Available on line www.jocpr.com

Journal of Chemical and Pharmaceutical Research

J. Chem. Pharm. Res., 2010, 2(4):442-448

ISSN No: 0975-7384
CODEN(USA): JCPRC5

Efficient production of Alpha – amylase from agro residues using *Bacillus subtilis*

M. Nagarajan, T. Deborah Paripuranam, S.Umamaheswari*

Department of Biotechnology,
Manonmaniam Sundaranar University,Alwarkurichi – 627 412,
Tamil Nadu, India

ABSTRACT

Two different agro residues were screened for alpha- amylase production using the Gram positive rod, Bacillus subtilis. Supplementation with different nitrogen and carbon source favoured the increased enzyme yield by submerged fermentation. Maximum enzyme yield titre was expressed as units as per micro mole of glucose yields. The objective of this study is to select a suitable strain for the production of amylase, screening of different agricultural by products as substrates for maximum enzyme production, application of different combinations of these substrates for enzyme production and optimization of cultural conditions for the production of amylase. Enzyme yield of Wheat bran and Rice bran were 51.04 ± 0.54 IU/g and 12.42 ± 0.15 IU/g respectively at the time of 40hr incubation at 37˚C of which the wheat bran (WB) was proved as the best substrate source.

Key word: Wheat bran, Rice bran, *Bacillus subtilis*, Alpha- amylase

INTRODUCTION

Alpha- amylases (indo-1, 4 α D- glucan glucanohydrolase) are extra cellular cellular endo enzymes that randomly cleave α-1, 4 linkages between adjacent glucose units in the linear amylase chain and ultimately generate glucose, maltose and maltotriose units. Enzymatic hydrolysis of starch has now replaced acid hydrolysis in over 75% of starch hydrolysing process due to many advantages. Microorganisms like fungi and bacteria have been extensively used for gluco – amylase production [1-3]. Several Bacillus species and thermostable *Actinomycetes* including *Thermonospora* and *Thermoactinomycetes vulgaris* are versatile producers of α-amylases [4]. These are extra cellular enzymes acting at the ends. The final products of α –

amylase are glucose and maltose. Alpha – amylase are used in various industries like starch industry to produce glucose, fructose and maltose by liquefaction of starch.

Sources of α – Amylases

Alpha – amylases are ubiquitous enzymes produced by plants, animals and microbes which play a dominant role in carbohydrate metabolism. Amylases from plant and microbial sources have been employed for centuries as food additives. Barley amylases have been used in the brewing industry. Fungal amylases have been widely used for the preparation of oriental foods. In spite of the wide distribution of amylases, microbial sources namely fungal and bacterial amylases are used for the industrial production due to cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization (Bruhan). Among bacteria, *Bacillus sp.* is widely used for thermostable α – amylase production to meet the industrial needs. *B. subtilis, B.stereothermophilus, B.licheniformis and B.amyloliquefaciens* are known to be good producers of α – amylase and these have been widely used for commercial production of the enzyme for various applications. Similarly, filamentous fungi have been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of extracellular proteins, they are widely exploited for the production of different enzymes including α-amylase. Fungi belonging to the genus *Aspergillus* have been most commonly employed for the production of α – amylase. Production of enzymes by Solid State Fermentation (SSF) using these moulds turned a cost effective production technique. Detailed literature is available on various microbial sources for the production of amylases [5-6].

EXPERIMENTAL SECTION

Selection of amylase producing bacteria

Amylase producing *Bacillus subtilis* was sub – cultured from the stock cultures maintained in the Department of Microbial Biotechnology, Sri Paramakalyani Centre for Environmental Sciences, Alwarkurichi. It was maintained in Nutirent Agar slants at 37ºC for 24hr and stored at 4ºC which was repeatedly sub – cultured at every 2 weeks. The selected *Bacillus subtilis* was optimized at different pH and temperature for its optimal growth.

Purification of amylase producing *Bacillus subtilis*

Bacillus subtilis was screened by performing various biochemical tests by following standard procedures [7].

Substrates

Wheat bran (WB) and rice bran (RB) were obtained from local mills at Ambasamudram Taluk, Tamil Nadu and ground into coarse powder with a blender.

Preparation of fermentation medium

In an attempt to choose a potential substrate for Solid State Fermentation (SSF) which supports amylase production, both agro residues like wheat bran and rice bran were screened individually and fermentation was carried our by optimizing medium with 12% wheat bran, 10% Sucrose, 0.5% urea, 0.25g/l magnesium sulphate and potassium chloride in 250ml conical flask. The flasks were mixed and autoclaved at 121ºC for 20 minutes into which 1ml of the culture broth was inoculated and incubated at 37ºC for 24hrs.

Optimization of culture conditions

The best substrates were employed for further optimization of process parameters viz., initial incubation time from 4hrs to 48hrs with an interval of 4hrs, at different incubation temperatures

(25º, 30º, 35º, 40º, 45ºC), initial pH (5, 6, 7, 8, 9) of the medium. Nutrient supplementation such as substrate optimization at 2%, 4%, 6%, 8%, 10%, 12% concentrations, inorganic nitrogen sources (ammonium fluoride, ammonium nitrate, ammonium per sulphate and ammonium chloride) and organic nitrogen source viz., urea was used in a concentration of 1% with varying carbon sources (Glucose, Fructose, Maltose, Sucrose, Lactose) were subjected to optimization. Crude enzyme was extracted by centrifuging the sample at 8000rpm for 20min and it was subjected to protein estimation.

Assay of protein concentration

The protein concentration was determined by the Lowry's method [8] using bovine serum albumin as the standard.

Statistical analysis

All the experiments were carried out in triplicates. Starch content was calculated by using glucose standard graph of the substrate. Starch was estimated by multiplying factor 0.9 with glucose content. α – amylase can be calculated by IU (International Unit) of enzyme which is the amount of enzyme releasing one micro mole of glucose equivalent per minute under the assay conditions. Glucose was used as a standard and was read at 560nm in spectrophotometer. The samples collected from each replicate were tested for amylase production and activity. Means of amylase activity and production were calculated.

RESULTS AND DISCUSSION

Optimization of pH and Temperature on *Bacillus subtilis*

The pH is known to affect the synthesis and secretion of α – amylase just like its stability [9]. The growth of *Bacillus subtilis* was generally in the pH range of 7 to 8. Reduced growth was observed at pH 5 whereas the growth was moderate at pH 6 and 9. The present study confirms the coincidence of optimum pH with bacterial cultures such as *B.subtilis, B.licheniformis* and *B.amyloliquefaciens* which required an initial pH of 7.0 [10-12]. Similarly, the temperature range from 30º - 35ºC was observed to be optimum for better growth of *B.subtilis*. Decreased growth was observed in temperatures maintained at 40ºC and above.

Table 1: Production of α amylase from wheat and rice bran substrates at different time intervals

Incubation Time (hours)	Amylase activity (IU/g)		
	Starch (control)	Wheat bran	Rice bran
4	46.70 ± 1.51	4.34 ± 0.09	3.41 ± 0.06
8	73.43 ± 2.02	5.98 ± 0.26	3.93 ± 0.21
12	109.79 ± 3.02	7.90 ± 0.59	3.64 ± 0.14
16	148.83 ± 12.35	11.57 ± 0.18	4.50 ± 0.24
20	170.04 ± 4.03	24.36 ± 1.35	5.27 ± 0.09
24	294.62 ± 7.31	27.92 ± 1.17	5.48 ± 0.09
28	319.75 ± 2.02	33.04 ± 0.76	6.53 ± 0.87
32	375.00 ± 19.66	36.56 ± 0.76	7.56 ± 0.81
36	621.86 ± 42.09	40.75 ± 0.97	11.71 ± 0.91
40	351.83 ± 13.11	51.04 ± 0.54	12.42 ± 0.15
44	190.35 ± 64.53	49.89 ± 0.08	11.69 ± 1.61
48	112.47 ± 7.81	46.83 ± 0.06	13.05 ± 0.23

Mean±SD

Effect of incubation time on amylase production

The maximum amylase activity for wheat and rice bran was observed to be 51.04 ± 0.54 and 12.42 ± 0.15 IU/g respectively at the 40th hour incubation after which the amylase activity decreased. (Table 1)

Fig 1: Influence of pH on production of α -amylase from wheat bran and rice bran substrates

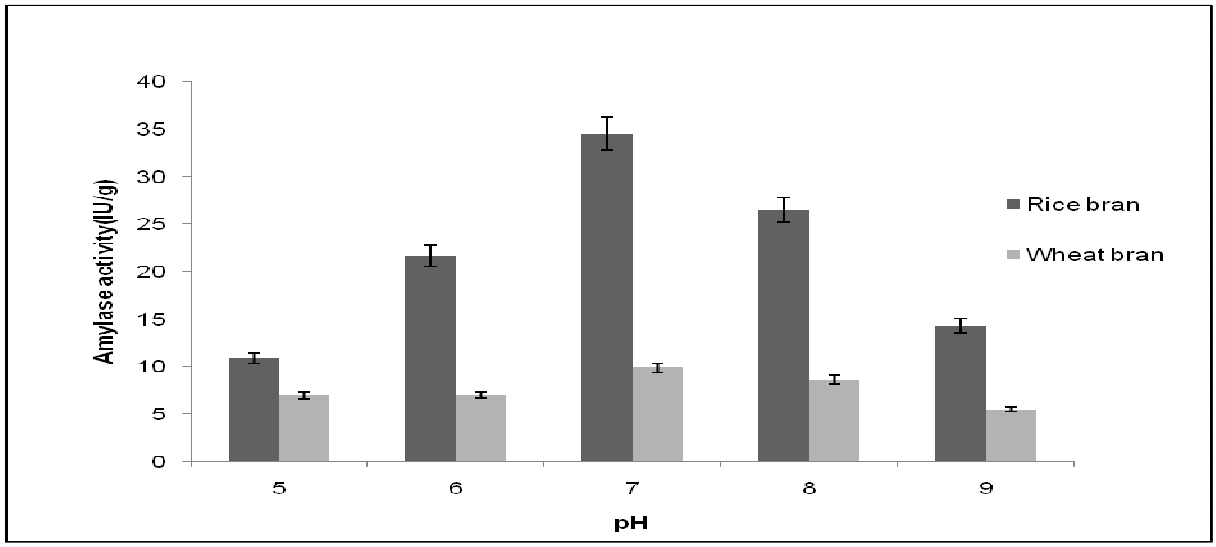
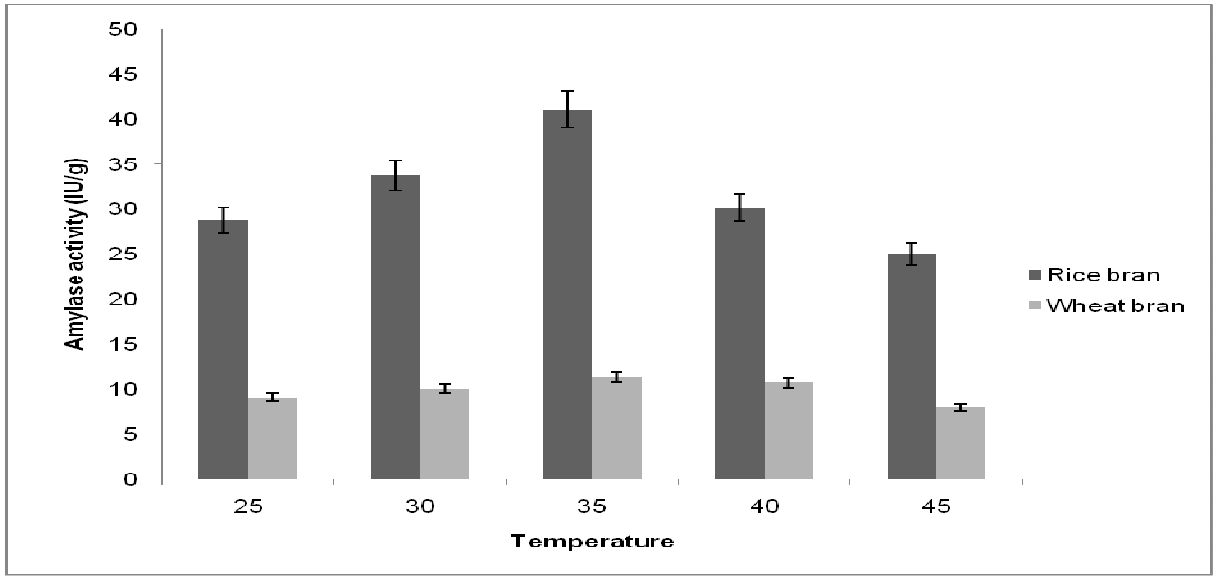


Fig 2: Influence of Temperature on production of α -amylase from wheat bran and rice bran substrates



Influence of pH and temperature on enzyme production

Temperature and pH are the most important factors which markedly influence enzyme activity. Thermophilic anaerobic bacterial *Clostridium thermosulfurogenes* gave maximum titres of α – amylase at pH = 7.0 [13] which corroborates with the enzyme production being with neutral pH (7.0) in the initial medium for both wheat and rice bran (34.53 ± 0.42 IU/g and 9.90 ± 0.79 IU/g)

respectively. Fig. 1 and 2 indicates maximum amylase production at 35º - 40ºC for both wheat and rice bran (41.09 ± 1.22 IU/g) and (11.29 ± 0.11 IU/g). Generally, bacterial amylases are produced at a much wider range of temperatures.

Influence of substrate concentration

Table 5 shows wheat bran (12%) and rice bran (10%) were optimal for better amylase production. It has been reported that high concentration of substrate suppresses the amylase production due to the mechanism of feedback inhibition (Table 2).

Table 2: Influence of substrate concentrations on the production of amylase from wheat and rice bran substrates

Substrate concentration (%)	Amylase activity (IU/g)	
	Wheat bran	Rice bran
2	6.43 ± 0.01	2.54 ± 0.26
4	9.46 ± 0.79	5.03 ± 0.18
6	16.16 ± 0.37	5.58 ± 0.25
8	23.37 ± 0.25	7.08 ± 0.10
10	33.44 ± 0.86	10.85 ± 0.30
12	44.68 ± 1.39	8.47 ± 0.08
14	32.19 ± 0.35	7.44 ± 0.42
16	18.87 ± 1.21	6.91 ± 0.14

Influence of nitrogen source on amylase production

Table 3 illustrates the maximum amylase production in urea as nitrogen source for both wheat and rice bran (39.58 ± 0.21 IU/g and 10.77 ± 0.47 IU/g) which was higher than control.

Table 3: Influence of various nitrogen sources on production of amylase from wheat and rice bran substrates

Various Nitrogen sources (0.1%)	Amylase activity (IU/g)	
	Wheat bran	Rice bran
Control	28.37 ± 0.06	7.62 ± 0.16
Urea	39.58 ± 0.21	10.77 ± 0.47
Ammonium nitrate	18.51 ± 0.44	5.641 ± 0.11
Ammonium chloride	23.25 ± 2.38	7.129 ± 0.03
Ammonium ferrous sulphate	13.41 ± 0.24	3.681 ± 0.11

Influence of carbon source on amylase production

Amylase production under the influence of carbon sources is indicated in Table 4. Higher amylase production was observed in Sucrose for both wheat and rice bran (33.08 ± 0.88 IU/g and 10.59 ± 0.45 IU/g) when compared to control.

Table 4: Influence of various carbon sources on production of amylase from wheat and rice bran substrates

Various carbon sources (0.1%)	Amylase activity (IU/g)	
	Wheat bran	Rice bran
Control	28.05 ± 1.15	7.08 ± 0.09
Glucose	32.69 ± 0.55	8.95 ± 0.10
Sucrose	33.08 ± 0.88	10.59 ± 0.45
Maltose	26.56 ± 0.23	7.50 ± 0.50
Fructose	29.45 ± 0.24	5.94 ± 0.06
Lactose	25.70 ± 0.10	5.60 ± 0.20

Protein concentration of enzyme extracts

The enzyme extract of starch substrate was found to have a maximum protein concentration of 0.448 ± 0.004% indicating the amylase production. It has also been proved by protein concentration of enzyme extracts of wheat and rice bran substrates (Table 5).

Table 5: Protein concentration of enzyme extracts in different substrates at 36 hour of incubation

Substrates	Protein concentration of enzyme extracts (%)
Starch (Control)	0.448 ± 0.004
Wheat bran	0.242 ± 0.002
Rice bran	0.138 ± 0.007

Mean ± SD

CONCLUSION

Study on the evaluation of wheat and rice bran as a substrate and analysing the effect of various fermentation parameters (Fermentation period and temperature, pH of the production medium, inorganic and organic nitrogen sources, carbon sources and substrate concentration for the production of α – amylase by *Bacillus subtilis* was carried out.

The enzyme is observed to be very sensitive to pH. Among the various pH studied, pH 7 to 8 was considered to be optimum for the better growth of *Bacillus subtilis.* Among the two substrates screened for amylase production, wheat bran gave highest amylase activity at 36 hours of incubation when compared with the rice bran due to the presence of higher starch content in it. The enzyme production was maximum when the initial medium was neutral (pH 7) for both wheat and rice bran with the temperature range from 30º - 40ºC as better for amylase production which also supported the growth of *B.subtilis.*

From this investigation, 12% of wheat bran concentration and 10% of rice bran concentration were considered as optimal for better amylase production. Among the inorganic nitrogen sources added to the medium, no higher yield of amylase production was noted compared with the control but organic nitrogen source of urea enhanced the amylase production in both the substrates. Higher amylase production was recorded in the carbon source incorporated medium than the control. From this investigation, wheat bran was recorded to have higher efficiency in amylase production among the agro residues studied. α –amylases are one of the most widely used enzymes required for the preparation of fermented foods. With an increase in its application spectrum, there exists a demand for the enzyme with specificity. Research is focused on

developing thermo tolerant and pH tolerant α-amylase from microbes, modifying them genetically or applying site – directed mutagenesis to acquire desired properties in the enzyme. Commercially most of the production of α – amylase is carried out in submerged fermentation but solid – state fermentation is being looked as a potential tool for its production, especially applying agro industrial residues as substrate.

Acknowledgement
The authors thank the Vice –Chancellor for providing the needed facilities for carrying over the work.

REFERENCES

[1] AK Gupta; SP Grautam. *World J. Microbiol & Biotechnology*., **1995**, 11, 193–195.
[2] Y Haolin; S Xin; Z Lina. *Weisheng Wuxve Zazhi*., **1996**, 16, 26.
[3] V Ivanova; D Yankov; L Kabaivanova; D Pashkoulov. *J. Biochem. Eng*., **2001**, 8, 61- 81.
[4] M Ben; AM Meghani; S. Bejar. *Enzyme. Microb. Technol*., **1999**, 24, 548 – 549.
[5] M Vihinen; P Mantasala. *Crit. Rev. Biochem. Mol. Biol*., **1989**, 24, 329 – 418.
[6] A Pandey; P Nigam; CR Soccol; VT Soccol; D Singh; R Mohan. *Biotechnol. Appl. Biochem*., **2000**, 31,135 – 152.
[7] JS Cappucino; Sherman, Microbiology – A Laboratory Manual, 6th Edition. Pearson Education, Dorling Kindersley Publishers, New Delhi, **2003**; pp-107.
[8] OH Lowry; NJ Rosebrough; AL Farr; RJ Randall. *J. Biol.Chem*., **1951**, 48, 17-25.
[9] MW Fogarty; Microbial Amylases. In: Microbial Enzymes and Biotechnology, WM. Forgarty (Edition), Applied Science Publishers Ltd, London, UK **1983**; pp -1-92.
[10] MS Tanyildizi; D Ozer; M Elibol. *Process Biochem*., **2005**, 40, 2291 – 2296.
[11] MJ Syu; YH Chen. *Chem. Eng. J*., **1997**, 65, 237 – 247.
[12] IU Haq; H Ashraf; J Iqbal; MA Qadeer. *Pak. Jour. Biological Sci*., **2002**, 2, 73 – 75.
[13] MV Swamy; G Seenayya. *Process Biochem*., **1996**, 31, 157 – 162.