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Full Length Research Paper

Mixture of residual fish hydrolysate and fish extract hydrolysate to activate *Bacillus licheniformis* 6346

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Microbes are first activated in appropriate media before cultivation/fermentation. *Bacillus licheniformis* 6346 activated in nutrient broth-starch was inoculated to a locally formulated solid medium (paddy husk, rice flour, $(NH_4)_2SO_4$, cuttle fish powder, murate potash, table salt, triple super phosphate, sesame oil, coconut oil and tap water) and the highest α-amylase activity was obtained on day 5 (1075 U g DBM^{-1}) at 42°C. Replacement of nutrient broth-starch with residual fish hydrolysate-starch led to the enzyme production to 1100.3 and 1180.4 U g DBM^{-1} on days 5 and 6, respectively. Supplementation of residual fish hydrolysate-starch with 3 gL^{-1} $(NH_4)_2HPO_4$ increased the α-amylase production to 1426 U g DBM^{-1}on the 4th day. Supplementation of residual fish hydrolysate-starch-$(NH_4)_2HPO_4$ with yeast extract reduced the α-amylase production to 877 U g DBM^{-1}. Mixing residual fish hydrolysate with fish extract hydrolysate in the volume ratio of 1: 32.6 and supplementing starch and $(NH_4)_2HPO_4$ increased the enzyme production to 2328 U g DBM^{-1}. Maintenance of amino acid content in terms of glycine or tyrosine in activation medium did not show significant correlation with α-amylase production by *B. licheniformis*. This study shows that, to activate *B. Licheniformis*, residual fish hydrolysate - starch - $(NH_4)_2HPO_4$ - fish extract hydrolysate could be used instead of nutrient broth-starch.

Key words: α–Amylase, *Bacillus licheniformis*, fish extract hydrolysate, paddy husk, solid state fermentation.

INTRODUCTION

Economic starch hydrolysis is a recognised priority in biotechnology research (Gosh and Chandra, 1984; Mahon et al., 1999). One of the ways to reduce the expense is cutting the cost of enzyme production in the process operations (Haq et al., 1999). The microbes stored in glycerol stock or lyophilized powder or in nutrient-agar slant need to be first activated and then the inoculum or starter culture is prepared to continue fermentation process (Haq et al., 1999; Anto et al., 2006; Aiyer, 2004; Chandra et al., 1980; Ramesh and Lonsane, 1987; Tonkova et al., 1994; Bierbaum et al., 1994). In such activation and inoculum preparations, the nutrients usually used are yeast extract, peptone, nutrient broth, MRS broth, etc. α-Amylases have been produced by submerged fermentation (Dey et al., 2001; Malhotra et al., 2000) and solid state fermentation (Swain and Ray, 2007; Mukaherjee et al., 2009; Shukla and Rita, 2006; Murthy et al., 2009) techniques. In this work *Bacillus licheniformis* which is widely used for thermostable α-amylase production has been selected. To activate *B. licheni-formis,* the medium in practice is the nutrient broth with soluble starch for α-amylase production (Haq et al., 1999; Aiyer, 2004; Chandra et al., 1980; Ramesh and Lonsane, 1987; Tonkova et al., 1994; Bierbaum et al., 1994; Pederson and Nielson, 2000). It would be economically advan-tageous if this nutrient broth is replaced with local sources. To avoid the import and transport barriers and to reduce the cost of the processes, locally available protein source was used to replace nutrient broth. This study presents the use of a cheap variety of locally available fish instead of nutrient broth to activate *Bacillus licheniformis* 6346. Further to avoid the barriers such as frequent interruption of electricity causing problems in continuous oxygen supply and mixing solid state fermentation was carried out. Here to test the efficacy of the activation medium, a solid medium formulated in the laboratory was used (Arasaratnam and Thayaananthan, 2009).

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Table 1. Different activation media used to activate *B. licheniformis* 6346.

Components	Activation Medium (1000 ml)													
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Nutrient broth (g).	25	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch (g).	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Residual Fish hydrolysate (mL).	-	1000	1000	1000	1000	1000	1000	145	-	6.675	13.35	20.025	26.7	33.35
$(NH_4)_2HPO_4$ (g).	-	-	1.0	3.0	10	30	3.0	3.0	-	3.0	3.0	3.0	3.0	3.0
Yeast extracts (g).	-	-	-	-	-	-	3.0	-	-	-	-	-	-	-
Fish extract hydrolysate (mL).	-	-	-	-	-	-	-	855	1000	925	925	925	925	925
Distilled water (mL)	1000	-	-	-	-	-	-	-	-	68.325	61.65	54.975	48.3	41.65
Total amino acids in terms of Glycine (gL^{-1}).	-	-	-	-	-	-	-	-	-	0.51	0.68	0.85	1.02	1.19
Total amino acids in terms of tyrosine (gL^{-1}).	-	-	-	-	-	-	-	-	-	0.31	0.33	0.34	0.37	0.39
Amino acid ratio in terms of glycine (Fish extract hydrolysate: Residual fish hydrolysate).	-	-	-	-	-	-	-	-	-	2:1	2:2	2:3	2:4	2:5
Amino acid ratio in terms of tyrosine (Fish extract hydrolysate: Residual fish hydrolysate).	-	-	-	-	-	-	-	-	-	16.1:1	8.1:1	5.34:1	4.03 :1	3.23: 1

Total amino acid content of residual fish hydrolysate in terms of glycine is 25.5 gL^{-1} and in terms of tyrosine is 2.75 gL^{-1}.
Total amino acid content of fish extract hydrolysate in terms of glycine is 0.3675 gL^{-1} and in terms of tyrosine is 0.32 gL^{-1}. Total amino acid content of nutrient broth in terms of glycine is 35.16 g kg^{-1} and in terms of tyrosine is 132 g kg^{-1}.

MATERIALS AND METHODS

Materials

Paddy husk, raw unpolished rice, fertilizers such as ammonium sulphate, murate potash and triple super phosphate, table salt (sodium chloride) and mullet fish (*Mugil cephalus L.*), sesame oil (*Sesamum indcus*, L.) and coconut oil (*Cocus nucifera*, L.) were purchased from local market. Cuttle fish (*Sepia* sp.) shells were collected from the fish market, washed and powdered. Raw unpolished rice was ground to powder in a domestic grinder. Nutrient broth was from Oxoid Limited, UK. Pepsin (91 Units mg^{-1} solid, Catalogue No. P7125) was from Sigma Chemical Company. Other chemicals were from standard sources.

Microorganism

B. licheniformis 6346 from Harriet-Watt University, UK was used.

Development of inoculum

Two loop full of the bacteria from stock culture was activated in 100 mL sterile nutrient broth (25 gL^{-1})-soluble starch (3.0 gL^{-1}) (Activation medium I, Table 1) and incubated at 42°C for 19 h in an orbital shaker (300 rpm). This was (40 mL) inoculated to 160 mL of the same activation medium, incubated for 5 h at 300 rpm to be used as inoculum for 1 kg medium.

Solid state medium

Sterile solid medium containing (g kg^{-1}): paddy husk, 250; rice flour, 62.5; fertilizer $(NH_4)_2SO_4$ (15.5 g) and cuttle fish (*S.* sp.) shell powder (12 g kg^{-1} medium); 15.5; K_2O (murate potash, 3.036 g kg^{-1}) and NaCl (table salt, 2.457 g kg^{-1}), triple super phosphate (16 g kg^{-1}) and (mL kg^{-1}) sesame oil, 23.4; coconut oil, 7.8 and tap water, 425 (Arasaratnam and Thayaananthan, 2009) was inoculated with 200 mL of activated *B. licheniformis* inoculum and incubated at 42°C. α-Amylase production was monitored.

Extraction and measurement of α-amylase activity

Enzyme was extracted by mixing 1 g of bacterial medium (wet) with 4 mL of tap water for 20 min and centrifuged (8325 g, 10 min). The supernatant was pre-incubated at 85°C for 3 min, mixed with 0.5 mL of 20 gL^{-1} starch in 0.1 M phosphate buffer (pH 7.0) and incubated for 5 min. Reducing sugar produced was measured by dinitrosalicylic acid method (Miller, 1959). One unit of α-amylase activity is the amount of enzyme that liberates 1µ mole of glucose in one minute. The activity is represented as U/g Dry Bacterial Medium (U g DBM^{-1}). All data are means of at least three samples which agreed with 5%

Preparation of residual fish hydrolysate and fish extract hydrolysate

Fish (200 g) suspended in 1000 mL normal saline was incubated for 72 h at 37°C and allowed to settle. The extract (975 mL) was treated with pepsin (27.3 x 10^3 U, 0.3

Table 2. Different activation media used to activate *B. licheniformis* 6346 with same total amino acid contents in terms of glycine.

Components	Activation Medium (1000 mL)			
	XV	XVI	XVII	IX
Starch (g)	3.0	3.0	3.0	3.0
$(NH_4)_2HPO_4$ (g)	3.0	3.0	3.0	3.0
Fish extract hydrolysate (mL)	-	66.5	-	1000
Residual fish hydrolysate (mL)	34.5	33.5	-	-
Nutrient broth (g)	-	-	25.0	-
Distilled water (mL)	965.5	9000	1000	-
Amino acid content in terms of glycine (gL^{-1})	0.88	0.88	0.88	0.3675
Amino acid content in terms of tyrosine (gL^{-1})	0.095	0.1133	3.3	0.32

Total amino acid contents of residual fish hydrolysate, fish extract hydrolysate and nutrient broths are shown in Table 1.

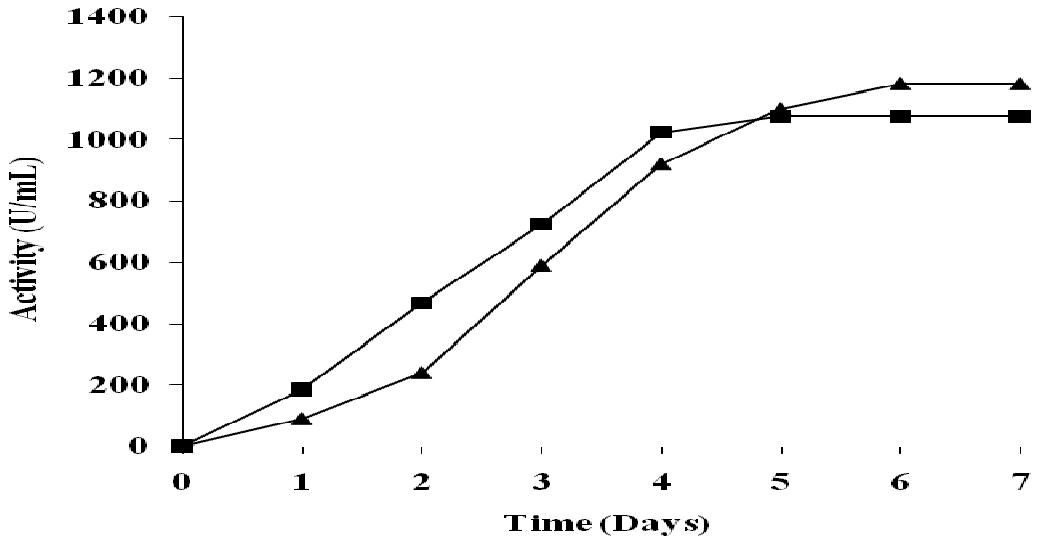

Figure 1. Production of α-amylase by *B. licheniformis* 6346 at 42°C in solid formulated medium inoculated with activation media prepared from nutrient broth- starch (Activation medium I) and with fish hydrolysate- starch (Activation medium II). Solid formulated medium (800 g) was inoculated with 200 mL of different activation media and incubated at 42°C.

g in 250 ml 0.5 N HCl) for 24 h at 37°C. This is the (1225 mL) fish extract hydrolysate. The residue was mixed with 6 N HCl (400 mL) and pressure cooked for 2 h. The extract was used as fish hydrolysate. Total amino acid contents of residual fish hydrolysate, fish extract hydrolysate and nutrient broths were measured as total glycine (Mann and Saunders, 1975) and tyrosine (Rick, 1974).

Substitution of nutrient broth in activation medium with different nutrients

Nutrient broth in the activation medium was replaced with different activation media prepared from residual fish hydrolysate, $(NH_4)_2HPO_4$, yeast extract, fish extract hydrolysate (Activation Media II – IX, Table 1) and different combinations of residual fish hydrolysate and fish extract hydrolysate (Media X - XIV, Table 1). Different activation media were also prepared with the same total amino acid content (Activation Media XV - XVIII and IX, Table 2).

RESULTS AND DISCUSSION

Nutrient broth is the most common growth medium used for the activation of *B. licheniformis* (Haq et al., 1999; Aiyer, 2004). The problem was faced in importing chemicals to Jaffna and hence as an alternative nitrogen source a cheap variety of fish, *Mugil cephalus* was selected and its hydrolysed products were used as substitutes for nutrient broth.

B. licheniformis was cultivated in a medium formulated in this lab (Arasaratnam and Thayaananthan, 2009). When nutrient broth-starch was used as the activation medium, the α-amylase production commenced on day 1 and maximum α-amylase activity obtained on day 5 (Figure 1). When nutrient broth was substituted with

Table 3. Production of α-amylase at 42°C by *B. licheniformis* 6346 activated in different activation media and inoculated to solid formulated medium. Solid formulated medium (800 g) was inoculated with 200 mL of different activation media and incubated at 42°C. Highest activity was produced on 4th day and presented.

Activation medium	Nutrients					α-Amylase activity ($UgDBM^{-1}$)
	Fish hydrolysate (mL)	Starch (gL^{-1})	$(NH_4)_2HPO_4$ (gL^{-1})	Yeast extract (gL^{-1})	Fish extract hydrolysate (mL)	
II	1000	3.0	0.0	0.0	0.0	920 ± 6.4 (1180.3 ± 0.2)
III	1000	3.0	1.0	0.0	0.0	1312 ± 0.9
IV	1000	3.0	3.0	0.0	0.0	1426 ± 0.4
V	1000	3.0	10.0	0.0	0.0	882 ± 0.25
VI	1000	3.0	30.0	0.0	0.0	900 ± 0.1
VII	1000	3.0	3.0	3.0	0.0	877 ± 0.3
VIII	145	3.0	3.0	0.0	855	1202 ± 0.1
IX	0.0	3.0	3.0	0.0	1000	1094 ± 8.9

*Highest activity was obtained on 6th day and given in bracket.
Total amino acid in terms of glycine in media IV, VIII and IX were 25.5, 7.5482 and 0.3675 gL^{-1}, respectively, and in terms of tyrosine were 2.75, 1.1014 and 0.32 gL^{-1}

residual fish hydrolysate (Activation Medium II, Table 1), the highest α-amylase activity (1180.4 U $gDBM^{-1}$) was produced on day 6 (Figure 1). *B. licheniformis* activated in residual fish hydrolysate-starch (Activation Medium II, Table, 1) showed higher α-amylase production than that activated in nutrient broth- starch on 6th day and it was 1.1 times higher (Figure 1). Even though there was a delay in the enzyme production, the enzyme production has reached the higher level on day 6 in Medium II. Hence, it was decided to use residual fish extract to activate *B. licheniformis* 6346. The hydrolysed fish products would have been present in the residual fish hydrolysate. Fish contains about 20% protein, fat and water (55 - 83%) varying widely (Wickramanayake, 1998). Fish protein comprises of ten essential amino acids in desirable concentrations (Srivatsava, 1985). Aspartic acid and cysteine were reported to be two important amino acids for α-amylase production. The order of amino acids correlated with increasing rate of the enzyme formation was cysteine = aspartic acid > arginine > alanine = phenyl alanine > isoleucine > serine (Chandra et al., 1980). Of these amino acids, fish protein contains arginine and isoleucine (Srivatsava, 1985), which would have promoted the growth of *B. licheniformis*. The nutrient content of the residual fish hydrolysate seems to be useful for the culture of *B. licheniformis*, but α-amylase production was delayed. Therefore, the activation medium with the residual fish hydrolysate needs to be improved.

When the residual fish hydrolysate- starch was supplemented with different amounts of $(NH_4)_2HPO_4$ (Activation Media III-VI, Table 3), the highest enzyme production time was reduced to 4 days (Table 3). $(NH_4)_2HPO_4$, of 3.0 gL^{-1} was observed to be the best concentration for the activation of *B. licheniformis* (Activation Medium IV, Table 3). $(NH_4)_2HPO_4$ has reported to influence the enzyme production (Ramesh et al., 1993; Ramesh and Lonsane, 1989) and phosphate ion (PO_4^{3-}) stimulates α-amylase production (Aiyer, 2004; Chandra et al., 1980; Dimitrovski and Doneva-Spaceska, 1995). The nutrient broth contains Lab Lemco powder, yeast extract, peptone and sodium chloride, where Lab Lemco powder and yeast extract contain phosphorous and phosphate. The residual fish hydrolysate is a hydrolysed product of fish and $(NH_4)_2HPO_4$ have provided the free amino acids and peptones, as those in nutrient broth.

Supplementation of yeast extract to residual fish hydrolysate-soluble starch-$(NH_4)_2HPO_4$ (Activation medium VII, Table 1) reduced the α-amylase production to 877 U g DBM^{-1} (Medium VII, Table 3). Previous results indicated that there is species variation in response to yeast extract (Dimitrovski and Doneva-Spaceska, 1995; Terezinha and Iracema, 1994). Therefore, yeast extract was omitted.

Even though residual fish extract hydrolysate activated *B. licheniformis* gave better α-amylase activity with supplementations, it was decided to use the fish extract hydrolysate along with residual fish hydrolysate because most of the soluble nutrients of the fish would have been extracted with this fraction. Mixing of residual fish hydrolysate and fish extract hydrolysate in a

Table 4. Production of α-amylase by *B. licheniformis* 6346 at 42°C in formulated solid medium inoculated with activation media prepared from mixtures of residual fish hydrolysate and fish extract hydrolysate in different ratios supplemented with 3.0 gL^{-1} $(NH_4)_2HPO_4$ and 3.0 gL^{-1} starch. Solid formulated medium (800 g) was inoculated with 200 ml of different activation media and incubated at 42°C. Highest activity was produced on 4th day and presented.

Activation medium	*Amino acid ratio (FEH: RFH)	Total glycine (gL^{-1})	Total tyrosine (gL^{-1})	FEH:RFH volume ratio	α-Amylase activity (U g DBM^{-1})
X	4:2	0.51	0.31435	138.6:1	1123 ± 0.4
XI	4:4	0.68	0.3327	69.3:1	1133 ± 0.8
XII	4:6	0.85	0.35105	46.2:1	1166 ± 0.4
XIII	4:8	1.02	0.3694	34.6:1	1202 ± 0.6
XIV	4:10	1.19	0.38775	32.6:1	2328 ± 0.2

*FEH: RFH -Fish extract hydrolysate: Fish hydrolysate amino acid ratio was based on glycine content.

Table 5. Effect of same total amino acid content (except in fish extract hydrolysate) in terms of glycine in different activation media on the activation of *B. licheniformis* 6346 and on α-amylase production in formulated solid medium at 42°C. Highest activity was produced on 4th day and presented.

Activation medium	Sample	Glycine (gL^{-1})	Tyrosine (gL^{-1})	α-Amylase activity (U g DBM^{-1})
IX	Fish extract hydrolysate	0.3675	0.32	1094 ± 0.9
XV	Diluted residual fish hydrolysate	0.88	0.095	1061 ± 0.4
XVI	Fish Extract hydrolysate: residual fish hydrolysate (2: 1)	0.88	0.1133	1294 ± 0.4
XVII	Nutrient broth	0.88	3.3	1188 ± 0.2

volume ratio of 145: 855 with starch and $(NH_4)_2HPO_4$ (Activation medium VIII, Table 1), led to the highest α-amylase activity of 1202 U g DBM^{-1} (Table 3) on the day 4. When fish extract hydrolysate-starch-$(NH_4)_2HPO_4$ was used as the inoculum (Activation Medium IX, Table 1) 1094 U g DBM^{-1} of α-amylase activity was produced on day 4 (Table 3). Mixing of residual fish hydrolysate and fish extract hydrolysate reduced the α-amylase activity to 84.3% of that obtained with Activation medium IV, while in fish extract hydrolysate-starch-$(NH_4)_2HPO_4$ the activity reduced to 76.7%. If residual fish hydrolysate and fish extract hydrolysate are mixed in a suitable ratio, better activity could be obtained.

The variation in α-amylase production could be due to the amino acid contents of the fish derivatives. If the *B. licheniformis* is activated in different media prepared with fish extract hydrolysate and residual fish hydrolysate by changing the amino acid contents, the importance of amino acids on α-amylase production can be studied. When fish extract hydrolysate was mixed with residual fish hydrolysate in different ratios (Activation Media X - XIV, Table 1) and supplemented with $(NH_4)_2HPO_4$ – starch, α-amylase production was increased with an increase in total amino acid content (Table 4). As the amount of residual fish hydrolysate supplementation increased, enzyme production did increase but not in the similar fashion (Table 4). Mixing fish extract hydrolysate to residual fish hydrolysate in the volume ratio of 32.6: 1 gave highest α-amylase production. To find whether the total amino acid content has any influence on *B. licheniformis*, activation media with same amino acid concentrations were prepared.

The activation media with residual fish hydrolysate, mixture of residual fish hydrolysate and fish extract hydrolysate (in volume ratio of 1:2) and nutrient broth were prepared with same glycine content (0.88 gL^{-1}) while fish extract hydrolysate was prepared with lower amount of total amino acid content (0.3675 gL^{-1}, Activation Media XV-XVII and IX, Table 2). α-Amylase production was highest when *B. licheniformis* was activated in the activation medium prepared from residual fish hydrolysate and fish extract hydrolysate (Activation medium XVI) followed with activation medium prepared from nutrient broth (Activation medium XVII), fish extract hydrolysate (Activation medium IX) and diluted residual fish hydrolysate (Activation Medium XV). Thus, the same total amino acid concentration in terms of glycine or tyrosine has no correlation with the activation of *B. licheniformis* (Table 5).

Therefore, the results indicated that for the activation of *B. licheniformis* more than the concentration of amino acid, the composition of amino acids present in the activation medium takes an important role. The amino acids present in fish extract hydrolysate were better for the activation of *B. licheniformis* than those present in residual fish hydrolysate and nutrient broth. When fish hydrolysate was used at higher concentration, the composition of amino acid needed for the activation

seems to be suitable. It is also important to note that α-amylase activity with nutrient broth supplemented with $(NH_4)_2HPO_4$ and starch activation medium (1188 Units g DBM^{-1}, Activation Medium XVII, Table 5) was higher than that obtained with the nutrient broth - starch activation medium (1075 Units g DBM^{-1} Activation Medium 1, Figure 1). The enzyme activity obtained with high concentrations of residual fish hydrolysate was able to activate *B. licheniformis*. However, fish extract hydrolysate to residual fish hydrolysate in the volume ratio of 32.6: 1 seems to be superior to all other activation media used in this experiment.

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REFERENCES

Aiyer PVD (2004). Effect of C: N ratio on alpha-amylase production by *Bacillus licheniformis* SPT 27. Afr. J. Biotechnol. 3(10): 519-522.

Anto H, Trivedi U, Patel K (2006). Alpha amylase production by *Bacillus cereus* MTCC 1305 using solid state fermentation. Food. Technol. Biotechnol. 44(2): 241-245.

Arasaratnam V, Thayaananthan K (2009). Substitutes for GPR grade salts for α-amylase production by *Bacillus licheniformis* 6346. Malaysian J. Biochem. Molec. Biol. 17(1): 1-4.

Bierbaum G, Karutz M, Weuster- Botz D, Wandrey C. (1994). Production of protease with *Bacillus licheniformis* mutants insensitive to repression of exoenzyme biosynthesis. Appl. Microbiol. Biotechnol. 40: 611-617.

Chandra AK, Medda S, Bhadra AK(1980). Production of extracellular thermostable α–amylase by *Bacillus licheniformis*. J. Ferment. Technol. 58(11): 1-10.

Dey N, Soni R, Soni Sk (2001). A novel thermostable α–amylase from thermophilic *Bacillus* sp. SN-1 and its application in the liquefaction of sorgum starch for ethanol fermentation. Asian J. Microbiol. Biotechnol.

Dimitrovski A, Doneva-Spaceska D (1995). Utilization of rice bran as substrate for α–amylase production by *Bacillus* sp. 1b1-3. Mikrobiologica, 32(2): 225-233.
Environ. Sci. 4: 1-6.

Gosh SB, Chandra AK (1984). Nutritional requirements and cultural characteristics of *Bacillus apiarius* CBML-152 for the production of α–amylase. Bibl. Microbiol. 139: 293-304.

Haq IU, Ashraf H, Iqbal J, Qadeer MA (1999). Production of alpha-amylase by *Bacillus licheniformis* using an economical media. Bioresour. Technol. 87: 57-61.

Malhotra R, Noorwez SM, Satyanarayana T (2000). Production and partial characterization of thermostable and calcium-independant alpha-amylase of an extreme themophilic *Bacillus thermooleovorans* NP 54. Let. Appl. Microbiol. 31: 378-384.

Mann FG, Saunders BC (1975). Estimation of amino acids. In: Mann FG, Saunders BC (eds) Practical Organic Chemistry. 4th Edition. Longman Group Ltd, London, pp. 463-464.

Mc Mahon HEM, Kelly CT, Forgarty WM (1999). High maltose producing amylolytic system of *Streptomyces* sp. Biotechnol. Lett. 21: 23-26.

Miller GL (1959). Use of dinitrosalicylic acid reagent for the determination of reducing sugar. Anal. Chem. 313: 426-428.

Mukaherjee AK, Borah M, Rai SK (2009). To study the influence of different components of thermostable substrates on induction of extracellular α–amylase synthesis by *Bacillus subtilis* DM-03 in solid-state fermentation and exploration of feasibility for inclusion of α–amylase in laundry detergent formulations. Biochem. Eng. J. 43: 149-156.

Murthy PS, Naidu MM, Srinivas P (2009). Production of -amylase under solid-sate fermentation utilizing coffee waste. J. Chem. Technol. Biotechnol. 84: 1246-1249.

Pederson H, Nielson J (2000). The influence of nitrogen sources on the alpha amylase productivity of *Aspergillus oryzae* in continuous culture. Appl. Microbiol. Biotechnol. 53(3): 278-281.

Pederson V, Thayaananthan K (2000). Substitutes for GPR grade salts for α–amylase production by *Bacillus licheniformis* 6346. Malaysian J. Biochem. Mol. Biol. 17(1): 1-4.

Ramesh MV, Chand N, Charyulu NCLN, Lonsane BK (1993). Cell growth in solid state fermentation system: the phenomenon and its mathematical description in nutritionally different media for production of alpha-amylase by *Bacillus licheniformis* M27. Chem. Mikrobiol. Technol. Lebensm. 15(5-6): 138-145.

Ramesh MV, Lonsane BK (1987). A novel thermostable alpha-amylase system produced under solid state fermentation. Biotechnol. Lett. 9(7): 501 -504.

Ramesh MV, Lonsane BK (1987). Solid state fermentation for production of α–amylase by *Bacillus megaterium* 16M. Biotechnol. Lett. 9(5): 323-328.

Ramesh MV, Lonsane BK (1989). Solid state fermentation for production of higher titres of thermostable alpha-amylase with two peaks for pH optima by *Bacillus licheniformis* M 27. Biotechnol. Lett. 11(1): 49-52.

Rick W (1974). Trypsin In: Bergmeyer HV (ed). Methods in Enzymatic Analysis. Academic Press, New York. 2: 1013-1015.

Shukla J, Rita K (2006). Potato peel as a solid state substrate for the thermostable α–amylase production by thermophilic *Bacillus* isolates. W. J. Microbiol. Biotechnol. 22: 417-422.

Srivatsava CBL (1985). Fish as a Food Commodity. In: Srivatsava CBL (ed). A Text Book of Fish Science and Indian Fisheries. KITAB Mahal, Allahabad. p. 48.

Swain MR, Ray RC (2007). Alpha amylase by *Bacillus subtilis* CM3 in solid state fermentation using cassava fibrous residue. J. Basic Microbiol. 47: 417-425.

Terezinha JGS, Iracema OMH (1994). Cell growth and α-amylase production characteristics of *Bacillus subtilis* ATCC 601. Development Food Engineering. In: Abstract: Proc 6th Int. Congr. Eng. Food. Toshimasa Y, Ryuichi M, Koza N, Blackie (eds) Glasgow. pp. 534-536.

Tonkova A, Ivanova V, Dobreve E, Stefanova M, Spasova D. (1994). Thermostable α–amylase production by immobilised *Bacillus licheniformis* cells in agar gel and on acrylonitrile/ acrylamide membrane. Appl. Micrbiol. Biotechnol. 41: 517-522.

Wickramanayake TM (1998). Other foods of Animal Origin. In: Wikramanayake TM (ed) 3rd ed. Food and NutritionHector Kobbekaduwa Agrigarian Research and Training Institute, Colombo, 7: 195-201.