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## Production and stabilization of amylases from *Aspergillus niger*

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Four isolates of *Aspergillus niger* and two of *A. flavus* were screened for  $\alpha$ -amylase production by submerged fermentation. *A. niger* RN (isolated from boiled rice) gave maximum  $\alpha$ -amylase yield. The pH and temperature optima of  $\alpha$ -amylase of *A. niger* RN were 4.6 and 40°C, respectively. The optimum incubation period, temperature and pH for maximum enzyme production were 7 days, 30°C and 4.5, respectively. Of the six different nitrogen sources used peptone at 0.2% N w/v gave maximum  $\alpha$ -amylase yield. The fermentation medium was varied with eight different carbon sources and the combination of soluble starch and maltose gave the best enzyme production. Shelf life could be improved up to 120 days with PEG at 1% and sodium azide at 0.001% concentration. Thermostability improved considerably with 30% sorbitol, 30% sucrose and 5% PEG.

Key words – *Aspergillus* spp. – submerged fermentation –  $\alpha$ -amylase – thermostability

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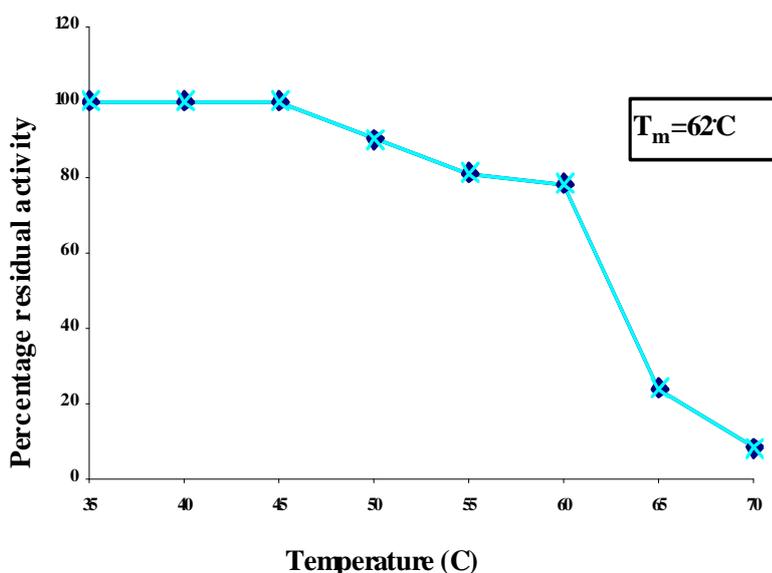
### Introduction

Amylases have potential applications in the food, fermentation, textile, paper and pharmaceutical industries. Amylases have been most widely reported to occur in microorganisms, although they are also found in plants and animals. Currently, they comprise about 30% of the world enzyme production (Vander et al. 2002). The main microbial sources for amylase production are *Bacillus* species (Nurmatov et al. 2001, Deutch 2002, Dey et al. 2002) and *Asperigillus* species (Goto et al. 1998, Gigras et al. 2002). Considerable information is available on the microbial production of  $\alpha$ -amylase in solid media (Ramachandran et al. 2004, Xu et al. 2008) and liquid media under stationary and submerged conditions (Francis et al. 2003, Kaur et al. 2003). Low yield of enzyme has always been a problem in the commercial production of amylases. Moreover, thermal stability is a desirable feature for

economic viability of enzymatic processes. Therefore, the present work was undertaken to screen various *Aspergillus* isolates for  $\alpha$ -amylase production, optimization of fermentation conditions for maximum yield, and to stabilize the enzyme in liquid state using various additives.

### Materials and methods

Isolates of *Aspergillus niger* from different sources along with a culture of *A. niger* MTCC-281, procured from Institute of Microbial Technology, Chandigarh, were screened for production of  $\alpha$ -amylase using Czapek Dox medium supplemented with soluble starch (3%) as carbon source. The cultures were grown for 7 days at 30°C. Amylase activity was determined in culture filtrates by measuring the amount of starch hydrolyzed in the reaction mixture by the iodine method (Manning & Campbell 1961).



**Fig. 1** – Effect of temperature on residual activity of  $\alpha$ -amylase by *Aspergillus niger* RN

One unit of enzyme activity has been defined as the amount of enzyme that hydrolyses 1 mg of starch/min under assay conditions (Manning & Campbell 1961). *A. niger* RN was the best producer for the enzyme and *A. niger* was thus selected for further study and cultural and nutritional parameters were optimized for enhanced production of  $\alpha$ -amylase. Under optimized conditions, stabilization of the enzyme preparation was attempted using various salts and polyhydric alcohols at different concentrations.

To find thermostability of  $\alpha$ -amylase, the enzyme preparation of *A. niger* RN (7.4 U/ml) was heat treated at different temperatures ranging from 35 to 70°C for 15 min and then held in ice bath immediately afterwards. The residual enzymatic activities were measured at 42.5°C, pH 4.6.

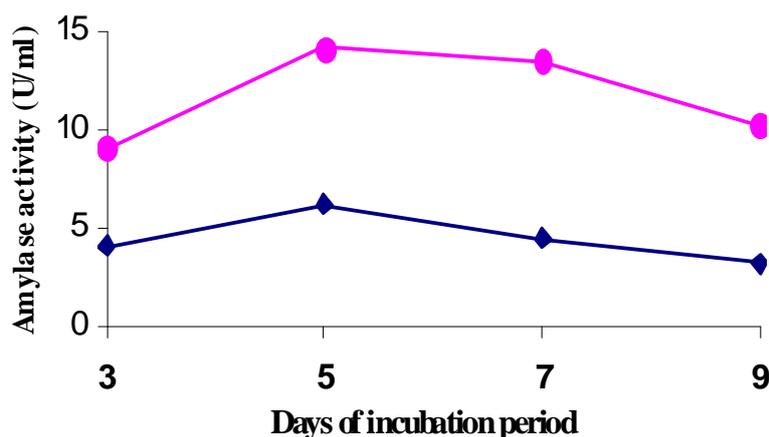
### Results and discussion

When grown on modified Czapek Dox broth *A. niger* RN produced 7.4 U/ml of enzyme followed by *A. niger* PN (4.4 U/ml), *A. flavus* AF (3.60 U/ml), *A. flavus* PF (3.0 U/ml), *A. niger* MTCC 281 (2 U/ml) and *A. niger* BN (1.9 U/ml). It was thus selected for further study and cultural and nutritional parameters were optimized for enhanced production of  $\alpha$ -amylase. Highest amylolytic activity of *A. niger* RN was between 40 and 45°C at pH 4.6. Ramachandran et al. (2004) recorded an optimum temperature of 50°C at pH 5 for enzyme production in *A. oryzae*.

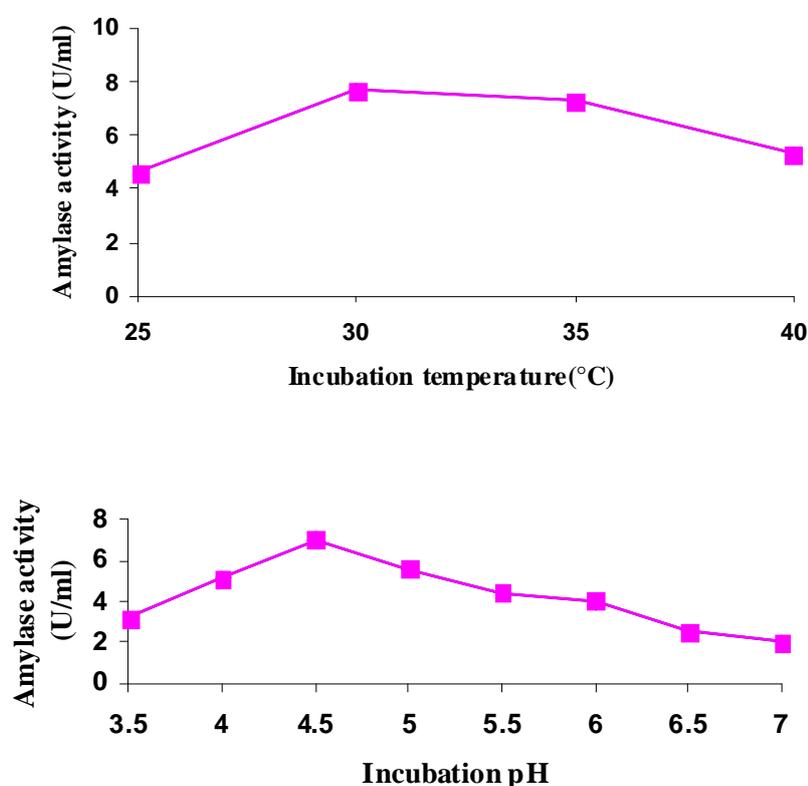
The temperature at which 50% of maximum enzyme activity was recorded was 62°C for *A. niger* RN (Fig. 1).

Fermentation was carried out under stationary and shake flask conditions for different incubation periods. Under shaking conditions (Fig. 2), maximum enzyme activity was obtained earlier i.e. on 5<sup>th</sup> day (6.0 U/ml) but was lower than in stills conditions (8.0 U/ml). Maximum production in still condition was achieved on 7<sup>th</sup> day (8.9 U/ml). Increasing incubation period beyond 7 days resulted in a decline in enzyme activity. Mukherjee & Majumdar (1973) also obtained maximum  $\alpha$ -amylase production from *A. flavus* after 7 days but maximum yield of enzyme was obtained in shake flask rather than in still conditions.

Optimum fermentation temperature was determined by growing the culture for 7 days under still conditions in different temperatures between 25°C and 40°C (Fig. 3). A temperature of 30°C favoured maximum  $\alpha$ -amylase production (8.7 U/mL). Incubation at lower (20°C) or higher (35°C, 40°C) temperature resulted in a substantial decrease in yield of enzyme, as was also reported by Mukherjee & Majumdar (1973). When the culture was grown in a medium with initial pH ranging from 3.5 to 7.0, maximum  $\alpha$ -amylase production was achieved at pH 4.5 (Fig. 3). Amylase production in *A. ochraceus* and *A. niger* UO 1 was optimum at pH 5 (Nahas & Waldemar 2002, Hernandez et al. 2006). However Goto et al



**Fig. 2** – Effect of shaking (◆) vs still (●) condition with incubation period on  $\alpha$ -amylase production by *Aspergillus niger* RN



**Fig. 3** – Effect of incubation temperature and incubation pH on  $\alpha$ - amylase production by *Aspergillus niger* RN

(1998) reported pH 6.0 to be optimum for  $\alpha$ -amylase production by *A. fumigatus*.

Several carbon sources were tried to optimize  $\alpha$ -amylase production. The combination of maltose + soluble starch was selected as the best carbon source for maximal  $\alpha$ -amylase production (8.6 U/mL) by *A. niger* isolate RN followed by soluble starch alone (7.7 U/mL), maltose (5.4 U/mL) and the others (Table 1).

Glucose greatly repressed enzyme synthesis. It appears that there is catabolic repression in  $\alpha$ -amylase activity by glucose. Mixed substrates of lactose and maltose have also been reported for  $\alpha$ -amylase production by Ramachandran et al. (2004). From the different nitrogen sources viz.  $\text{NaNO}_3$ ,  $\text{NH}_4\text{NO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{NH}_4\text{Cl}$ , peptone and urea, used in production medium at the level of 0.2% N w/w, peptone was the

**Table 1** Effect of different carbon and nitrogen sources on  $\alpha$ -amylase production by *Aspergillus niger* RN after 7 days of incubation at 30°C.

Carbon Source (3% w/v)	$\alpha$ -Amylase activity Uml <sup>-1</sup>
Soluble starch	7.7
Maltose	5.4
Sucrose	3.3
Lactose	2.4
Xylose	3.7
Sorbitol	2.1
Mannitol	2.6
Glucose	1.4
Maltose+Soluble starch	8.6
Nitrogen Source (0.2% N w/v)	$\alpha$ -Amylase activity Uml <sup>-1</sup>
Peptone	8.2
Sodium nitrate	7.0
Urea	5.4
Ammonium nitrate	4.4
Ammonium sulphate	3.2
Ammonium chloride	2.8

best nitrogen source for  $\alpha$ -amylase production by *A. niger* (8.2 U/ml) followed by sodium nitrate (7.0 U/ml). Ammonium chloride and ammonium sulfate were poor nitrogen sources for amylase production (Table 1). Peptone as best nitrogen source for  $\alpha$ -amylase production has also been reported earlier (Marlida et al. 2000, Hernadnez et al. 2006). However  $\alpha$ -amylase production by *A. niger* NCIM 1248 showed NaNO<sub>3</sub> as best nitrogen source (Kaur et al. 2003).

By optimizing the incubation conditions of  $\alpha$ -amylase production from *A. niger* RN could be enhanced more than 2 fold (Table 2)

To stabilize the enzyme preparation for shelf life, enzyme was kept for storage at 15–25°C with and without additive. The additives used were sodium azide (0.001%), polyethylene glycol (PEG 1%), glycerol (30 and 50%), sorbitol (20 and 30%) and a combination of sodium azide and PEG. Residual activities were recorded at different intervals of time. Results indicated that sodium azide and PEG

**Table 2**  $\alpha$ -amylase production by *Aspergillus niger* RN under optimized conditions.

Cultural conditions	Control	Optimized
Incubation period	7 days	7 days
Temperature	30°C	30°C
pH	4.5	4.5
Carbon source	Soluble starch	Maltose+soluble starch
Nitrogen source	Sodium Starch	Peptone
Supplement	Nil	Yeast
Shaking/Still	Still	Still
<b>Production (U/ml)</b>	<b>7.2</b>	<b>15.2</b>

had a profound effect on the shelf life of enzyme (Table 3). Back et al. (1979) observed 100% stability at -4°C for 165 days with the addition of polyhydric alcohols like 2M xylitol and 2M sorbitol.

To improve the thermostability various polyhydric alcohol such as glycerol, sorbitol and carbohydrates such as sucrose and xylose at varied concentrations were tried as additives. The residual activities (Table 4) recorded after subjecting these to a selected temperature clearly indicated that PEG at 5%, sucrose at 30% and sorbitol at 30% level had a reasonable protective effect in improving thermostability of the enzyme preparation. Also there was no initial loss in the enzyme activity on addition of the additive. Polyols as thermostabilizing agents for  $\alpha$ -amylase activity at 60°C have been earlier reported (Graber & Combes 1987). The stabilizing effect of various additives on polygalactouranase II from *A. carbonarium* showed that except for ethylene glycol, the other polyhydric alcohols enhanced thermal stability in a concentration dependent manner (Devi & Rao 1998).

Since *A. niger* RN has simple nutritional requirements and can produce a good enzyme yield, this isolate could be exploited for enzyme production in still conditions using maltose + soluble starch as carbon source at 3% level and peptone as the nitrogen source with incubation at 30°C and an initial pH of 4.5. The shelf life of enzyme can be improved greatly using sodium azide at 0.001% and PEG at 1%. Moreover, addition of sorbitol at 30%

**Table 3** Effect of additives on the shelf life of  $\alpha$ -amylase from *Aspergillus niger* RN at 12–15°C at different intervals of time.

Time Interval (Days)	Without additive	% Residual activity with different additives						
		Glycerol		Sorbitol		PEG	Sodium azide	PEG+ Sodium azide
		30%	50%	20%	30%	1%	0.001%	1%+0.001%
0	100	100	100	100	100	100	100	100
30	92	90	96	98	97	99	100	100
60	88	87	90	89	87	97	99	99
90	74	76	81	85	84	97	98	98
120	60	68	78	80	78	95	96	97

**Table 4** Effect of additives on the thermostability of  $\alpha$ -amylase preparation from *Aspergillus niger* RN.

Additive		% Residual activity*
PEG	2%	59
	5%	70
Sorbitol	20%	65
	30%	76
Glycerol	20%	48
	30%	49
Xylose	20%	58
	30%	67
Sucrose	20%	49
	30%	75

\*Activity after thermal treatment at 62°C T<sub>m</sub> for 15 min.

level can be extremely useful in improving thermostability.

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