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EXPERIMENTAL ARTICLES

Induction and Carbon Catabolite Repression of Isoamylase Production in *Rhizopus oryzae* PR7[1]

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Abstract—The growth and the extracellular isoamylase production by *Rhizopus oryzae* PR7 MTCC 9642 were studied in a stationary culture at 28°C, with maximum isoamylase production obtained after 72 hours. Glycogen was found to be the best inducer for isoamylase synthesis, followed by maltose and dextrin. The enzyme was found to be repressed by glucose and this repression was not overcome by the addition of cGMP. The abrupt reduction in enzyme synthesis after the addition of exogenous glucose in a glycogen-induced culture medium confirmed the repressive action of glucose. An almost similar rate of repression was found to be exerted by α- and β-cyclodextrins. The inhibition of enzyme production after the addition of cycloheximide, a translation blocker, indicated the existence of de novo synthesis of the enzyme.

Keywords: isoamylase, repression, inhibition, *Rhizopus oryzae*

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Starch is a major storage product and a large-scale starch processing industry has emerged in the last century with the increased use of starch-converting enzymes in the production of maltodextrin, modified starches, or glucose syrups. Apart from the use in starch hydrolysis, starch-converting enzymes are also used in a number of other industrial applications, such as laundry and porcelain detergents or as anti-staling agents in baking [1]. The conversion of raw starch is brought about by starch-degrading enzymes that belong to a single family: the amylase family, of which isoamylase (glycogen-6-glucanohydrolase, EC 3.2.1.68) hydrolyses 1,6-α-D-glycosidic bonds of glycogen, amylopectin and α and β limit dextrins, producing linear malto oligosaccharides [2]. Isoamylase is used primarily in the production of food ingredients from starch (e.g., glucose, maltose, trehalose, and cyclodextrins) [3]. In comparison to other amylases, isoamylase is known to be produced by very few microorganisms, mainly *Pseudomonqs* sp. [2, 4] and other bacterial [1, 5] and yeast sources [6–8]. These isoamylases differ from one another in their substrate affinity, the major end products of amylolysis, and other characteristic features [9]. Moreover, conflicting results have been reported with respect to the suggested mechanism of amylase synthesis control in fungi [10].

Generally, induction of isoamylase requires a substrate having an α-1,6-glycoside bond, such as glycogen, amylopectin, dextrin, or starch. Glucose, as a final product of the enzymatic reaction of substrate hydrolysis, represses enzyme synthesis by a well-known mechanism of catabolite repression [11]. However, it was found in several microorganisms that the carbohydrate used as a carbon source was not essentially required for induction of amylase production according to the proposed models, showing that it is a constitutive enzyme [12].

According to Magasanik [13] catabolite repression is a paradigm for the studies concerned with global and specific gene control mechanisms. To our knowledge, the existence of induction and carbon catabolite repression of isoamylase synthesis in the fungus *Rhizopus oryzae* has not been investigated. The study of growth and isoamylase production in this fungus may contribute to the understanding of the mechanism of action of this fungus in the environment.

The goal of the present study was to investigate the control of the production of isoamylase (glycogen-6-glucanohydrolase, EC 3.2.1.68) by *Rhizopus oryzae* PR7 in terms of the effect of the carbon catabolites in the culture medium.

MATERIALS AND METHODS

Microorganism and cultivation conditions. *Rhizopus oryzae* PR7 MTCC 9642, a saccharifying isoamylase-producing strain [14] was isolated from soil enriched with decaying vegetation in Eastern India and screened as a potent isoamylase producer out of about 30 fungal isolates. The strain was identified as

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Abbreviations: Cyclic guanosine 3',5'-monophosphate (cGMP).

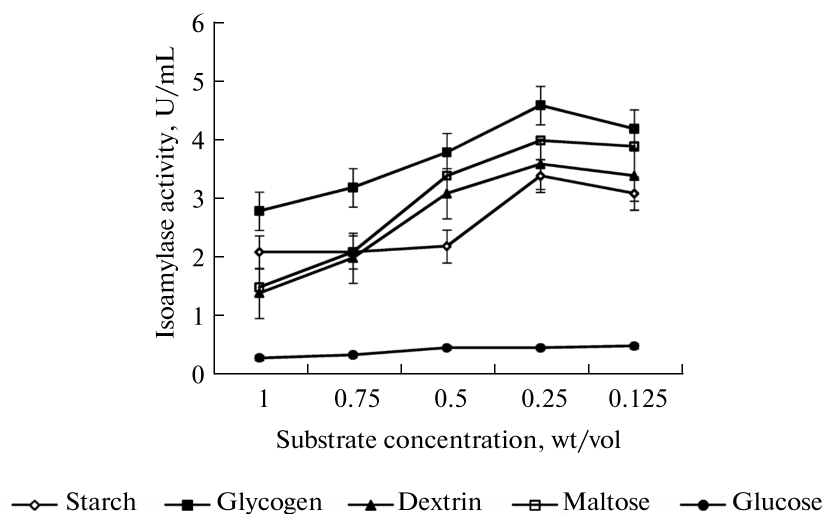

Fig. 1. Effect of various concentrations of different carbon sources.

Rhizopus oryzae and deposited to Institute of Microbial Technology, Chandigarh, India. The strain was grown on starch agar slants (pH 8.0) and stored at 4°C. For submerged fermentation, the strain was grown in 250-mL Erlenmeyer flask containing 50 mL of Basal medium (BM) containing the following (g/L): peptone, 0.9; $(NH_4)_2HPO_4$, 0.4; KCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.1 (pH 8.0). The carbon sources (soluble potato starch, glycogen, dextrin, maltose, or glucose) were added at a concentration of 0.5 g/L, unless otherwise mentioned. The medium was autoclaved at 121°C for 15 min. Each culture flask (250 mL) was inoculated with a single hyphal disc (0.5 cm diameter) scooped out from a starch agar spread plate containing 48 h old culture [15]. After different time intervals (18, 24, and 36 h), chemicals, such as glucose and cGMP, were added aseptically into the flasks. The activity of the enzyme synthesized in the presence of glucose (0.05%, wt/vol) and cGMP (2.0 mM) at different time intervals (0, 6, 12, 18, 24, 30, 36, 42, and 48 hr) was measured. To study the effect of various additives on the induction of isoamylase synthesis, the medium was supplemented with 1.0 mM of additives (fluconazole, griseofulvin, ethidium bromide, α- and β-cyclodextrin, and cycloheximide). In order to specifically determine the inhibitory effect of cyclodextrins and cycloheximide on glycogen-induced culture, different concentrations of these two chemicals (0.125 to 1%, wt/vol) were supplemented in the production media.

Chemicals. All chemicals used were of analytical grade and were purechased from Merck (Germany) and Himedia (India). The chemicals used were starch, glycogen, peptone, KCl, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2HPO_4$, dextrin, maltose, glucose, cGMP, α- and β-cyclodextrin, cycloheximide, fluconazole, griseofulvin, and EtBr (ethidium bromide).

Enzyme extraction and assay. For in vitro detection of isoamylolytic activities, the culture broth was filtered through Whatman no. 1 filter paper and the clear filtrate was used as a crude enzyme. The enzyme was properly diluted. The substrate used was 1% (wt/vol) glycogen (Oyster) in 0.1 M acetate buffer (pH 5.0). The isoamylase activity was measured by incubating the assay mixture (1 mL) containing equal volumes of properly diluted enzyme (500 μL) and 1% (wt/vol) glycogen substrate (500 μL) without any agitation at 55°C for 5 min. The reducing sugar was measured spectrophotometrically at 540 nm by the 3,5-dinitrosalicylic acid method [16], with glucose as the standard. One unit of isoamylase activity was defined as the amount of enzyme which catalyzed the liberation of 1 mmol of glucose per minute per mL under optimal conditions. Each experiment was done in triplicate and their values were averaged.

RESULTS AND DISCUSSION

Effect of various concentrations of different carbon sources. *Rhizopus oryzae* PR7 was found to synthesize extracellular isoamylase when grown in the presence of starch or related substrates, of which 0.25% (wt/vol) glycogen acted as the best inducer for the enzyme synthesis (Fig. 1). This was in accordance to reports by Ara et al. [1] that mentioned glycogen as the best carbon source for isoamylase production by *Bacillus* sp. The production kinetics indicated that the highest production of the enzyme could be achieved at 72 h of growth in glycogen-induced medium with no significant change in pH of the culture medium (Fig. 2). As the strain could not produce isoamylase under starvation condition (Fig. 2), it could be concluded that isoamylase in the present strain was not synthesized constitutively. As the pH of the glycogen-induced culture slowly decreased from 8.0 to 6.0 after 96 h, the inducer uptake might have operated through facilitate diffusion rather than proton symport.

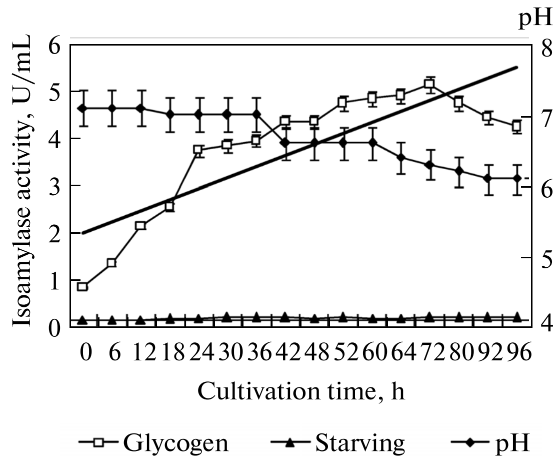

Fig. 2. Production kinetics.

Effect of glucose in glycogen-induced media. As glucose was found to be the major end product of isoamylase action [17], glucose was added to the medium to test the existence of an end product inhibition of isoamylase synthesis. As glucose could not induce isoamylase synthesis (Fig. 1) and the addition of glucose along with glycogen blocked the enzyme synthesis, glucose could be regarded as a potent inhibitor of isoamylase in the present strain, a contrary to the results of Ueda and Nanri [18], but similar, to those of Lai and Liu [19]. This inhibitory effect existed throughout the early and mid-exponential phases of growth, since addition of glucose at any time instantly inhibited isoamylase activity (Fig. 3). Even addition of the cyclic nucleotide guanosine 3',5'-monophosphate (cGMP) together with glucose was not able to overcome the inhibitory effect. Immediate repression of isoamylase synthesis after addition of glucose indicated that this might be due to the catabolite inhibition of the enzyme synthesis or due to inducer exclusion. Similar repression of amylase synthesis by glucose was also reported in various fungal strains [20, 21].

Effect of cyclodextrins. Mot and Verachtert [22] reported cyclodextrins to have a pronounced stimulatory effect on the production of extracellular amylases namely α-amylase and glucoamylase, but in the present strain the repressor action of cyclodextrins was found to be almost similar to that of glucose in glycogen-induced culture media. On increasing concentration of cyclodextrin, isoamylase activity was progressively repressed, resulting in a more than sevenfold decrease in the enzyme activity (Table 1). Since similar to other isoamylases [1, 8, 23], the present isoamylase was not inhibited by cyclodextrins, it could be concluded that cyclodextrins as such did not affect the isoamylase activity but exerted a repressive action on isoamylase synthesis. Cyclodextrin, being the end product of starch hydrolysis by cyclodextrin glucanotransferase, an enzyme belonging to the same family as isoamylase [24], showed the same mechanism of end product inhibition similar to that of isoamylase synthesis by glucose.

Effect of additives. Although the antifungal agent fluconazole was found to inhibit isoamylase activity to a certain extent, griseofulvin showed no pronounced effect (Fig. 4). Addition of potent inhibitors of transcription (ethidium bromide) and translation (cycloheximide) led to the inhibition of enzyme synthesis. This indicated that secretion of isoamylase in this fungal species was not due to the liberation or activation of the preformed enzyme. Increase in cycloheximide concentration caused a decrease in isoamylase activity by more than 2.5-fold (Table 2) which confirmed the

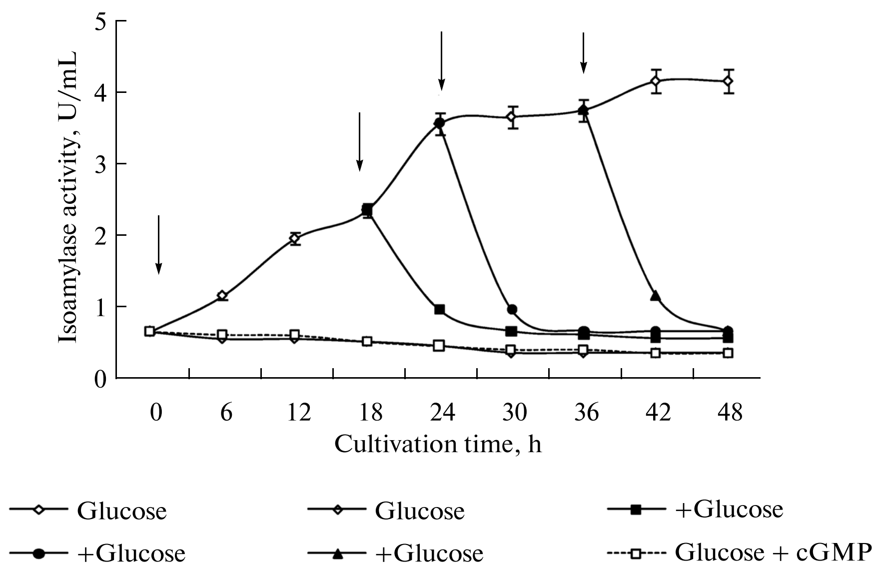

Fig. 3. Effect of glucose in glycogen-induced media.

Table 1. Effect of cyclodextrins

Carbon source	Isoamylase activity (U/mL)	
	α-cyclodextrin	β-cyclodextrin
Glycogen	4.5 ± 0.06	4.5 ± 0.01
Glycogen + cyclodextrin (0.125%)	1.34 ± 0.05	1.21 ± 0.03
Glycogen + cyclodextrin (0.25%)	1.14 ± 0.02	1.11 ± 0.01
Glycogen + cyclodextrin (0.5%)	0.6 ± 0.09	0.7 ± 0.05
Glycogen + cyclodextrin (0.75%)	0.6 ± 0.08	0.6 ± 0.08
Glycogen + cyclodextrin (0.10%)	0.6 ± 0.09	0.5 ± 0.07

Glycogen: 0.25% wt/vol, culture time: 48 h.

Table 2. Effect of cycloheximide concentrations

Carbon source	Isoamylase activity (U/mL)
Glycogen (0.25%)	4.5 ± 0.06
Glycogen (0.25%) + cycloheximide (0.125%)	2.2 ± 0.05
Glycogen (0.25%) + cycloheximide (0.25%)	2.1 ± 0.09
Glycogen (0.25%) + cycloheximide (0.5%)	1.8 ± 0.05
Glycogen (0.25%) + cycloheximide (0.75%)	1.7 ± 0.05
Glycogen (0.25%) + cycloheximide (1%)	1.7 ± 0.06

Glycogen: 0.25% wt/vol, culture time: 48 h.

de novo synthesis of isoamylase by *Rhizopus oryzae* PR7. Similar inhibitory phenomena after blockage of transcription found in case of synthesis of α-amylase in *Aspergillus oryzae* [25] and β-amylase production in *Bacillus megaterium* B_6 [26] were interpreted as the result of de novo synthesis rather than a modification of any preformed protein.

Very low levels of isoamylase were found to be synthesized at the basal level in the present strain constitutively; the synthesis was induced only in the presence of specific inducers of which glycogen proved to be best. Addition of glucose at any time during the growth of glycogen-induced cultures resulted in an abrupt decline in the enzyme synthesis, which clearly indicated that a classical glucose effect was operative in this organism [27].

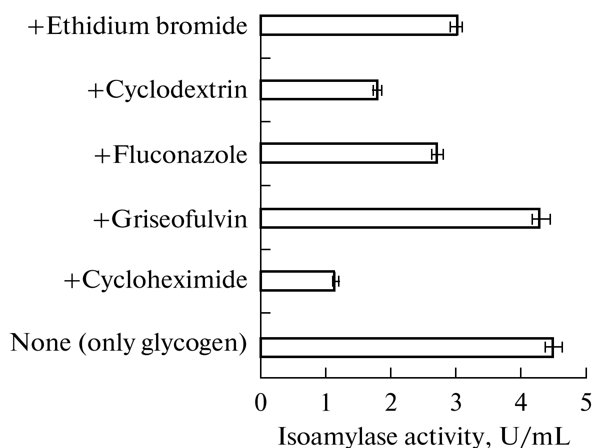

Fig. 4. Effect of additives (1.0 mM).

Addition of cyclodextrins, the cyclic oligosaccharides consisting of glucose monomers, to the induced medium repressed the enzyme synthesis, probably in the manner similar to that of glucose repression.

Although cycloheximide inhibited the enzyme synthesis, the repressive effects of glucose and cyclodextrins were more pronounced which indicated that the repressive action of glucose and cyclodextrins probably affected the mRNA formation [4] whereas repression of enzyme synthesis by cycloheximide was mediated only at the translational level.

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