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INDUCING EFFECT OF SALICIN FOR EXTRACELLULAR ENDOGLUCANASE SYNTHESIS IN *Rhizopus oryzae* PR7 MTCC 9642

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The induction of endoglucanase of *Rhizopus oryzae* PR7 MTCC 9642 has been observed in cultivation medium using a batch-fermentation technique. The effect of various experimental parameters such as use various carbon sources as inducers, concentration of inducer and effect of different additives were investigated. Salicin at concentrations from 0.25 to 0.75% (w/v) was found to bring about a remarkable increase in endoglucanase synthesis when used as a sole carbon source and thought to act as a gratuitous inducer of the enzyme. Endoglucanase activity increased within 24 h after the addition of salicin, reached maximum after 48 h and maintained high level even after 120 h of fungal growth. Repression of enzyme synthesis by glucose could partially be restored by addition of salicin at an early phase of growth. The carboxymethyl cellulose induced enzyme was inhibited by cyclohexamide and ethidium bromide and partially recovered by the salicin which indicated that salicin might act at the transcriptional but not translational level.

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Cellulose is the most abundant compound that contains only α-1,4-linkages [1]. An important feature of cellulose, relatively unusual in the polysaccharide world, is its crystalline structure [2] and the insoluble, recalcitrant nature of cellulose represents a challenge for cellulase systems. Cellulase enzymes, which can hydrolyze cellulose forming glucose and other cello-oligosaccharides, can be divided into 3 types: endoglucanase (endo-1,4-α-D-glucanase, EC 3.2.1.4); cellobiohydrolase or exoglucanase (exo-1,4-α-D-glucanase, EC 3.2.1.91) and β-glucosidase (1,4-β-D-glucosidase, EC 3.2.1.21) [3]. Cellulase systems are not merely an agglomeration of enzymes representing these enzyme groups but rather act in a coordinated manner to efficiently hydrolyze cellulose [2].

Microorganisms have adapted different approaches to effectively hydrolyze cellulose, naturally occurring in insoluble particles or imbedded within hemicellulose and lignin polymers [4]. Cellulases from aerobic fungi have received more study than any other physiological group, and fungal cellulases currently dominate the industrial applications of cellulases [5–7]. Cellulases are induced in most of fungi only when cellulose or an inducer exists [8]. Cellulolytic filamentous fungi (and actinomycete bacteria) have the ability to penetrate cellulosic substrates through hyphal extensions, thus often presenting their cellulase systems in confined cavities within cellulosic particles [9].

Cellulolytic capability is well represented among the various subdivisions of aerobic fungi of which Ascomycetes, Basidiomycetes, and Deuteromycetes contain large numbers of cellulolytic species and the genus *Mucor* of Zygomycetes was found to possess significant activity of this enzyme [2], however very little is known about the induction of cellulase system in *Rhizopus*.

Since the natural inducer cellulose is insoluble, several studies were performed to determine how an insoluble polymer, which cannot transfer into the cell, would initiate cellulase production [10]. It was postulated [11–13] that low basal level of cellulases is formed, which can start the degradation of cellulose and thereby release small amounts of oligosaccharides, which can induce further cellulase biosynthesis. After the degradation of cellulose, the liberated end product causes catabolite repression which prevents the fungus from synthesizing an excess amount of cellulose [2] through a negative regulation at the transcriptional level. Although a number of inducers were reported for fungal cellulase synthesis, effect of non cellulosic substance in uninduced or in catabolically repressed fungus was not investigated. The aim of the study was the induction and catabolite repression of endoglucanase synthesis in *Rhizopus oryzae* highlighting the role of salicin as inducer with its plausible mechanism of action.

MATERIALS AND METHODS

Microorganism. *Rhizopus oryzae* PR7 MTCC 9642, an endoglucanase producing strain was isolated from the decaying vegetation enriched soil of India.

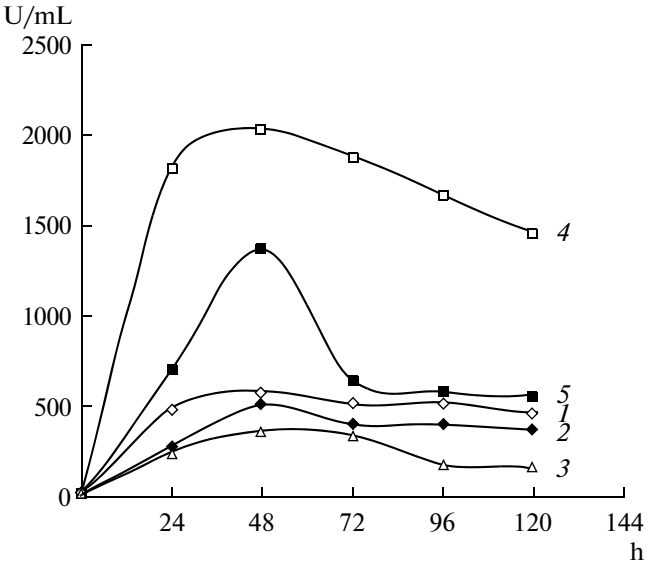

Fig. 1. Production of the *R. oryzae* PR7 MTCC 9642 endoglucanase in presence of various carbon sources: CMC (*1*), cellobiose (*2*), glucose (*3*), salicin (*4*), and CMC+salicin (*5*) in the cultivation medium.

The strain was identified by Institute of Microbial Technology (IMTECH), Chandigarh, India and deposited in Microbial Type Culture Collection as *Rhizopus oryzae* MTCC 9642.

Chemicals. All chemicals used were of analytical grade.

Cultivation of the fungus. The strain *R. oryzae* PR7 MTCC 9642 was cultivated in 100 mL Erlenmeyer flasks each containing 20 mL of basal medium (**BM**) composed of (g/L): peptone – 0.9; $(NH_4)_2HPO_4$ – 0.1; KCl – 0.1; $MgSO_4 \cdot H_2O$ – 0.1 and carboxymethyl cellulose (**CMC**) – 0.5 (pH 6.0).

Preparation of inocula. The fungus was grown in 1% PDA plates, for 48 h at 28–30°C. The inocula were prepared by making hyphal discs (0.5 cm diameter). Each disc was used to inoculate 20 mL of medium [14].

Effect of various factors. The culture flasks (100 mL) with 20 mL medium were cultivated at various temperatures (4°C–40°C) for 0–144 h. To determine the effect of carbon sources, CMC was replaced by various polysaccharides and simple sugars. BM was supplemented with various additives (at concentration 10 mM). Antifungal agents (0.25 μg/mL) were added in the culture flask to test the susceptibility of the fungus.

Enzyme assay. The culture broth of filamentous form of fungus was filtered through filter paper (Whatman No. 1, USA) and filtrate was used as crude enzyme whereas the culture broth of the yeast form was centrifuged at 10000 rpm for 5 min and the supernatant was used as the crude enzyme. To measure the activity of endoglucanase, the assay mixture (1 mL) containing an equal volume of properly diluted enzyme and 1.0% (w/v) CMC in 50 mM phosphate buffer (pH 7.0) was incubated at 37°C for 10 min. The reducing sugar released was measured by the dinitrosalicylic acid method [15] taking glucose as standard. Blanks were prepared with inactivated enzymes treated by exposing at 60°C for 15 min. One unit of endoglucanase was defined as that amount of enzyme that liberated 1 μmole of glucose per mL per min of reaction.

Protein estimation. The extracellular protein was estimated by the Lowry method. The supernatant of yeast form was scanned spectrophotometrically at 250–300 nm range.

Each experiment was performed in triplicate and their values were averaged.

RESULTS AND DISCUSSION

R. oryzae was found to produce all components of enzyme complex hydrolyzing cellulose, namely endoglucanase [16], exoglucanase [17] and β-glucosidase [18], of which endoglucanase was the most pronounced. The regulation of endoglucanase synthesis by induction and catabolite repression in *R. oryzae* PR7 MTCC 9642 studied in batch fermentations indicated that a basal level of endoglucanase was synthesized by the fungus even in absence of any carbohydrate source. The observation was similar to that of Hulme and Stranks [19], who working with several cellulolytic fungi, came to the conclusion that cellulase is not formed as a response to any inducing substance. According to Carle-Urioste with coworkers [13] and Suto and Tomita [8], a basal level of constitutive cellulase might degrade cellulose into cello-oligosaccharides, and they trigger the induction after entry into the cell.

It has been proposed that a low constitutive level of cellulase expression in *R. oryzae* PR7 MTCC 9642 is responsible for the formation of an inducer from cellulose. In this fungus, low constitutive level of endoglucanase expression could be induced by CMC followed by gentibiose, similar to that reported earlier for microorganisms like *Hypocrea jecorina*, *Penicillium purpurogenum and Chaetomium erraticum* [8, 20]. It was revealed that salicin caused about a 3.5 times increase in this level.

On the other hand, although CMC acted as the inducer, it was thought that it could not trigger the induction directly because of its insolubility [8] and could not lead to that degree of endoglucanase synthesis like that of salicin (Fig. 1). The level of induction was much higher in the salicin-supplemented culture medium than that of the medium supplemented with salicin along with CMC, which indicated CMC or its derivative must have some repressive effect that actually controlled the enzyme synthesis.

The concentration of salicin played an important role in inducing the *R. oryzae* endoglucanase. The best concentration of salicin was found to be 0.75% (w/v), after which it gradually declined (Fig. 2) indicating

that above a certain saturation level it failed to operate the induction mechanism of the enzyme.

The maximum induction of endoglucanase of *R. oryzae* PR7 MTCC 9642 in presence of different types of carbon sources was revealed after 48 h of cultivation, although salicin could brought about an abrupt increase in enzyme production (Fig. 1). Salicin ($C_{13}H_{18}O_7$) being an alcoholic β-glucoside containing D-glucose could maintain the enzyme synthesis at a high level even after 120 h of growth. This fact indicated that it must have acted as a non-metabolizable trigger that could not be degraded and hydrolyzed by the cellulase system of the fungus.

Cultivation of the fungal cell in presence of only glucose or cellobiose, failed to enhance the endoglucanase synthesis from (Fig. 1), but these compounds could not be regarded as the end product inhibitors, rather as catabolite repressors that act at transcription level. Addition of glucose in CMC-induced culture sharply decreased the enzyme activity from 570 U/mL to 390 U/mL probably through carbon catabolite repression and the repressive action of glucose could be overcome at least partially by the addition of salicin (870 U/mL).

The addition of salicin in an already glucose repressed culture could bring about an increase of the endoglucanase activity probably by bringing about a repression of the cellulase gene expression [21, 11].

Failure of saligenin and salicylic acid, the major breakdown product of salicin to individually enhance endoglucanase synthesis (data not shown), indicated that probably salicin by its molecular configuration could induce the endoglucanase synthesis or acted on some other enzyme system related to increase the β-linked disaccharide permease activity of the cells [22].

As cellobiose failed to boost up endoglucanase synthesis, it could be concluded that the breakdown product of salicin i.e, β-glucosides were not responsible for enzyme induction. This was against the suggested model of induction through the enhanced transcription of the gene encoding the permease [23]. Therefore, the probability of the effect of salicin on permease biosynthesis [24] might be ruled out.

As no other carbon source could induce the endoglucanase at the same level as that by the salicin itself, it could be easily concluded that probably salicin acted as the gratuitous inducer for endoglucanase synthesis in *R. oryzae* PR7 MTCC 9642 by directly regulating the transcription of the related gene.

Griseofulvin, a fungal mitotic inhibitor, at a concentration of 0.5% added at 0 h of growth, did not affect the enzyme synthesis even after 48 h of growth, which indicated that endoglucanase was formed as a proenzyme and later modified. Addition at 0 h of growth of 0.5% ethidium bromide and 0.5% cycloheximide, affecting transcription and protein synthesis in the fungus, respectively, resulted in the serious reduction in endoglucanase synthesis. Addition of salicin could recover the inhibition if added with ethidium bromide but failed to do so if protein synthesis was blocked by cyclohexamide. This indicated that salicin might have exerted its role at transcription level, not at translational level.

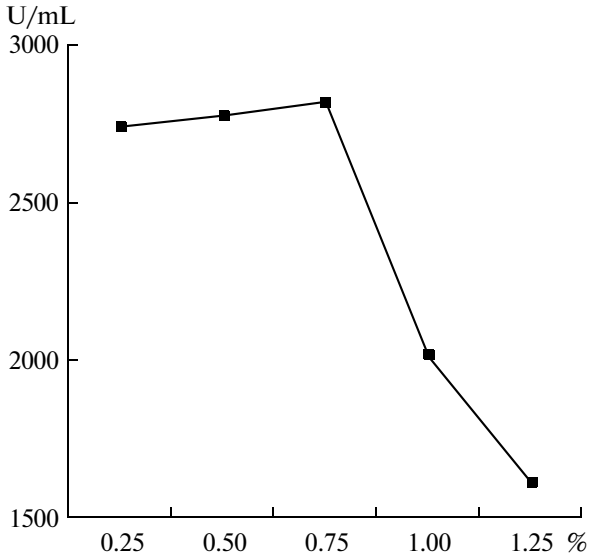

Fig. 2. Effect of the salicin (%) concentration on the production of the *R. oryzae* PR7 MTCC 9642 extracellular endoglucanase.

Therefore, it can be presumed that in the present system, salicin (a lower molecular weight compound than cellulose) after entering the cell triggers full scale transcription of the endoglucanase gene mediated by activator protein or activating element, totally following a scheme proposed by Suto and Tamita [8].

The amount of extracellular protein detected from salicin supplemented culture (Fig. 3) was found to be at least 10 times more than that of only CMC-supplemented culture. It might be presumed that salicin or other inducers regulate *cbh* gene transcription which was correlated with the change of enzyme activities [25].

Although, salicin utilization in the *E. coli* bglB strain was found to require a two-step activation process of definite operons [26] the existence of this operon like regulatory system was not proved for filamentous fungi. Although Somers with coworkers [27] proposed a model for the assimilation of salicin in *A. irakense* and Faure *et al.* [28] presumed the presence of the specific receptor "OMR SalC" *Azospirillum irakense* the specific cytoplasmic protein that could allow this type of signal transmission is still unknown. The glucose repression was found to be mediated by the Cys_2His_2 type transcription factor CreA/CREI in numerous filamentous fungi [10], a salicin-specific phosphotransferase enzyme IIB component and either the structural gene for the β-glucoside-permease or a regulatory gene. The glucose permease PtsG is known to transport sucrose and salicin

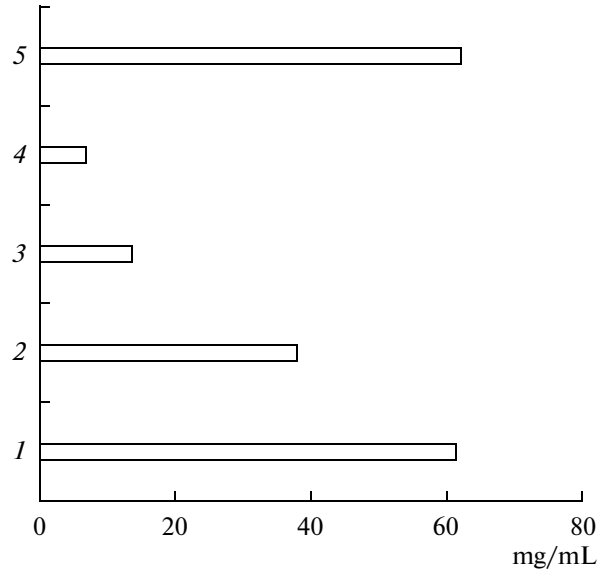

Fig. 3. Extracellular protein (mg/mL) concentration of *R. oryzae* PR7 MTCC 9642 induced by various carbon sources: salicin (*1*), glucose + salicin (*2*), glucose (*3*), CMC (*4*), and CMC + salicin (*5*).

in addition to glucose [29, 30] but if glucose was added after 24 h of growth of *R. oryzae* PR7 MTCC 9642 to the medium, it could not be totally taken up by the strain possibly due to a nutrient saturation (Table).

However, salicin was proved to be the most effective inducer for endoglucanase synthesis and could be used as the sole carbon source for bulk production of the enzyme by *R. oryzae* PR7 MTCC 9642.

Effect of the salicin and glucose addition in induced and repressed cultures of *R. oryzae* PR7 MTCC 9642 at different cultivation points

Carbon source	After 48 h of cultivation	
	Endoglucanase activity, U/mL	Unused sugar in the medium, mg/mL
0.5% salicin-supplemented medium	2010	–
+1% glucose at 0 h	900	–
+1% glucose at 5 h	1206	–
+1% glucose at 24 h	1552	6.72
+1% glucose at 30 h	1206	8.10
1% glucose-supplemented medium	360	–
+1% salicin at 0 h	900	–
+1% salicin at 5 h	750	–
+1% salicin at 24 h	450	–
+1% salicin at 30 h	375	–

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