2003) 1665–1668.
6. Arasaratnam V, Mylvaganam K & Balasubramaniam K,. Glucoamylase production by *Aspergillus niger* in solid state fermentation with paddy husk as support. J Food Sci Technol, 38 (2001) 334–338.
7. Kocher G S, Kaur P & Grewal H S, Production of a-amylase by Aspergillus niger using wheat bran in submerged and solid state fermentations, Indian J Microbiol, 43 (2003) 143–145
8. Suganthi R, Benazir JF, Santhi R., Ramesh kumar V, Anjana H, Nitya M, Nidhiya K A, Kavitha G & Lakshmi R,. Amylase production by *aspergillus niger* under solid state fermentation using agroindustrial wastes, Int J Eng Sci Tech, 3 (2011) 1756-1763
9. Alva S, Anupama J, Savla J, Chiu Y Y, Vyshali P, Shruti M, Yogeetha B S, Bhavya D, Purvi J, Ruchi K, Kumudini B S, & Varalakshmi K N, Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture, African J Biotechnol, 6 (2007) 576-581
10. Raper K B & Fennell D J The Genus Aspergillus. Williams and Wilkins, Baltimore, USA, (1965) pp: 686.
11. Lingappa K, Vivek babu C S,. Siddalingeshwar K G, & Pramod T,. Isolation, screening and rapid confirmation of

lovastatin producing strains of *Aspergillus terreus*. Ind J Microbiol, 44 (2004) 133-135
12. Sadasivam S & Manickam A, Estimation of starch by antrone reagent. Biochemical Methods: New Age International Publishers, second ed, (1996) pp. 11–13.
13. Miller G L, Use of dinitrosalicylic acid reagent for determination of reducing sugars, Anal Chem, 31(1959) 426–428
14. Lowry O H, Rosebrough N J, Farr A L & Randall R J Protein measurement with the folin-phenol reagent, J Biol Chem, 193(1951) 265– 275.
15. El-Safey EM and Ammar M. S. Purification and characteraization of -α-amylase isolated from *Aspergillus falvus var. columnaris* Ass. Univ. Bull. Environ. Res. 7 (1), 2004 , 93-100.
16. Laemmli U K, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680-685.
17. Fogarty, W.M. and C.T. Kelly, 1979. Starch degrading enzymes of microbial origin. Prog. Ind. Microbiol., 15: 87-150.
18. Iverson, W.G. and Millis NF (1974). A method for the detection of starch hydrolysis by bacteria. J. Appl. Bacteriol. 16, 126-130.
19. Pandey A, Ramachandran S, Patel K A, Nampoothiri K M, Francis F, Nagy V & Szakacs G. Coconut oil cake—a potential raw material for the production of a-amylase, Biores. Technol, 93(2004) 169–174
20. Mulimani V H, Patil G & Ramalingam N, α-Amylase production by solid state fermentation: a new practical approach to biotechnology courses, Biochem Educ, 28 (2000) 161–163
21. Pacheco de Delahaye E, Jime´nez P, Perez E (2005). Effect of enrichment with high content dietary fibre stabilized rice bran flour on chemical and functional properties of storage frozen pizzas. J. Food Engr. 68: 1-7.
22. Heuzé V., Tran G., Lebas F., Lessire M., Renaudeau D., 2013. *Wheat grain*. Feedipedia.org. A programme by INRA, CIRAD, AFZ and FAO. http://www.feedipedia.org/node/223 *Last updated on May 17, 2013, 14:15*
23. Anto H, Ujjval T, & Kamlesh P, Alpha amylase production by *Bacillus cereus* MTCC 1305 Using solid state fermentation, Food Technol Biotechnol, 44 (2006) 241-245
24. Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P & Srinivasula B, Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergilu*s species, Process Biochem, 38 (2002) 615–620.
25. Mukerjee S K & Majumdar S K. Fermentative production of alpha amylase by *Aspergillus flavus*, Indian J Exp Biol, 11(1993) 436-438.
26. Chimata M K, Sasidhar P & Challa S,. Production of extracellular amylase from agricultural residues by a newly isolated *Aspergillus* species in solid state fermentation, African J Biotechnol , 9 (2010) 5162-5169
27. Nahas E & Waldermarin M M, Control of amylase production and growth characteristics of *Aspergillus ocharaceus*, Microbiologia, 44 (2002) 5–10.
28. Varalakshmi K N, Kumudini B S, Nandhini B N, Solomon J, Suhas R, Mahesh B and Kavitha A P Production and characterization of alpha amylase from *Aspergillus niger* JGI 24 isolated in Bangalore, Polish J Microbiol. 58 (2009) 29-36.
29. Hernandez M S, Rodriguez M R, Guerra N P & Roses R P, Amylase production by *Aspergillus niger* in submerged cultivation on two wastes from food industries. J Food Eng, 73 (2006) 93-100.
30. Gomes E, Souza S R, Grandi R P & Silva E D, Production of thermostable glucoamylase by newly isolated *flavus* A. 1.1 and *Thermomyces lanuginosus* A 13.37, Braz J Microbiol, 36 (2005) 75-82.
31. Peixoto S C, Jorge J A, Terenzi H F & Polizeli T M. *Rhizopus microsporus* var. rhizopodiformis: A thermotolerant fungus with potential for production of thermostable amylases, Int Microbiol, 6 (2003) 269-273.
32. Frolova G M, Silchenko A S, Pivkin M V & Mikhailov V V, Amylases of the fungus *Aspergillus flavipes* associated with *Fucus evanescens*, Appl Biochem Microbiol, 38 (2002) 134-138.
33. Shaista K, Asghar M, Rehman K, Asad M J & Adedyo Q, Bio- Processing of Banana peel for alpha amylase production by *B. subtilis*, Int J Agric Biol, (2003). 1560-8530/05-1-36-39.
34. Okolo B N, Ire S, Ezeogu li, Anyanwu C U & Odibo F J C, Purification and some properties of a novel raw starch-digesting amylase from

Aspergillus carbonarius, J Sci Food Agric, 81 (2001) 329-336.
35. Aquino A C, Jorge J A, Terenzi H F & Polizeli M L, Studies on a thermostable α-amylase from the thermophilic fungus *Scytalidium thermophilum*, Appl Microbial Biotechnol, 61(2003) 323-328.
36. Asgher M, Asad M J, Rahman S U & Legge R L, A thermostable - amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing, J Food Eng, 79 (2007) 950-955.
37. Babu K R & Satyanarayana T, Extracellular calcium-inhibited alpha-amylase of *Bacillus coagulans* B 49, Enzyme Microb Technol, 15 (1993) 1066–1069.
38. Chakraborty K, Bhattacharyya B K & Sen S K, Purification and characterization of a thermostable α-amylase from *Bacillus stearothermophilus*, Folia Microbiol, 45 (2000) (3): 207-210.
39. Sidkey N M, Abo-shadi M A, Balahmar R, Sabry R & Badrany G, Purification and characterization of α-amylase from a newly isolated *Aspergillus flavus* F2Mbb, Int Res J Microbiol, 2 (2011) 096-103