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Amylase production under solid state fermentation by a bacterial isolate W74

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This study was concerned with the screening of a suitable isolate and optimization of cultural conditions for the biosynthesis of thermostable amylase under solid state fermentation (SSF). Twenty seven isolates were screened for amylase production out of which one isolate designated as W74 showed maximal amylase activity at 70°C and a pH of 6.5 and selected for further optimization of cultural conditions under SSF. Among the different carbon and nitrogen sources supplemented to wheat bran, starch (96.7 U/g) and casein (107.3 U/g) enhanced maximum amylase production. Addition of exogenous glucose repressed secretion of amylase, demonstrating that a classical glucose effect was operative in this organism. Cultural optimization was undertaken to evaluate the effect of main process parameters as incubation period (144 h), moisture (66.7%), inoculum size (40%), and initial medium pH (6.5) on enzyme production. The enzyme was optimally active at 70°C and in pH range of 5.5-6.5.

Key words: Thermostable amylase, solid state fermentation, wheat bran, enzyme

INTRODUCTION

Enzymatic hydrolysis of starch is carried out under temperatures up to 100°C, normal pressure, and pH of medium around 6.0 to 8.0. However, enzymes are relatively expensive and above all thermally unstable at higher temperatures as reviewed in Hossain et al. (2006). Attempts are now being made to find enzymes from thermophilic microorganisms. Extreme environments (high temperature and acidic environments) harbor a wide range of acidophilic hyperthermophilic organisms including members of both bacteria and archaea prokaryotic subdivisions (Worthington et al., 2003). These properties imply extremely important industrial and biotechnological implycations due to the fact that enzymes from such microorganisms can be employed for use in harsh industrial conditions where their specific catalytic activity is retained (Haki and Rakshit, 2003). A new strain of Bacillus sp.

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I-3 was isolated from natural soil samples by Soni et al. (2005) and the crude α-amylase extract showed maximum activity at 70^0C, pH 7. It has been reported by Mamo et al. (1999) that a thermostable amylase producing microbe, *Bacillus* sp. WN11, was isolated from Wondo Genet hot spring. Similarly, Haki and Rakshit (2004) isolated bacterial colonies from Ethiopian hyperthermal springs at Arbaminch, Awassa, Nazreth, Shalla and Abijata, Wendo Genet and Yirgalem. The thermostability experiments showed that more thermotolerant enzymes were isolated from Shalla and Abijata, followed by Awassa, where the temperatures of the water were also the highest. The bacterial colonies were identified as *B. stearothermophilus* and *B. licheniformis* by Haki and Rakshit (2004). Furthermore, Muluye Teka (2006) has isolated a *Bacillus* sp. from Lake Chitu and found amylases with optimum temperature of 80^0C. The present study was aimed to isolate and screen bacterial species from hot spring soil samples for the production of thermostable amylases under SSF.

EXPERIMENTAL METHODS

Bacterial Isolation

The bacterial strains used in this study were isolated by directly inoculating 1 g of soil samples from Gendysony thermal spring located in Arbaminch area, Ethiopia, into 10 g solid substrate (wheat bran) containing 0.5 g soluble starch. The inoculated bran was statically incubated at 37°C. After 5 days of incubation a sample was taken, serially diluted and spread plated on to starch agar containing 0.5% soluble starch as used by Elnasser et al. (2007). Screening of isolates for amylolytic activity was carried out by growing the organisms on starch agar plates containing 0.5 %(w/v) starch and subsequently staining with iodine solution (1% I_2 (w/v) in 2% (w/v) KI). The presence of a halo around the colony was indicative of amylolytic activity. The composition of starch agar used in the study was as follows: starch (0.5%), bacteriological peptone (0.2%), $MgSO_4$ (0.02%), $CaCl_2$ (0.02%), K_2HPO_4 (0.1%) and agar 1.5%. All the materials and reagents used in this study were obtained from microbiology laboratory at Addis Ababa University, Science Faculty.

Screening for thermostable bacterial amylases

The isolated strains were further screened for their ability to produce thermostable amylases, with assay temperatures ranging from 50 to 90°C at 10°C intervals and incubation period of 10 min. The individual isolates were re-inoculated into solid media and after 5 days of incubation, enzyme was extracted with 100 mL distilled water for assaying. The isolate with the best enzyme activity at 70°C was selected and taken for further investigations.

Inoculum preparation

For inoculum preparation, 250 mL Erlenmeyer flasks containing 50 mL of starch broth were inoculated with a loop full of cells from a 24 h slant and kept in a rotary shaker (120 rpm) at room temperature. After 24 h of incubation, 3 mL of culture were used as the inoculum. By serial dilution and plating, the number of viable colonies in the inoculum was determined.

Enzyme production in SSF

The SSF process was carried out in 250 mL Erlenmeyer flasks using 10 g of wheat bran solid substrate (Table 4). After proper agitation of the substrate, it was autoclaved at 121°C for 15 min, allowed to cool to room temperature, and inoculated with 3 mL of 24 h old culture. Substrate moisture ratio was adjusted to 1:2. Subsequently, incubation was carried out statically at 37°C for five days. The SSF media flasks were gently shaken after every 24 h for uniform mixing up of substrate and inoculum.

Enzyme extraction

The extracellular enzymes from the fermented bacterial bran were extracted with distilled water (100mL) after agitated on a rotary shaker at 120 rpm for 30 min. The content was filtered and squeezed out through a cotton cloth. The filtrate was centrifuged at 10,000 × g for 10 min to separate small particles, cells, and spores. The brown, clear supernatant was used in enzyme assay as the crude enzyme.

Amylase activity assay

Amylase activity was determined by the procedure of Anto et al. (2006) using wheat starch, gelatinized on a heater, as a substrate. The reaction mixture containing 0.9 mL of 1% substrate in 0.01 M KH_2PO_4/K_2HPO_4 buffer, pH 6.5, and 0.1 mL of enzyme extract was incubated for 10 min at 50°C. The reaction was stopped by adding 2 mL of 3, 5-dinitrosalicylic acid solution (DNS) followed by heating in a boiling water bath for 5 min and cooling to room temperature. The absorbance of each solution containing the brown reduction product was measured at 540 nm. Enzyme assay was performed in triplicates and the average was calculated. One unit (U) of α-amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar as glucose per minute, under the assay conditions and expressed as U/g of dry substrate (Anto et al., 2006). The composition of the DNS reagent used in the study was as follows (g/L): phenol 2; sodium sulfite 0.5; sodium-potassium tartrate 20; NaOH 10; and dinitrosalicylic acid (DNSA) 10.

Optimization of fermentation process under SSF

The SSF of wheat bran for production of extracellular amylase was optimized by varying process conditions like time course, moisture level, inoculum size, carbon and nitrogen additives and initial medium pH. The strategy followed was to optimize each parameter, independently of the others and, subsequently, optimal conditions were employed in all experiments. In all optimization procedures, enzyme assays were performed in triplicates and the average result used in data analysis.

Time course of enzyme production

Growth media containing 10 g of wheat bran were incubated for

Table 1. Enzyme activities for the newly isolated bacterial colonies (W74 and W120) under various temperature ranges.

Temperature (°C)	Enzyme activity (%)	
	Isolate W74	Isolate W120
50	66	72
60	95	100
70	100	90
80	23	22

70°C was taken as 100 % for W74 and 60°C for W120

varying time periods (24-240 h) at 37°C with 3mL of a 24 h old culture as inoculum. Individual flasks were withdrawn with 1 day interval from 24-240 h to assess for enzyme production. Five day incubation was formerly employed for screening bacterial strains with amylolytic potential.

Initial moisture content

Substrate moisture ratio (w/v) was maintained as 1:1, 1:1.5, 1:2, 1:2.5 and 1:3, and incubated for 144 h (optimum) and inoculum level 30% at 37°C.

Effect of inoculum size

The wheat bran media were inoculated with different inoculum levels (10, 20, 30, 40 and 50% (w/v) and SSF was carried out for 144 h with 66.7% moisture content as pre-optimized growth conditions and incubated at 37°C. Inoculum size was determined by counting colony forming units using serial dilution and plating techniques. A sample (1mL) was taken from 24 h inoculating culture, serially diluted (10^{-1} -10^{-9}) and spread on to starch agar. Colonies were counted from clearly visible dilutions and log number of cells/g bran calculated.

Effect of carbon and nitrogen additives

Various carbon sources (0.05 g/g dry substrate) such as monosaccharides (xylose, glucose and fructose) and disaccharides (lactose, sucrose and maltose) were evaluated for their effect on amylase production by replacing starch in the production medium. The flasks were inoculated with 30% inoculum and incubated at 37ºC for 144 h in a 1:2 substrate-moisture ratio. The optimum carbon source was found by analyzing the results of amylase production. A starch supplemented and a control without additional carbon sources was also included for comparison. The production medium was supplemented with different nitrogen sources (0.02 g/g dry substrate) of NH_4Cl, $NaNO_3$, $(NH_4)_2SO_4$, peptone, yeast extract, casein and urea to check their effect on enzyme production.

Effect of initial pH on enzyme production

In order to investigate the effect of pH on extracellular amylase production, the pH of the starch solution (in duplicates) was adjusted with 1 N NaOH and 1 N HCl at values 4, 5, 6, 7, 8, 9, 10, 11 and 12 prior to sterilization. When wheat bran was moistened with the above starch solution, the respective final pH recorded in one duplicate was 4=5.6, 5=5.6, 6=5.9, 7=6, 8=6.2, 9=6.5, 10=6.7, 11=7.0, and 12=7.7. The final medium pH (after adding wheat bran) was taken as initial medium pH. The media were then inoculated with 30% inoculum and fermentation was carried out at 37°C for 144 h with 66.7% moisture content. The optimum initial pH of the solid substrate was determined by the standard assay procedures described above.

Effect of repeated washes on amylase extraction

The fermented bran was washed with distilled water (100 mL) for five consecutive times. Enzyme activity in the respective washes was assayed using the standard assay procedures.

Spotting starch digest on thin-layer chromatography (TLC) for identifying the type of amylase

The products liberated by the action of amylase on soluble starch were identified by spotting the starch digest and standard sugars (glucose and maltose) on a silica gel plate activated at 105°C for 10 min. About 2.5 µl of the starch digest, glucose and maltose standards were spotted on TLC plates. The plates were developed in butanol: ethanol: water (50: 30: 20) and TLC was run four times to concentrate the bands. After air drying the plates, sprayed with 30% H_2SO_4 in ethanol and dried at 105°C for 10 min.

RESULTS AND DISCUSSION

Bacterial isolation

A total of 190 bacterial colonies were taken out of which 27 showed clear halos on starch agar plates. The 27 colonies were again inoculated into wheat bran and checked for enzyme production. The crude enzyme extract for all 27 isolates was assayed at 50 and 70°C, pH = 6.5. Among the 27 isolates two of them designated as W74 and W120 showing better activity at 70°C were further analyzed for their activity at various pH and temperature ranges (Tables 1 and 2). Thus, isolate W74 showed a better activity at 70°C and was selected for further optimization experiments.

Table 2. Effect of pH on enzyme activities by isolates W74 and W120 at 50°C.

pH		3.5	4.5	5.5	6.5	7.5	8.5
Enzyme Activity (%)	Isolate W74	13	62	93	100	89	78
	Isolate W120	22	63	100	54	43	25

pH 6.5 was taken as 100 % for W74 and 5.5 for W120

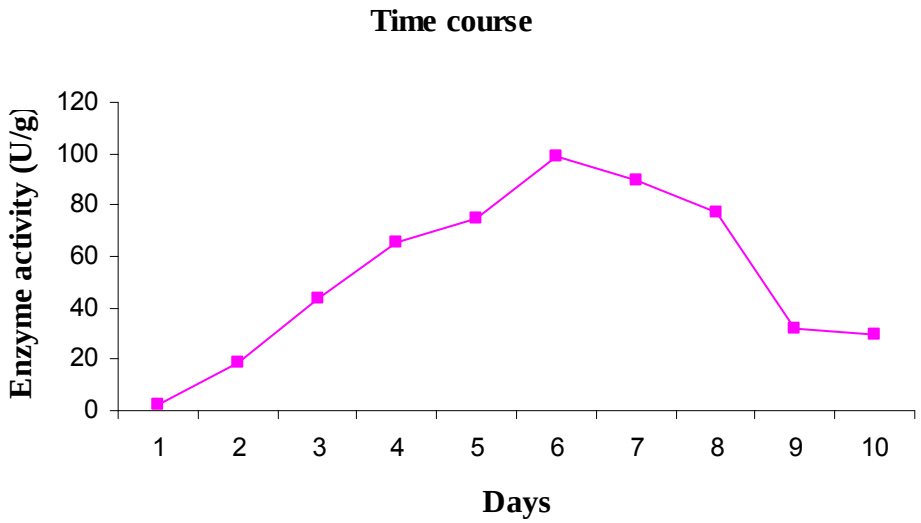

Figure 1. Time course of amylase production under solid state fermentation by isolate W74.

Optimization of cultural parameters

Time course of enzyme production

The incubation time was found to affect enzyme production (Figure 1) as it was related to the growth of the organism. There was a gradual increase in enzyme production through 24 and 48 h and maximum at 144 h (98.8 U/g). This may be because the cultures might be at stationary phase as Malhotra et al. (2000) showed that enzyme production was maximal when cells entered stationary phase. The lowest enzyme production was found at 24 h (2.6 U/g) and was 29.8 U/g at the 10^{th} day. The decline in enzyme production with prolonged incubation may be due to loss of moisture, slower growth, and lower enzyme production rates etc (Anto et al., 2006; Gangadharan et al., 2006).

Moisture levels

Moisture content changes during SSF as a result of evaporation and metabolic activities, therefore, adjusting moisture level can be very important. Enzyme production profiles with varying moisture levels (Figure 2) showed that SSF medium adjusted at 66.7% moisture content resulted in higher enzyme synthesis (114.1 U/g). Above 66.7%, enzyme production decreased. The effect of moisture level in SSF was also reviewed by Anto et al. (2006), Gangadharan et al. (2006) and Baysal et al. (2008). High moisture content might resulted in decreased substrate porosity, change wheat bran structure, promoting in development of stickiness, reducing gas volume, which in turn prevents oxygen penetration (Anto et al., 2006; Gangadharan et al., 2006).

Inoculum size

Inoculum size was found to be detrimental to enzyme production (Anto et al., 2006; Baysal et al., 2008; Gangadharan et al., 2006). Inoculum levels of 0.726, 0.756, 0.773, 0.786 and 0.795 (log number of cells/g bran) were assayed to determine their effect on enzyme

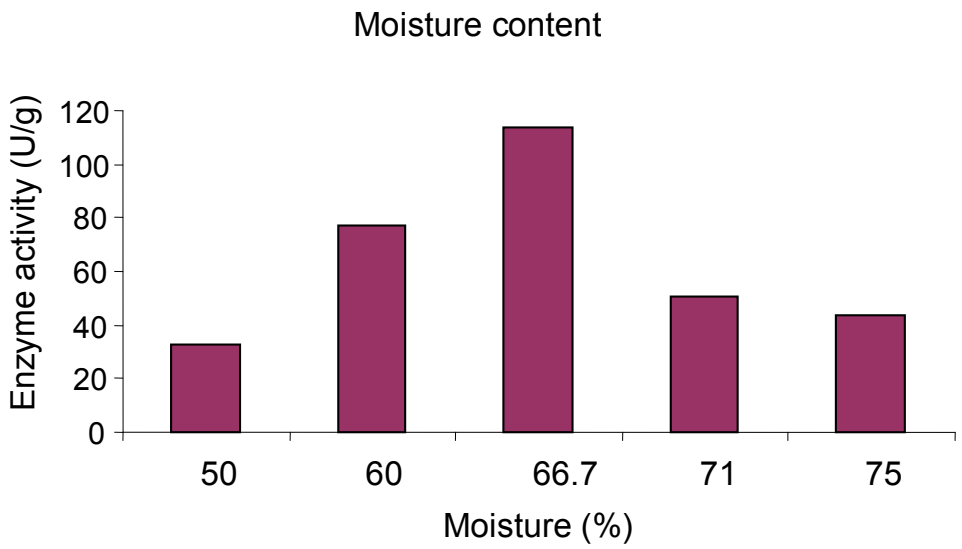

Figure 2. The influence of moisture content on amylase production under SSF.

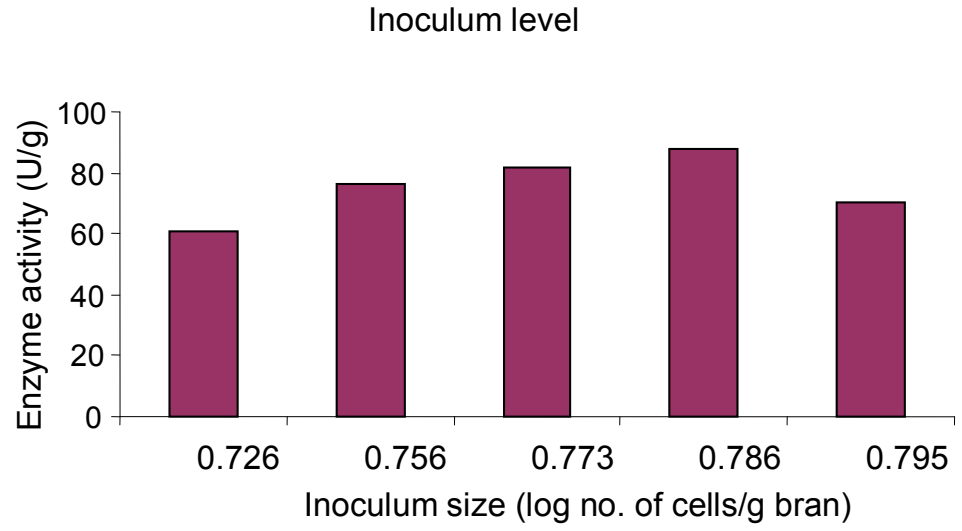

Figure 3. Influence of inoculum size of α-amylase production under SSF.

production. As shown in Figure 3, the maximum enzyme production (87.7 U/g) was observed at 0.786 (optimum) and the lowest (68.9 U/g) at 0.726. Enzyme production decreased with further inoculum increments. When inoculum size was varied from 0.726-0.795, enzyme production was between 69-93% with regard to the optimum which was taken as 100%. Low inoculum size 0.726 (log number of cells/g bran) resulted in relatively lower enzyme yield. The reduction in yield at this inoculum size could be due to lower number of viable cells for fermenting the given amount of substrate for the specified time interval. The maximum enzyme yield was obtained when 0.786 inoculum size was used. After that, there was a reduction in enzyme yield when inoculum size was increased to 0.795. This may be due to the limiting nutrients at higher inoculum size. With serial dilution and spread plating on starch agar, inoculum size (colony count) was found to be 1.8×10^7 CFU/mL, thus an inoculum size of 0.786 corresponds to 7.2×10^7 CFU/mL.

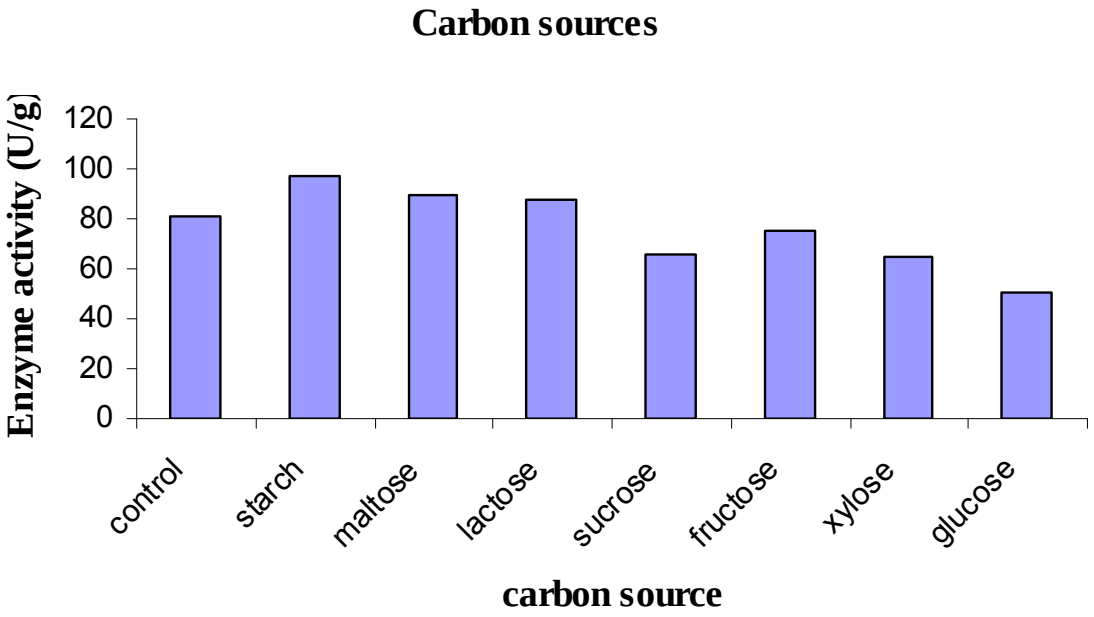

Figure 4. Effects of carbon sources on amylase (U/g) production by isolate W74 under solid state fermentation using wheat bran as substrate.

Effect of supplementation of carbon sources

The highest amylase production was obtained in a medium containing starch (96.7 U/g) (Figure 4). Starch was a generally accepted nutritional component for induction of amylolytic enzymes (Mamo and Gessesse, 1999; Kiran et al., 2005; Narang and Satyanarayana, 2001; Rasooli et al., 2008). It was also observed that maltose (89.5 U/g) and lactose (87.3 U/g) favored amylase production, whereas sucrose (65.6 U/g), fructose (74.8 U/g), xylose (64.6 U/g) and glucose (50.1 U/g) gave lesser results as compared to the control which yielded 81.3 U/g. It was evident that 84% (compared to the optimum) enzyme production was recorded from the control and only 51.8% was produced when glucose was added to the fermentation medium. It has also been reported that the synthesis of amylolytic enzymes is subjected to catabolic repression by glucose (Ezeji et al. 2005; Haseltine et al. 1996; Teodoro and Martins, 2000). Presumably the same phenomenon might justify this finding.

Effect of nitrogen source on amylase production

In the investigation of the effects of various nitrogen sources on amylase production, casein (107.3 U/g) (optimum) was found to be the most promising one, followed by yeast extract (82.7 U/g) and urea (81.3 U/g) (Figure 5). Relatively lower enzyme yields were recorded with addition of inorganic nitrogen sources and 45% reduction was recorded when $(NH_4)_2SO_4$ was used as nitrogen source. The synthesis of α-amylase was reported to be stimulated or inhibited by the type of amino acids present in the growth medium (Aguloglu et al., 2000; Park et al., 1996). In agreement with this study, organic nitrogen sources have been reported as a better inducer of amylase production than inorganic ones (Ul Qader et al., 2006; Nguyen et al., 2000).

Initial medium pH

Among the physicochemical parameters, pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme secretion. Results showing the effect of pH on amylase production by isolate W74 in SSF of wheat bran are presented in Figure 6. The maximum activity of amylase (192.7 U/g) was observed in the fermentation medium adjusted to pH 6.7. At pH 7.7, comparatively lower enzyme production was observed. The enzyme production was much better around neutral pH ranges and only 15% was produced at pH 5.6. Microbial product formation decreases on either side of the optimum pH value (Sudharhsan et al., 2007; Ul Qader et al., 2006). In the current study, amylase production was found to be very sensitive to initial pH of the fermentation medium that is

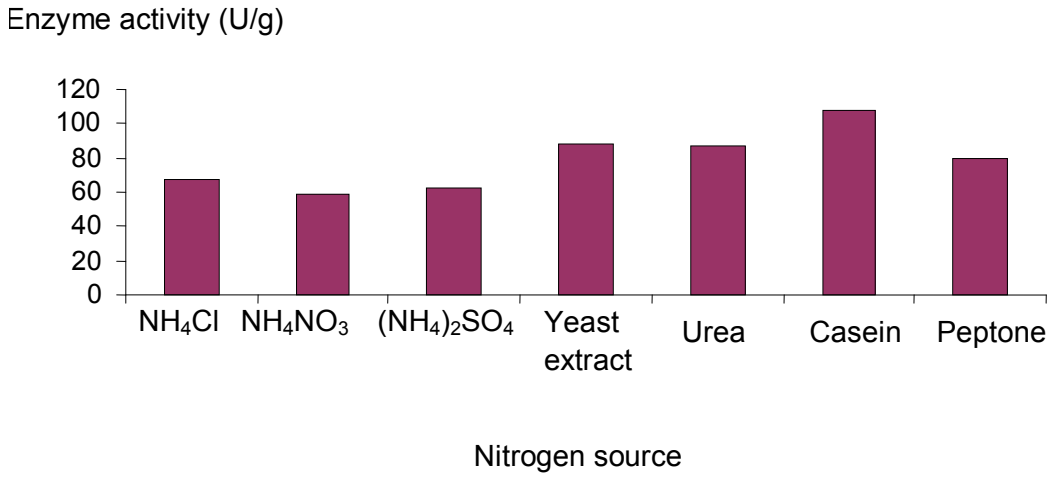

Figure 5. Effect of nitrogen source on amylase yields under SSF by isolate W74.

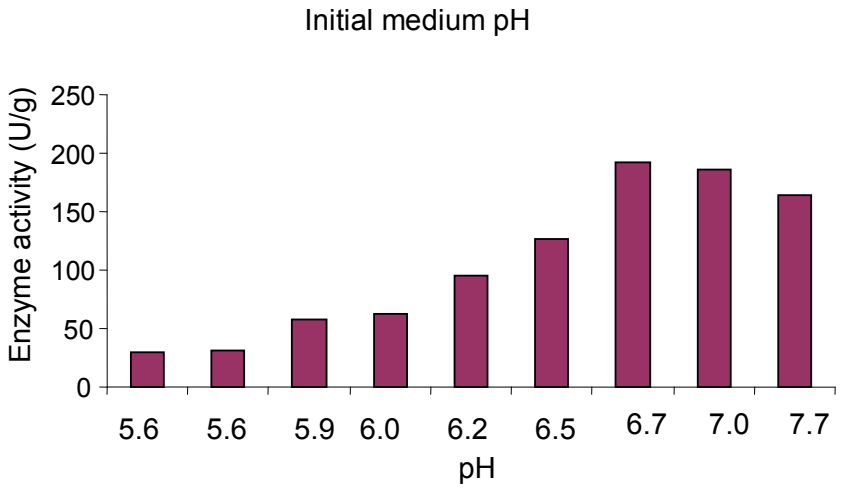

Figure 6. Effect of initial medium pH on α-amylase production by isolate W74 under SSF.

why less than 50% yield was recorded at fermentation medium adjusted to pH 6.2. It was reported by Raimbault (1998) that in SSF systems, the nature of the substrate has a strong influence on pH kinetics, due to the buffering effect of lignocellulosic materials which was similar with current study. Thus, wheat bran was examined to have a great buffering capacity.

Effect of repeated washes on amylase extraction

Extraction efficiency is critical to fully exploit the enzyme produced (Palit and Banerjee, 2001). For efficient leaching of the enzyme from the fermented biomass, the bacterial bran was socked for 30 minutes in five consecutive washes. With repeated washes, it was observed that 146.5 U/g and 14.2 U/g of enzyme extracted in the 1st and 2nd washes, respectively and only 4.5 U/g was recovered in the 3rd wash (Table 3). With the fourth and fifth washes, an almost negligible amount of enzyme was recovered. The subsequent washes did not have significant effect on extraction suggesting that most of the enzyme was leached out in the first wash. Finally, an assessment was undertaken to identify the type of amylase produced by this strain. The chromatogram indicated the formation of a range of oligosaccharides (data not shown) from wheat starch indicating that the enzyme was an α-amylase. Similar findings reported that α-amylases

Table 3. The effect of number of washes on α-amylase extraction from fermented bacterial bran.

Number of washes	Enzyme activity (U/g)	Percentage (%)
1st	146.5	88.3
2nd	14.2	8.6
3rd	4.5	2.7
4th	0.49	0.3
5th	0.15	0.09

Table 4. Composition (g) of solid state fermentation used for α-amylase production.

Parameter	Value (g)
Wheat bran	10
Starch (wheat)	0.5
KH_2PO_4	0.2
NaCl	0.25
$MgSO_4.7H_2O$	0.02
$CaCl_2.7H_2O$	0.02
$(NH_4)_2SO_4$	0.1

Distilled water was added to adjust the required moisture level

degrade starch in random fashion producing various maltooligosaccharide mixtures (Mamo et al., 1999; Sarikaya and Gurgun, 2000; Kiran and Chandra, 2008).

Conclusion

The results in this study indicated that isolate W74 was a potential strain for α-amylase production under solid state fermentation using wheat bran as a substrate. Its growth at neutral pH medium range and mesophilic growth temperature make isolate W74 a potential strain for future use. The extracted enzyme showed optimum activity at 70°C, pH 6.5 and was found to have a better activity between moderately acidic and neutral pH values (5.0-7.0). Therefore, research need to be undertaken to exploit the potential use of isolate W74. The utilization of wheat bran as solid substrate had a great advantage in buffering pH and its low cost could lead to large-scale production of this enzyme for industrial use in starch liquefaction.

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