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Production of Isoamylase from *Rhizopus oryzae* in Submerged Fermentation

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Abstract

This work mainly focus on factors relevant for improvement of enzyme hydrolysis by using *Rhizopus oryzae* MTCC 9642 in submerged fermentation. Several cultural conditions were examined to access their effect in optimizing enzyme production. The *Rhizopus oryzae* PR7 MTCC 9642 showed best growth at 28°C within a broad pH range of 6.0 to 10.0 with an optimal temperature and pH of isoamylase synthesis at 28°C and 8.0 respectively. Maximum growth and enzyme production was observed at 72 hours of cultivation, using carbon sources like carbon sources like starch, glycogen, amylopectin and dextrin of which dextrin acted as the best inducer for isoamylase synthesis. Tryptone was proved to be the best nitrogen source and addition of amino acids like Histidine HCl, Cysteine HCl and Tryptophan could remarkably enhance the enzyme synthesis .Amongst the additives thiol compounds like Dithiothreitol (DTT) and β-marcaptoethanol (B-ME) could increase enzyme synthesis. The strain could utilize native starch molecules from waste food stuffs, of which rice extract followed by bread dust showed best growth and isoamylase inducing activity.

Keywords: *Rhizopus oryzae*, Isoamylase, waste starches.

Introduction

Bioconversion of starch and allied substrates can be accomplished by a number of amylases, of which isoamylase (E.C.3.2.1.68, glycogen-6-glucanohydrolse) hydrolyses 1, 6- α-D-glycosidic linkages of glycogen, amylopectin and α and β limit dextrins, producing linear malto oligosaccharides (Fang *et al,* 1994). It completely splits the branches forked by α-1, 6 glucosidic linkages in glycogen in an exofashion (Yokobayashi *et al,* 1970). Isoamylase is used primarily in the production of food

ingredients from starch like glucose, maltose, trehalose and cyclodextrins (Olempska-Beer, 2007) and has immense significance in saccharide manufacturing and allied industries.

Extra cellular isoamylase was found to be produced by numerous strains of *Pseudomonas amyloderamosa* (Wu *et al*, 1994, Fang *et al,* 1994; Kainuma *et al*, 1978), *Cytophaga* sp (Gunja Smith, 1974), *Xanthomonas maltophila* (Yamada *et al,* 1994) *Flavobacterium* sp (Horwarth *et al*, 1977; Evans *et al,* 1979; Sato and Park 1980), *Bacillus circulans* (Castro *et al,* 1992), *Bacillus sp (*Ara *et al,* 1993,), *Sulfobolus* sp (Fang *et al*, 2005). *Escherichia intermedia* (Ueda and Nanri, 1967), *Lipomyces* sp (Spencer-Martins, 1982) and *Saccharomyces cerevisae* (Ma *et al*, 2000) but so far the literature survey is concerned no such report is obtained from *Rhizopus sp* (Lim et al, 1987).

As optimization of cultivation conditions is a more convenient and effective strategy to ameliorate the enzyme production and a pre requisite for adding economy in enzyme production process, the objective of this work was optimization of factors for growth and production of isoamylase by a strain of *Rhizopus* oryzae .

Materials and methods

Micro organism and culture conditions

Rhizopus oryzae PR7 MTCC 9642, a soil isolated strain with high endoglucanolytic activity (Karmakar & Ray,2010), was grown on starch peptone medium at pH-7. Batch cultures were carried out in a basal medium (BM) containing (g/l): peptone, 0.9 ;($NH4)_2HPO_4$, 0.4; KCl, 0.1; MgSO4 .$7H_2O$, 0.1 and soluble potato starch 0.5 (pH-7.0) .Each culture flask (250ml) was inoculated by single hyphal disc (0.5 cm diameter) scooped out from a 48 hr. growth plate (Ray & Chakraverty, 1998).

Chemicals

All chemicals used were of analytical grade and were purchased from Merck, Germany and Himedia, India.

Biomass measurement

The growth was measured by filtering the submerged culture and weighing the dried (at 80°C for 120 min) mycelial mat on dried pre weighed filter paper (Whatman No1).

Enzyme extraction and assay

For *in vitro* detection of isoamylolytic activities the culture broth (48-72 hour old) was filtered through Whatman no 1 filter paper and the clear filtrate as used as crude enzyme. The isoamylase activity was measured by incubating the assay mixture (1ml) containing equal volume of properly dilute enzyme and 1% (w/v) glycogen (Oyster) in 50 mM phosphate buffer (pH-5) at 55° C for 5 mins. The reducing sugar was measured spectrophotometrically at 540 nm by the 3, 5 dinitrosalicylic acid. (Bernfeld, 1955). One unit of isoamylase activity was defined as the amount of enzyme which catalyzes the liberation of 1m mole of glucose per min. per ml. under optimal conditions.

Study on the effect of culture conditions

A study on the effect of different carbon and nitrogen sources, additives, antifungals on isoamylase synthesis was examined .The effect of pH on isoamylase production was studied by cultivating the strain at different initial pH(4-10)under static condition at 28°C. The culture was grown at various temperatures (4°-37°C) to study the effect of cultivation temperature on isoamylase production. Cultivation period for maximum production of isoamylase was also quantified along with the effect of carbon, nitrogen source, amino acids, metal ions and various additives on the productivity of the enzyme.

Study on salt tolerance

The fermentation media in different culture flasks were supplemented with various concentrations (10mM -200mM) of NaCl to determine the enhancing effect of this salt on enzyme production.

Effect of static and agitated condition

To check the effect of agitation on growth and isoamylase production, two sets of experiments were maintained keeping all other factors constant, one cultivated in orbital shaker incubator at 120 rpm and another in static condition.

Fermentation of waste substrate for isoamylase production

With a view to replace starch or glycogen, a costly substrate of isoamylase synthesis, various starchy wastes, collected from kitchen effluents like rice extract, pop corn, corn cob, bread dust, flat rice, banana peel, pulse powder were tried as sole carbon sources in place of soluble potato starch (Merck).The rice extract was obtained from the discarded starchy material after cooking of rice and was added in the medium at a concentration of 0.5%(v/v). The bread dust (prepared by crushing the thrown away part of the hand made bread) , and other substrates after drying overnight and pulverizing at 40 mesh particle size were supplemented at a concentration of 0.5%(w/v) in the fermentation medium.

Results and Discussion

Rhizopus oryzae PR 7 was found to synthesize extra cellular isoamylase when grown in presence of starch or allied substrates of which Dextrin acted as the best inducer for growth and enzyme synthesis (Table 1). dextrin was also acted as the best inducer for isoamylase synthesis by *Pseudomonas amyloderamosa* MU 1174 (Olempska Beer,2007).Starch (1-3%) and maltose (0.05-25) were found to be the preferred carbon sources for production of isoamylases by *Lipomyces* sp (Spancer Martin,1982), *Flavobacterium* (Takahashi *et al*, 1996) and *E. intermedia* (Ueda and Nanri ,1967), *Pseudomonas amyloderamosa* WU 2130 (Fang *et al*,1994) respectively. Ara *et al*, 1993 mentioned glycogen to be the best carbon source for isoamylase production by *Bacillus* sp. In the present strain, cyclodextrin acted as potent repressor, whereas glucose although did not affect the growth but acted as an inhibitor of enzyme synthesis, a report contrary to that of Ueda and Nanri, 1967 but similar to that

of Tsyy Lai and Shen Lin, 1996.

The optimum temperature for isoamylase production by the present strain was found to be at 28°C, above which the strain showed a tendency toward formation of pellet due to thermal dimorphism and the enzyme synthesizing ability decreased remarkably (Graph 1).Other isoamylase producing bacterial strains showed almost similar range of cultivation temperature of 28-30°C. (Olempska-Beer,2007, Ueda & Nanri, 1967, Ara *et al*, 1993, Fang *et al*, 1994,Takahashi *et al*, 1996).Lipomyces, an yeast strain showed highest enzyme production at slightly lower temperature of 25 C (Spancer –Martins,1982).

Although the strain showed good filamentous growth at a broad pH range of 4-8, highest growth and enzyme production was found at an alkaline pH of 8.0 (Graph 2). The enzyme synthesis slightly decreased at pH 9.0, but dropped down with further increase in alkalinity. Similar preference towards alkalinity was found in the isoamylase production medium of *Bacillus* sp (Ara *et al,* 1993).But other bacterial strains like *Flavobacterium* sp, *Micrococcus* sp and *Arthrobacter* sp showed optimum enzyme production at a pH of 6.8 (Horwarth *et al*, 1977).

The present strain, achieved highest biomass at 96^{th} hour of growth but showed highest isoamylase synthesis within 72 hours of growth (Graph 3), which slightly decreased afterwards. Although some bacterial strains produced isoamylase within 2 days (Ueda & Nanri, 1967, Fang *et al*, 1994) but *Bacillus* took 3 days (Ara *et al*, 1993), similar to that of the present strain, whereas a longer time of 5 days was taken for isoamylase production by Lipomyces (Spancer-Martins, 1982) .

Amongst the nitrogen sources tested, tryptone showed the highest promoting action for enzyme synthesis followed by gelatin (Graph 4). Peptone and meat extract alone acted as the best nitrogen source for isoamylase synthesis in *E. internmedia* (Ueda and Nanri, 1967) and *Xanthomonas (*Yamada *et al,* 1994) respectively ,whereas a mixture of these two gave a satisfactory result in *Cytophaga* sp (Gunja Smith *et al,*, 1974).

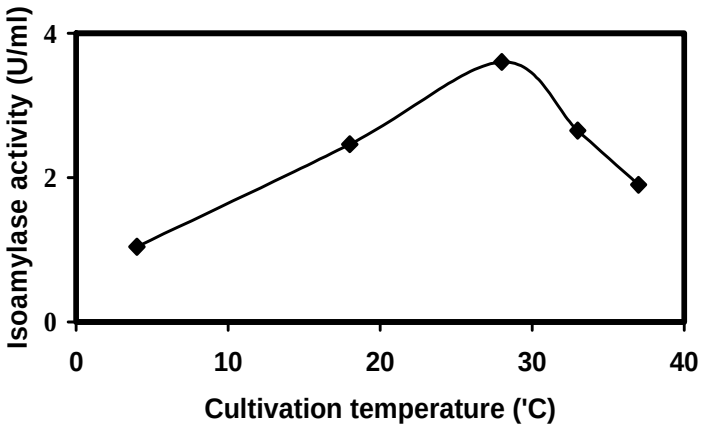
Among the amino acid supplemented the imidazole amino acid, Histidine HCl could remarkably increase the enzyme synthesis followed by cysteine HCl (Table 2).Addition of exogenous thiols like dithiothreitol (DTT), reduced glutathione (GSH), β-marcaptoethanol , cysteine HCl increased the production(Graph 5), whereas thiol inhibitor (pCMB) and metal ions like Cu^{2+},Sn^{2+}, K^{+} reduced the enzyme production (Table 3). Only Na^{+} was found to have a tendency to enhance the production of isoamylase , which could be further demonstrated by supplementing the media with gradually increasing concentration of Na^{+}. Results indicated that the enzyme synthesis became highest at a Na^{+} concentration of 100mM, above which enzyme synthesizing ability gradually decreased (Graph 6). This enhancing effect of Na^{+} ions might be due to their probable positive effect on cellular permeability.

The isoamylase production was slightly affected by the antifungals like flucanozole and griseofulvin but severely reduced in presence of cotrimazole. Although enzyme synthesis was not interfered by surfactant like Tween 20 and chelator like EDTA but decreased by surfactants like SDS, Tween 40, 80 and Triton X 100 (Graph 5), an observation contrary to that found during α-amylase production by *Aspergillus niger* (Gupta *et al*, 2008).

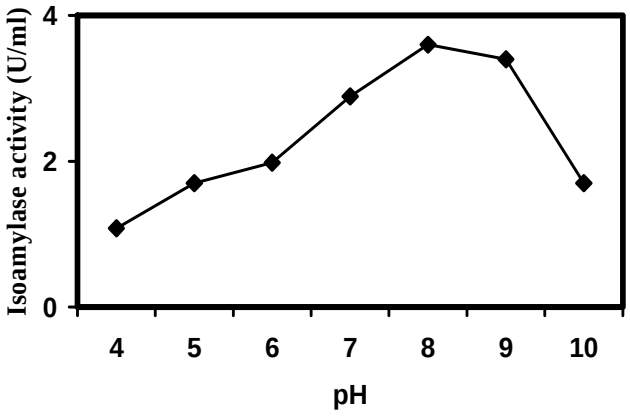
The strain preferred to grow in its mycelial form in static condition but tend to form pellet structure in agitated condition with eventual loss of enzyme synthesizing ability (data not shown).

With a view to replace pure and expensive starch and allied substrates for isoamylase production, various cheap and abundantly available indigenous starches have been tried as carbon sources of which rice extract followed by bread dust showed most satisfactory result (Table 4).This could be attributed to the relatively high starch content and easy accessibility of the starch molecules present in these starchy foodstuffs.The ability of the strain to digest the starchy wastes could be effectively utilized for the production of the enzyme at a nominal cost.

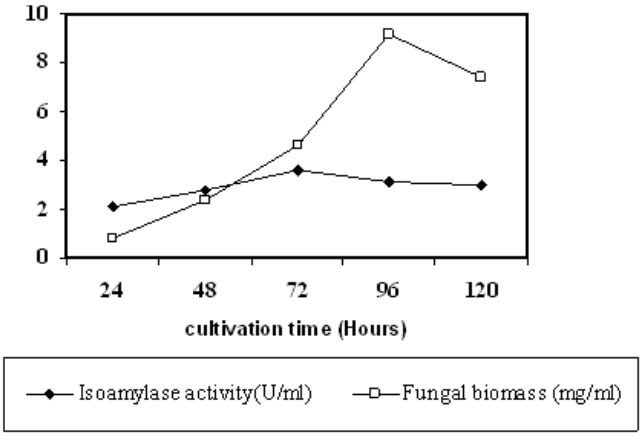
The conventional isoamylases were unsatisfactory for use on an industrial scale (Yamada *et al*, 1994) due to various disadvatages, the present strain with relatively moderate cultivation time, high enzyme activity and ability to utilize inexpensive waste starches might be a successful alternative.



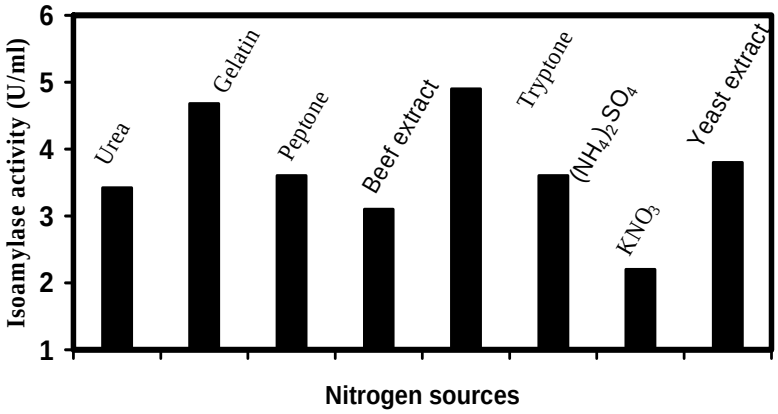
Graph.1. Effect of cultivation temperature on Isoamylase production in *Rhizopus oryzae*.



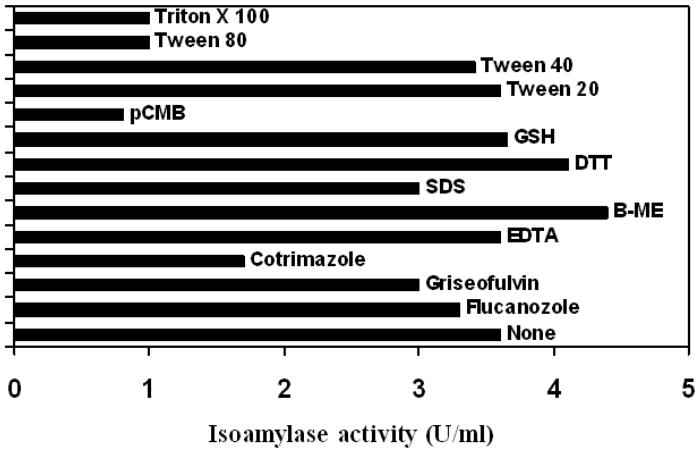
Graph .2. Effect of cultivation pH on Isoamylase production in *Rhizopus oryzae*.



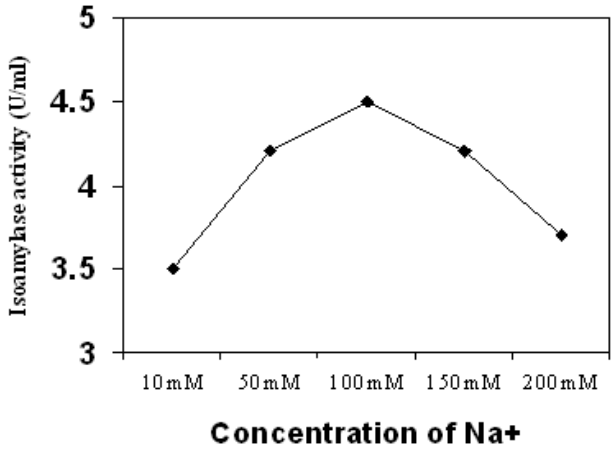
Graph 3. Effect of cultivation time on Isoamylase production and fungal biomass in *Rhizopus oryzae*.



Graph 4. Effect of nitrogen sources on Isoamylase production in *Rhizopus oryzae*.



Graph 5. Effect of various additives on Isoamylase production in *Rhizopus oryzae*.



Graph 6. Effect of various concentration of Na^+ on Isoamylase production in *Rhizopus oryzae*.

Table 1. Effect of carbon sources as inducer of growth and isoamylase production by *R.oryzae*

Carbon sources	Isoamylase activity (U/ml)	Fungal biomass (mg/ml)
Starch	3.6	4.6
Dextrin	9.0	4.9
Glycogen	4.7	4.8
Amylose	2.7	4.1
Amylopectin	4.0	4.7
CMCellulose	0.9	4.0
Xylan	1.0	2.8
Maltose	4.0	3.9
Glucose	2.7	4.0
α-cyclodextrin	0.2	1.1

Table 2. Effect of metal ions on Isoamylase production in *Rhizopus oryzae*

Ions (10mM)	Isoamylase activity (U/ml)
None	3.6
Na^+	3.7
K^+	1.2
Ca^{2+}	2.8
Mg^{2+}	1.4

Mn^{2+}	2.3
Fe^{3+}	2.7
Zn^{2+}	0.9
Sn^{2+}	1.0
Sr^{2+}	1.8
Ba^{2+}	2.0
Pb^{2+}	2.0
Cu^{2+}	1.0
Cd^{2+}	2.0
Hg^{2+}	1.4

Table 3. Inducing effect of amino acids on isoamylase production by *Rhizopus oryzae* PR 7

Amino acid (10mM)	Isoamylase activity (U/ml)
None	3.6
Arginine HCl	3.8
Glycine	2.9
Serine	4.2
Phenyl alanine	4.4
Leucine	4.2
Tryptophan	5.3
Aspartic acid	3.5
Proline	3.1
Hydroxy proline	4.6
Tyrosine	3.0
Histidine HCl	6.8
Methionine	4.1
Cysteine HCl	6.0
Alanine	3.8
Threonine	3.5
Glutamic acid	3.2
Valine	3.1
Isoleucine	3.2
Nor isoleucine	3.7
Cystine	3.2
DOPA	4.0
Lysine	2.9

Table 4. Role of starchy food wastes on growth and isoamylase production by *R.oryzae.*

Starchy wastes	Growth	Isoamylase activity (U/ml)
Potato starch (Merck)	++++++	3.6
Corn cob	+++	2.5
Pop corn	+++	2.5
Rice extract	++++++	5.4
Banana peel	+++	1.8
Bread dust	++++	4.5
Flat rice	++++	1.9
Pulse powder	+++	1.7

++++++: 4.4mg biomass per ml. of culture medium.

Acknowledgement

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