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Production and characterization of α-amylase from mango kernel by *Fusarium solani* NAIMCC-F-02956 using submerged fermentation

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Abstract

Microbial production of enzymes using low valued agro industrial wastes is gaining importance globally. Mango is one of the major fruit processed into a variety of products. During processing 40-50% of solid waste is generated in form of peel and stones. After decortications of mango stone, kernel is obtained which is a rich source of starch (upto 60%). It was utilized as a substrate for α-amylase production using *Fusarium solani*. Maximum α-amylase production (0.889 U g^{-1}) was recorded using a substrate concentration of 5% (w/v), pH-4 and temperature 30^{0}C on 9th day of incubation. Supplementation of production medium with micronutrients viz., Ca^{2+}, Fe^{2+} or Mg^{2+} improved the enzyme production while, Zn^{2+}, B^{3+} or Mn^{2+} ions exhibited inhibitory effect. The extracellular protein was precipitated by ammonium sulphate up to 70% saturation, dialyzed and purified (27.84 fold) by gel-exclusion (Sephadex G-75) chromatography. Protein profiling on 12% SDS-PAGE revealed three bands corresponding to 26, 27 and 30 kDa molecular sizes. The optimum amylase activity was achieved at pH 5.0 at 40^{0}C. The Michaelis constant (KM), Vmax and activation energy (-Ea) were found to be 3.7 mg ml^{-1}, 0.24 U mg^{-1} and 42.39 kJ $mole^{-1}$, respectively.

Key words

α-Amylase, *Fusarium solani,* Mango waste, Mango kernel

Introduction

Extracellular amylases are starch hydrolyzing enzymes derived from different sources including plants, animals and microorganisms. Three major classes of amylases such as α-amylase, glucoamylase and β-amylase are reported in microorganisms (Aedeniran *et al.,* 2008; Abu *et al.,* 2005). Alpha-amylases (endo-1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.1) are extracellular enzymes that randomly cleave starch at 1,4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. Microbial enzymes have greater importance and diversified commercial applications in food, fermentation, textile and paper industries (Pandey *et al.,* 2000; Selvakumar *et al.,* 1996). Currently, a large number of microbial amylases are available commercially and they have almost completely replaced the chemical hydrolysis in starch processing industry (Pandey *et al.,* 2000a; Kunamneni *et al.,* 2005). There are reports of α-amylase production using various micro-organisms under solid state and submerged fermentations conditions (Abu *et al.,* 2005; Pandey *et al.,* 2000). During industrial production of microbial enzymes, use of synthetic media is very expensive and makes production uneconomical. Therefore, the current use of low value agricultural by-products and fruit processing wastes are being explored.

Mango is a fruit which is processed into a variety of products and after processing about 40-50% of solid waste is generated in form of peel and stones (Garg and Prakash, 2006). Mango kernel, obtained after decortications of mango stone, is a rich source (upto 60%) of starch (Kaur *et al.*, 2004) which is not used for any purpose commercially excepting for its use as flour supplements in some parts of

Orissa (India). In the current study, kernel was used as a substrate for α-amylase production using an amylolytic fungus, *Fusarium solani*. Enzyme production conditions were optimized, followed by purification and biochemical characterization.

Materials and Methods

Mango kernel (dried and powdered) was used as substrate for α-amylase production. Thirty two amylolytic microorganisms were isolated from different soil samples, compost and other degrading starch rich substrates. The isolates were further purified by sub-culturing on starch-agar plates (Alva *et al.,* 2007). Primary screening of the microbial isolates for amylase production was carried out following the method of Thippeswamy *et al*. (2006). The starch agar plates containing microbial growth (after incubation at 60°C for 48 hr) were flooded with a solution of 0.5% (w/v) I_2 and 5.0% (w/v) KI. Colonies exhibiting halo starch hydrolysis were picked up. The zone of clearance was recorded. Five microbial isolates showing higher zone of clearance were subjected to secondary screening using di-nitro salicylic acid (DNS) method (Bernfeld, 1955). The best microorganism selected after from the primary and secondary screening experiments was used for enzyme production and was identified as *Fusarium solani* (identified as NAIMCC-F-02956). The production medium (500 ml in triplicate) was autoclaved and inoculated with 10^7 spores of *F. solani*. The flasks were incubated at 30°C under stationary conditions, and samples were withdrawn at specific time intervals of 7 days unless otherwise stated.

Substrate concentrations ranging from 1-10% (w/v) with increments of 2 %; pH of production medium varying from 3.0-7.5 with increments of 0.5; fermentation temperature 20-70ºC with increasing 10°C and fermentation period (1-12 day with 2 day interval). Effect of supplementation of production medium with inorganic salts individually (as detailed in table 1) on enzyme production was observed. The salts and fine chemicals used in the experiments were of analytical grade. All the experiments were conducted in triplicates, repeated twice and the mean values were calculated.

After the completion of fermentation, the extracellular protein fraction was extracted from the culture filtrate by gradient ammonium sulphate precipitation (upto 70% saturation) and dialyzed twice against 50mM acetate buffer (pH 5) to remove the excess salts. The protein concentration of crude as well as partially purified enzyme was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard. The reaction mixture containing 0.4 ml of 1% soluble starch (Hi-media, India) in 0.05M acetate buffer (pH 5.0) and 0.1 ml of the enzyme solution was incubated at 30ºC for 60 min. Alpha amylase activity was quantified by measuring the amount of reducing sugars maltose released in the medium (Miller, 1959). One unit of α-amylase is the amount of enzyme that produced one μ mole of maltose min^{-1} ml^{-1} under assay conditions and was expressed as U g^{-1} of dry substrate.

The concentrated protein was then fractionated using gel filtration chromatography column (1.0X40 cm) packed with Sephadex G-75 matrix pre-equilibrated with 50mM acetate buffer (pH 5). Two ml fractions were collected at the flow rate of 10ml hr^{-1}. The protein values were monitored spectrophotometrically at 280 nm. The enzyme kinetics experiment was performed by measuring the initial velocity at different temperature, pH and substrate (starch) concentrations in 50mM acetate buffer (pH 5.0). Kinetics of the enzyme was analyzed using Lineweaver-Burk and Michaelis-Menten plots (Sanghi *et al.,* 2010). The Michaelis constants (K_M), maximum velocity (V_{max}), and activation energy ($-E_a$) were calculated by Lineweaver-Burk and Arrhennis plots.

Protein profiling was performed by electrophoresis in 10% native polyacrylamide gel and 12% denaturating Sodium dodecyl sulphate-Polyacrylamide (SDS-PAGE) gel in discontinuous buffer systems as described by Laemmli (1970) in order to determine the molecular sizes of the enzyme isoforms. After electrophoresis, the protein bands were visualized by silver staining (Blum *et al*., 1987).

Results and Discussion

Potential of several microorganisms for α-Amylase production using various agro-horticultural wastes as substrate has already been reported (Balkan and Ertan, 2007; Mohamed *et al.,* 2009; Chen *et al.,* 2010; Oshoma *et al.,* 2010). Garg and Ashfaque (2010) and Kumar *et al.* (2012) reported the use of mango peel for pectinase and cellulose

Table 1 : Optimization of condition for maximum enzyme production by *Fusarium solani* under submersed fermentation conditions

Step	Condition optimized	Optimum condition	Enzyme production (U g^{-1})
a	Only substrate	Mango kernel (5% w/v)	33.2±5.1
b	a+ pH	pH 4	34.9±3.2
c	b+ Fermentation temperature	30ºC	44.83±7.3
d	c+ Micro-nutrients	Ca^{2+}, Mg^{2+} and Fe^{2+}	50.7±5.3
e	d+ Day of incubation	9^{th} day	88.9±2.5

production, respectively. There is no report available on amylase production using mango kernel. Bhatti *et al.* (2005, 2007) used *F. solani* for glucoamylase production.

The primary and secondary screening results revealed that a fungus isolated from degrading potato tuber, *Fusarium solani* NAIMCC-F-02956, expressed maximum amylase production (30 ±1.7U g^{-1}) over other isolates. Hence, it was selected for amylase production. Optimization studies indicated that as independent variable, substrate (mango kernel) concentration of 5% (w/v), pH-4 and incubation temperature of 30°C were found optimum for amylase production by *F. solani*. The results were confirmed in the integrated optimization experiment as reflected in Table 1 which indicated that the variables contributed synergistically for maximum α-amylase production (44.8±7.0 U g^{-1}). Similar result has been reported for glucoamylase production by *F. solani* using wheat bran as substrate (Bhatti *et al.,* 2007). Addition of Ca^{+2}, Mg^{+2} and Fe^{+2} to basal medium resulted in enhanced enzyme production to the extent of 1.38, 1.16 and 1.12 fold while Zn^{+2}, B^{+3}, or Mn^{+2} showed negative effect (Table 2). *α*-amylase production started only after 3rd day of incubation, and reached maximum (88.9±2.5 U g^{-1}) on 9th day. Production decreased thereafter, which may probably be attributed to catabolite repression.

The ammonium sulphate precipitation at 70% saturation resulted in 4.08 fold purification and 38.33 % yield. Dialysis followed by size exclusion chromatography brought about purification to an extent of 27.84 fold with a yield of 2.87% (Table 3). The specific activity of the purified enzyme was found to be 2.55 U mg^{-1}. Characterization of the purified enzymes by SDS-PAGE showed three bands corresponding to 26, 27 and 30 kDa molecular sizes (Fig. 1), while, it was observed as single band on 10% Native gel. Since, it is a heteromeric multienzyme complex, the three bands might correspond to different isoforms of amylase enzyme complex in *F. solani*. Earlier, 36 kDa amylase was reported in *Scytalidium thermophilium* (Aquino *et al.,* 2003).

The purified enzyme exhibited highest activity in acidic and near neutral condition. Maximum activity (0.26±0.02 U ml^{-1}) of the enzyme was found at pH 5.0, while, it dropped almost 50% at pH 4.0 (0.13±0.02 U ml^{-1}) and 6.0 (0.15±0.05 U ml^{-1}). Further fluctuation in the pH significantly hampered the activity of enzyme. The result suggested that the enzyme would be useful in processes requiring wide range of pH (4.0-6.0). The pH range of 5.0-6.0 has been reported to be optimum for amylase activity in *Trichoderma matsutake* (Mizuho *et al.,* 2003), while, it is 5-8 for *Aspergillus niger* and *Penicillium olsonii* (Omemu *et al.,* 2004; Afifi *et al.,* 2008).

Table 2 : Effect of micronutrient addition to basal medium* on amylase relative production

Micronutrient	Micronutrient Quantity µg 100 ml^{-1} basal medium (w/v)	Enzyme production (U g^{-1})	Relative enzyme production (fold)
Control	0	27.0±0.1	1
Fe^{2+}	100	30.4±5.7	1.12
Ca^{2+}	100	37.4±8.6	1.38
Mo^{3+}	1	25.8±5.0	0.95
Mg^{2+}	20000	31.4±8.6	1.16
Cu^{2+}	5	24.0±2.8	0.89
Mn^{2+}	1	19.3±9.2	0.71
Zn^{2+}	7	05.4±3.5	0.19
B^{3+}	1	09.9±4.8	0.36

*Basal medium (composition g 100ml^{-1} distilled water): mango kernel-5, KH_2PO_4- 0.4, Na_2HPO_4-6.0, $(NH_4)_2SO_4$-2.0

Table 3 : Purification profile of α-amylase

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification Fold	Yield (%)
Culture filtrate	1000	424.69	38.98	0.09	1.000	100.00
[a]Precipitate	100	39.88	14.94	0.37	4.08	38.33
[b]Sephadex G-75	2	0.43	1.01	2.55	27.84	2.82

[a]Ammonium sulphate precipitation (50-70%); [b]Active fraction collected from size exclusion chromatography; * One unit of endoglucanase is the amount of enzyme that produced one µmol of glucose min^{-1} ml^{-1} under assay condition

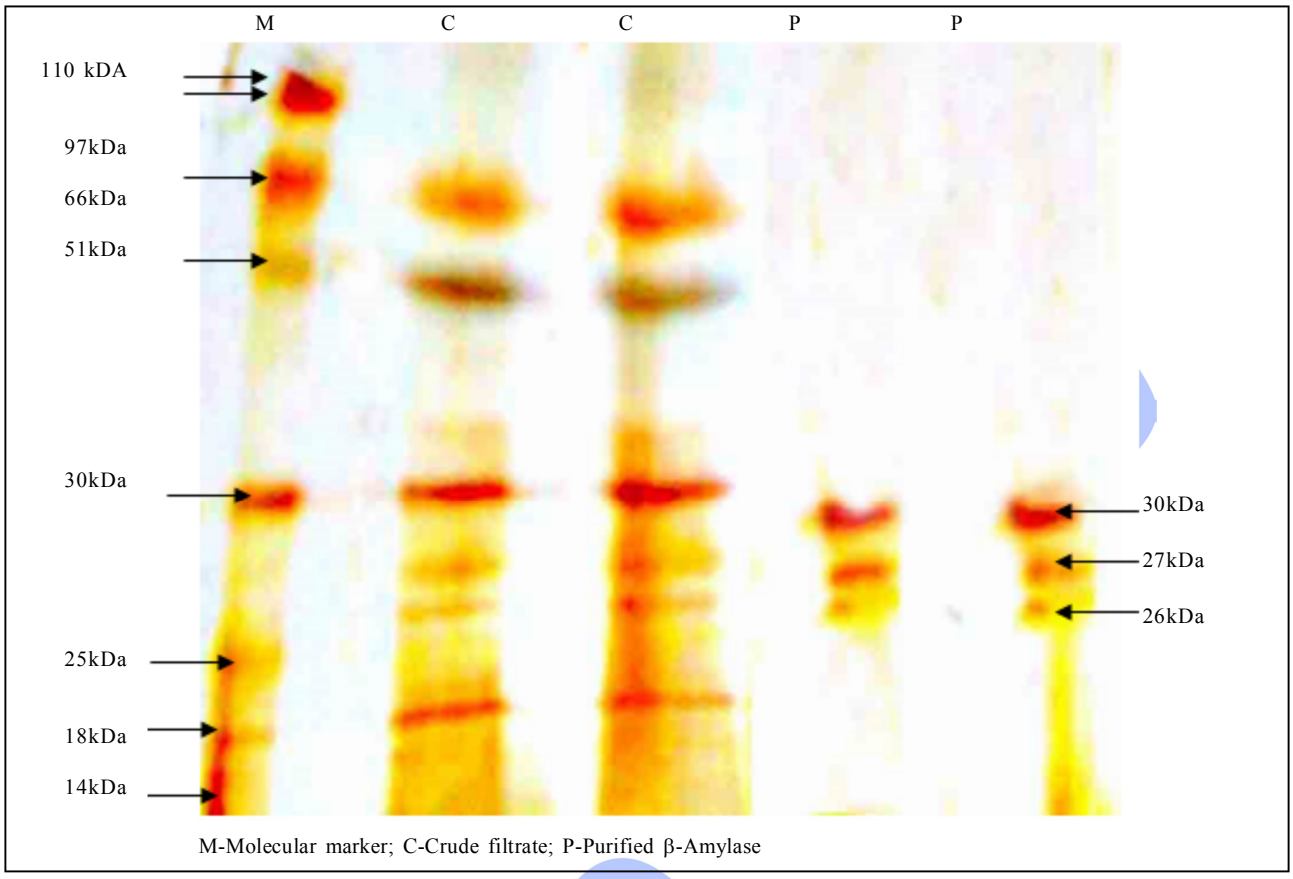

Fig. 1 : SDS-PAGE (12%) profile of crude protein and purified amylase. Abbreviations: M-Marker (β-Galactosidase(110 kDa), Phaphorylase (97 kDa), Bovine Serum albumin(66 kDa), Amylase e.merck (51 kDa), Carbonic anhydrase (30 kDa), BamHI (25 kDa), Lactoglobulin (18 kDa), Lysozyme (14 kDa); C-Crude protein, PU-Purified Amylase. Amylase bands: 30, 27 and 26 kDa

Most amylases are reported to exhibit maximum activity in the temperature range of 40-60ºC (Afifi *et al.,* 2008; Mahdavi *et al*., 2010). Higher enzyme activity was obtained at 40ºC (0.34±0.6 U ml^{-1}) compared to that 30ºC, (0.27±0.055 U ml^{-1}) and 50ºC (0.27±0.1 U ml^{-1}) respectively. A optimum temperature of 40ºC was in agreement with earlier observations of De Silva *et al.* (2009). Beyond the optimum temperature, there was gradual decrease in enzyme activity with increase in temperature. Similar amylase activity was found in *Bacillus cereus* which had optimum temperature 50ºC (Mahdavi *et al*., 2010). The thermal stability of the enzyme was found in the range of 50-70ºC up to 60 min. The enzyme lost about 79.3-81.1% of its activity at temperature 30ºC and 50ºC, respectively. At higher temperatures, for instance, activity was either very low (28.19%) at 70ºC, and virtually undetectable at 80ºC.

The effect of the substrate concentration on the enzyme activity showed that amylase activity increased with increasing the starch concentration up to 3.0 mg ml^{-1} but decreased thereafter, following a sigmoid pattern. Enzyme kinetics was worked out by Lineweaver-Burk plot which revealed the reciprocal of initial velocity and substrate concentration. Michaelis constant (K_M) values were found to be 3.37 mg ml^{-1} (Fig. 2). The K_M value was lower to that (5.75 mg ml^{-1}) reported in *Aureobasidium pullulans* (Li *et al.,* 2007), while, V_{max} was found to be 0.24U mg^{-1} which was equal to that reported by the same workers. Using Arrhenius plot with starch as substrate at 40-80°C in 50 mM acetate buffer pH 5.5, the activation energy ($-E_a$) was calculated to be 42.39 kJ.$mole^{-1}$ (Fig. 3). The enzyme properties of amylase produced by *F. solani* using mango kernel as substrate is superior to the amylases earlier reported by other organisms using various agro substrates (cassava, yam, banana, and plantain peels and brewery spent grains) (Aedeniran *et al.,* 2008; Bhatti *et al.,* 2005; Bhatti *et al.,* 2007) in terms of pH stability and relative activity.

This property makes the enzyme suitable for use in paper and food industry. The work supports the concept of making wealth through eco-friendly and cost effective waste utilization technology using mango kernel as substrate.

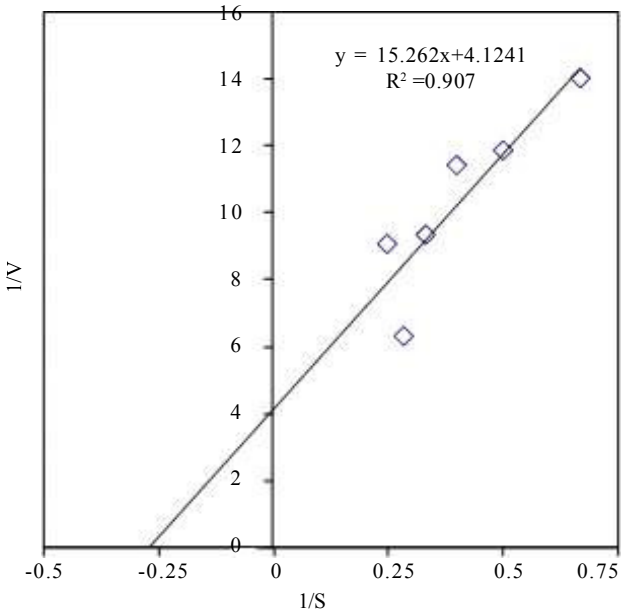

Fig. 2 : Lineweaver-Burk plot showing the Michaelis–Menten-type kinetics of the enzyme amylase on the starch under the stranded assay condition specified, indicating the K_M value under maximum velocity (V_{max})

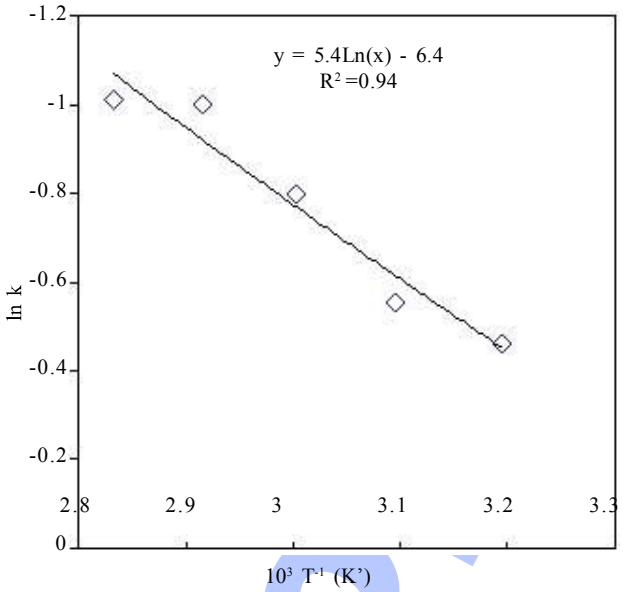

Fig. 3 : Arrhenius plot for the determination of activation energy (-E_a). Soluble starch were used substrate at 40-80 °C in 50 mM acetate buffer pH 5.5, the activation energy (-E_a) was calculated to be 42.39 kJ.mole^{-1}. There R^2 value was found higher than 0.9

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References

Abu, E.A., S.A. Ado and D.B. James: Raw starch degrading amylase production by mixed culture of *Aspergillus niger* and *Saccharomyces cerevisiae* grown on Sorghum pomace. *Afr. J. Biotechnol.,* **4**, 785-790 (2005).

Aedeniran, H.A., S.H. Abiose and A.O. Ogunsua: Production of fungal β-amylase and amyloglucosidase on some Nigerian agriculture residue. *Food Bioprocess Technol.*, **5**, 693-698 (2010).

Afifi, A.F., E.M. Kamel, A.A. Khalil, M.A. Foaad, E.M. Fawzi and M.M. Houseny: Purification and characterization of α-amylase from *Penicillium olsonii* under the effect of some antioxidant vitamins. *Global J. Biotechnol. Biochem.*, **3**, 14-21 (2008).

Alva, S., J. Anupama, J. Savla, Y.Y. Chiu, P. Vyshali, M. Shutri, B.S. Yogeetha, D. Bhavya, J. Purvi, K. Ruchi, B.S. Kumudini and K.N. Varalakshmi: Production and characterization of fungal amylase enzyme isolated from *Aspergillus sp.* JGI 12 in solid state culture. *Afr. J Biotechnol.,* **6**, 576-581(2007).

Aquino, A.C., J.A. Jorge, H.F. Terenzi and M.L. Polizeli: Studies on a thermostable amylase from the thermophilic fungus *Scytalidium thermophilum*. *Appl. Microbiol. Biotechnol.*, **61**, 323-328 (2003).

Balkan, B. and F. Ertan: Production of α-amylase from *Penicillium chrysogenum* under solid state fermentation by using some agricultural by product. *Food Technol. Biotechnol.,* **45**, 39-442 (2007).

Bernfeld, P.: Amylases, alpha and beta. In: Methods in Enzymology, (Eds.: S.P. Colowick and N.O. Kaplan) Vol. **1**. Academic Press, New York, pp.149-158 (1955).

Bhatti, H.N, M.H. Rashid, R. Nawaz, M. Asgher, R. Perveen and A. Jabbar: Purification and characterization of a novel glucoamylase from *Fusarium solani*. *Food Chem.,* **103**, 338-343 (2007).

Bhatti, H.N., A. Zia, R. Nawaz, M.A. Sheikh, M.H. Rashid and A.M. Khalid: Effect of copper ions on thermal stability of glucoamylase from *Fusarium sp. Int. J. Agr. Bio.,***7**, 585–587 (2005).

Blum, H., H. Beier and H.J. Gross: Improved silver staining of plant-proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis,* **8,** 93–99 (1987).

Chen, L., Z.M. Chi, Z. Chi and M. Li: Amylase production by *Saccharomycopsis fibuligera* A11 in solid state fermentation for hydrolysis of cassava starch. *Appl. Biochem. Biotechnol.*, **162**, 252-263 (2010).

De Silva, S.N.T., D.P.S.T.G. Attanayaka, S.F. Nirosha and A.M.W.S. Aththanayaka: Isolation of raw starch hydrolysis fungi and purification of α-amylase from *Geotrichum candidum* CMSS 06. *J. Natn. Sci. Foundation Sri Lanka.*, **37**, 93-98 (2009).

Garg, N. and Om Prakash: Biodegradation of mango kernel by *Syncephalastrum racemosum* and its biological control. *Biocontrol.,* **51**, 353-361 (2006).

Garg, N. and M. Ashfaque: Mango peel as substrate for production of extra cellular polygalacturonase from *Aspergillus fumigatus*. *Ind. J. Hort.* **67**, 140-143 (2010).

Kaur, M., N. Singh, S.K. Singh and H.S. Guraya: Physicochemical, morphological, thermal and rheological properties of starches separated from kernels of some Indian mango cultivars (*Mangifera indica* L.). *Food Chem.*, **85**, 131-140 (2004).

Kumar, D., M. Ashfaque, M. Muthukumar, M. Singh and N. Garg: Production and characterization of carboxymethyl cellulase from *Paenibacillus polymyxa* using mango peel as substrate. *J. Env. Biol.*, **33**, 81-84 (2012).

Kunamneni, A., K. Permaul and S. Singh: Amylase production in solid state fermentation by the thermophilic fungus *Thermomyces lanuginosus*. *J. Biosci. Bioeng.*,**100**, 168-171

(2005).
Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685 (1970).
Li, H., Z. Chi, X. Wang, X. Duan, L. Ma and L. Gao: Purification and characterization of extracellular amylase from the marine yeast *Aureobasidium pullulans* N13d and its raw potato starch digestion. *Enzyme Microb. Technol.*, **40**, 1006-1012 (2007).
Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J Randall: Protein measurements with the Folin phenol reagent. *J. Bio. Chem.*, **193**, 265-275 (1951).
Mahdavi, A., R.H. Sajedi, M. Rassa and V. Jafarian: Characterization of an α-amylase with broad temperature activity from an acid-neutralizing *Bacillus cereus* strain. *Iranian J. Biotech.*, **8**, 103-111 (2010).
Miller, G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.*, **31**, 426-428 (1959).
Mizuho, K., N. Masaru, C.H. Tae, U. Mitsuhiro and T. Takao: Purification of some properties of α-amylase from an ectomycorrhizal fungus, *Trichoderma matsutake. Mycoscience*, **44**, 311-317 (2003).
Mohamed, S.A., A.L. Al-Malki and T.A. Kumosani: Partial purification and characterization of five α-amylases from a wheat local variety Balady during germination, *Aust. J. Basic Appl. Sci.,* **3**, 1740-1748 (2009).
Omemu, A.M., I. Akpan, M.O. Bankole and O.D. Teniola: Hydrolysis of raw tuber starches by amylase of *Aspergillus niger* AM07 isolated from the soil. *Afr. J. Biotechnol.,* **4**, 19-25 (2004).
Oshoma, C.E., E.E. Imarhiagbe, M.J. Ikenebomeh and H.E. Eigbaredon: Nitrogen supplements effect on amylase production by *Aspergillus niger* using cassava whey medium. *Afr. J. Biotechnol*., **9**, 682-686(2010).
Pandey, A., C.R. Soccol and D.Mitchell: New developments in solid state fermentation: I-Bioproceses and products. *Process Biochem*., **35**, 1153-1169 (2000a).
Pandey, A., P. Nigam, V.T. Soccol, D. Singh and R. Mohan: Advances in microbial amylases. *Biotechnol. Appl. Biochem.,* **31**, 135-152 (2000).
Sanghi, A., N. Garg, V.K Gupta, A. Mittal and R.C. Kuhad: One-step purification and characterization of cellulase-free xylanase produced by alkalophilic *Bacillus subtilis* ash. *Braz. J. Microbiol.,* **41**, 467-476 (2010).
Selvakumar, P., L. Ashakumary and A. Pandey: Microbial synthesis of starch saccharifying enzyme in solid state fermentation. *J. Sci. Ind. Res.*, **55**, 443-449 (1996).
Thippeswamy, S., K. Girigowda and H.V. Mulimami: Isolation and identification of α-amylase producing *Bacillus sp.* from dhal industry waste. *Indian J. Biochem. Biophys*., **43,** 295-298 (2006).