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α-L- rhamnosidases Produced under Solid State Fermentation by Few *Aspergillus* Strains

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Authors' contributions

This work was carried out in collaboration between all authors. Author SY designed the study, performed the statistical analysis, wrote the protocol and first draft of the manuscript. Author DK and KDSY managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

The production of α-L-rhamnosidase from *Aspergillus ochraceous MTCC -1810, A. wentii MTCC-1901, A. sydowii MTCC- 635, A. foetidus MTCC-508* under solid- state fermentation using easily available agro- industrial residues such as corn cob, rice bran, sugarcane bagasse, wheat bran and citrus peel as substrate. Among these, sugarcane bagasse in combination with naringin and sucrose were found to be the best substrate. The α-L-rhamnosidase production was highest after the 4th day of incubation at 30ºC and a substrate to moisture ratio of 1:1 w/v. Supplementation of the medium with 10% naringin caused the maximum production of the enzyme. The temperature optima and pH optima of α-L-rhamnosidases were determined in the range of 50-60ºC and 4.0-5.0 respectively. The α-L-rhamnosidases secreted from the above fungal strains is suitable for the debittering of orange fruit juice.

Keywords: α-l rhamnosidase; solid state fermentation; Naringin; corncob; Aspergillus.

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1. INTRODUCTION

Solid- state fermentation [SSF] has become more popular in recent years, due to the enormous potential for the production of enzymes [1-3]. The food processing and the agricultural industry annually generate a great amount of wastes worldwide, which causes a serious disposal problem. The composition of such wastes is usually rich in sugars, which are easily assimilated by the microorganism due to their organic nature. This makes them highly suitable as the raw materials for the production of microorganism for industrial significant enzymes [4]. Solid- state fermentation is the microbial cultivation process in the absence or near absence of free water in the substrate. Among the different α-L-rhamnosidase production processes namely, submerged system, solid state fermentation is more economical due to the cheap and abundant availability of agricultural wastes which can be used as substrates [5].

The α-L-rhamnosidase [EC.3.2.1.40] cleaves terminal α-L-rhamnose specifically from natural glycosides such as naringin, rutin, hesperidin, quarcitrin and dioscin [6-8]. The enzyme is biotechnologically important for having potentials for applications in debittering of citrus fruit juices [9], enhancement of wine aroma [10] and derhamnosylation of natural glycosides containing terminal α-L-rhamnose to produced L-rhamnose and pharmaceutically important compounds like prunin from naringin, quercetin from quercetrin [11,12] etc. These different applications require α-L-rhamnosidases with different pH optima. Thus there is a need to search new sources of the enzyme with different pH optima suitable for different applications and to make those α-L-rhamnosidases available in large amounts for industrial applications [6]. It has been found that not all α-L-rhamnosidases are suited for all applications. For example, α-L-rhamnosidases which have pH optima in the range 3-4 pH unit are best suited for debittering of citrus fruit juices and elimination of hesperidin crystals because orange fruit juices have a pH near the same range [13]. The α-L-rhamnosidases which have pH optima in the alkaline range are more suited for the derhamnosylation of natural glycosides containing terminal α-L-rhamnose for the preparation of important pharmaceutical compounds like prunin from naringin, quercetin glucoside from rutin, diosgenin from diosgene, quercitin from quercetrin, hesperitin from hesperidin [14-19]. α-L-rhamnosidases which have pH optima in the range 5-6 are more suited for the enhancement of wine aroma [20]. Thus there is a need to search α-L-rhamnosidases with different properties suitable for different applications and make them available in large quantities for commercial utilisation.

Fungal strains belonging to *Aspergillus* genera are generally rich sources of enzymes. Moreover, more than 100 indigenous fungal strains belonging to the genera *Aspergillus* have been isolated, purified, identified and deposited at Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology Chandigarh [21] by Indian Scientists. The potentials of these indigenous fungal strains as α-L-rhamnosidases secretors are not known. Keeping these points in mind, this project was proposed on the studies of α-L-rhamnosidases of indigenous fungal strains belonging to the genera *Aspergillus.*

2. MATERIALS AND METHODS

2.1 Chemicals

p-Nitrophenyl-α-L-rhamnopyranoside, naringin, L-rhamnose, rutin, CM cellulose were purchased from Sigma Chemical Company, St. Louis (USA). All the chemicals including the protein molecular weight markers used in the polyacrylamide gel electrophoresis were procured from Bangalore GENEI Pvt. Limited Bangalore (India). All other chemicals were either from Merck Limited Mumbai (India) or from s.d. – fine CHEM limited Mumbai (India) and were used without further purifications. Bagasse, rice brain, orange peel, corn cob etc. were procured from local market.

2.2 Microorganism

Aspergillus ochraceous MTCC -1810, A. wentii MTCC- 1901, A. sydowii MTCC- 635, A. foetidus MTCC-508 were procured from MTCC Center and Gene Bank, Institute of Microbial Technology, Chandigarh (India) and was maintained in the laboratory on the agar slant as mentioned in the MTCC catalogue-2000 and store at 4 ºC.

2.3 α-L-rhamnosidase Production by SSF and Enzyme Extraction

The secretion of α-L-rhamnosidases by the fungal strains wares studied in the reported liquid culture medium which consisted of $CaCl_2$1.0 g, $MgSO_4.7H_2O$ 3.0 g, KH_2PO_4 20.0 g, $N(CH_2COONa)_3$ 1.5 g, $MnSO_4$ 1.0 g,

$ZnSO_4.7H_2O$ 0.1 g , $CuSO_4.5H_2O$ 0.1g, $FeSO_4.7H_2O$ 0.1 g, H_3BO_3 10.0 mg, sucrose 40.0 g, ammonium tartarate 8.0 g. water (MilliQ)1000 mL. The solid substrates and liquid culture medium were separately autoclaved. One mL of the spore suspension (spore density 5 x 10^6 spores/mL) from the agar slant was inoculated aseptically into the liquid culture medium (100 mL) kept in 250 mL culture flask. Aliquots of one ml of the liquid culture growth medium were withdrawn at regular intervals of 24 hrs. It was filtered through Millex syringe filters (0.22 µm) and was analysed for the presence of α-L-rhamnosidase activity by reported method [22]. Three sets of α-L-rhamnosidase secretion of experiment were performed. In the first sets, the effect of the presence of sucrose, glucose, rhamnose and fructose in the liquid culture medium for the secretion of α-L-rhamnosidase were studied. Keeping in view, the medium with no carbohydrate was used as the control [13]. In the second set, the effects of the presence of hesperidin, naringin, rutin, naringenin and quercetin in the liquid culture growth medium containing 4.0% sucrose on the secretion of α-L-rhamnosidase were studied. In the third set of the experiment, the solid substrate moistened with 10 mL nutrient solution contained sucrose and naringin in Erlenmeyer flasks (100 ml). The four fungal strains were inoculated with 5 x 10^6 spores/mL from the different agar slants in different Erlenmeyer flasks, and flasks were incubated at 30ºC in BOD under stationary conditions. The solid substrate of growth medium (2-3 particles) were withdrawn at regular intervals of 24 hrs and were extracted in 1 ml of sodium acetate / acetic acid buffer solution pH 4.5 and by agitation (200 rpm) at 4ºC . The slurry was filtered and centrifuged at 2000 g for 5 min and the supernatant was the source of crude enzyme. The experiments were performed in triplicates, and the data points were the average of three measurements, and the standard deviation was less than 5%.

2.4 Enzyme Assay

The activity of α-L-rhamnosidase was determined using p-nitrophenyl-α-L-rhamnopyranoside as the substrate following the reported method [22]. 1.0 ml reaction solution consisted 0.4 mM p-nitrophenyl-α- L-rhamnopyranoside as the substrate in 0.2M sodium acetate/acetic acid buffer pH 4.5, maintained at 50°C. 0.1 ml of the enzyme extract was added to the above solution. 0.1 mL aliquot was withdrawn immediately and was diluted to 3.0 mL of 0.5 M NaOH solution. Then 0.1 mL aliquot was withdrawn at regular intervals of 2.0 min and was treated similarly. The samples were kept in standing position at the room temperature at least for 30 minutes, and the absorbance was measured spectro-photometrically at 400 nm using UV/Vis spectrophotometer Hitachi (Japan) model U-2000. The molar extinction coefficient value of 21.44 $mM^{-1}cm^{-1}$ of p- nitrophenol was used for the calculation of enzyme unit. One unit of enzyme activity was defined as the amount of enzyme required to release one µmol of p-nitrophenol per min in the reaction mixture under the above assay conditions. The least count of the absorbance measured was 0.001 absorbance unit. The steady state velocity measurements were reproducible within 5% standard deviation.

2.5 Effect of Different Carbon Sources on Enzyme Production

The carbon sources of glucose, rhamnose and fructose (monosaccharide) and sucrose (disaccharide) were used to determine the best carbon source for optimum enzyme production. All the carbohydrates were used at the concentration of 2% and 5% and replaced the carbon source of the basal medium and naringin in the basal medium was also used at the optimum concentration (0.75%). All the other conditions were maintained the same as the basal medium.

2.6 Effect of Inducer on the Naringinase Production

Naringin, rutin, hesperidin, quercetin and naringenin were used as an inducer in this experiment. The concentration of inducers was only changed in a liquid broth of control medium, and the concentration ranged from (w/v) 0.25% to 1.25% with 0.25% interval. The pH of the medium and all the other conditions for the fermentation were maintained same as basal medium. Fermentation was allowed for nine days and the optimum enzyme activity was obtained on the 4^{th} to 7^{th} days at room temperature.

3. RESULTS AND DISCUSSION

3.1 Secretion of α-L-rhamnosidase

The enzyme activity produced at several cultivation times using rhamnose, glucose, sucrose and fructose as carbohydrates and carbon source is shown in Table 1. The effect of

sucrose concentration on enzyme yield was a better carbon source than rhamnose glucose and fructose [13]. Table 2 shows that the effect of naringin, rutin, hesperidine, quercetn and naringenin as inducers and carbon source on the secretion of α-L-rhamnosidase by *Aspergillus ochraceous MTCC -1810, A. wentii MTCC- 1901, A. sydowii MTCC- 635, A. foetidus MTCC-508.* It can be seen that naringin was the better inducer than the other inducers.

The effect of the carbon source is important to produce a higher amount of enzyme. Carbon source will act as a source of energy and carbon. The glucose is widely utilised as a carbon source but other carbon sources like sucrose, maltose, lactose, rhamnose etc. have been used [14-15]. The sucrose gave prominent α-L-rhamnosidase enzyme production than glucose and rhamnose [23]. Another study indicates that sucrose and molasses can exhibit maximum α-L-rhamnosidase production but maltose and lactose produce a low level of α-L-rhamnosidase [24]. The study of α-L-rhamnosidase production by *Micrococcus* shows that increasing concentration of sucrose from 0.25% to 0.5%, increases the α-L-rhamnosidase enzyme and then the rate declines [25]. Sucrose is the best carbon source for α-L-rhamnosidase production by *Streptomycetes* than glucose [26]. The present study also indicates that sucrose at 4.0% concentration is the better carbon source than glucose.

Inducers increased the α-L-rhamnosidase secretion by *Aspergillus ochraceous MTCC -1810, A. wentii MTCC- 1901, A. sydowii MTCC-635, A. foetidus MTCC-508* and this studies indicate that naringin could be one of the best inducer among rhamnose, naringenin, rutin and hesperidin. The activity of α-L-rhamnosidase from *Aspergillus foetidus* MTCC-508 was highest (2.68U/ml) on the 7th day after incubation of fungal spore in the liquid culture medium.

3.2 α-L-rhamnosidase by Solid State Fermentation

Effect of naringin concentration was studied, which concluded that stepwise addition of smaller concentration of naringin to the medium was more effective than the addition of a higher amount of naringin at initial [23]. Hence the concentration of naringin should be considered during α-L-rhamnosidase production from the microbes. The enzyme activity of α-L-rhamnosidase produced by *Streptomycetes* was high at low concentrations of naringin, and the activity of α-L-rhamnosidase increased from 7th day to 14th day [24]. Hence inducer is important for the microorganism to secrete the enzyme. The experiment was done to increase the α-L-rhamnosidase production and using the low- cost agro wastes, plant products, machinery, equipments, raw materials and also using less labour. The solid- state fermentation (SSF) might be a good solution for this than the submerged fermentation system [27]. The solid support should be an optimum surface area for oxygen diffusion, nutrient absorption and assimilation. The paddy husk functions as a good support for solid state fermentation [28,29]. The filamentous fungi were used to produce α-L-rhamnosidase in SSF using grapefruit rind and orange rind as support but the grapefruit rind functioned as the best support [30]. The possibilities are found to produce α-L-rhamnosidase enzyme by solid state fermentation by *Aspergillus ochraceous MTCC -1810, A. wentii MTCC- 1901, A. sydowii MTCC- 635, A. foetidus MTCC-508* using sugarcane bagasse, rice grain, corn cob, wheat brain and orange peel which are capable of producing α-L-rhamnosidase enzyme. Table 3 shows the production of α-L-rhamnosidase using solid state fermentation using sugarcane bagasse as the best inducer. To compare the results, the activities of α-L-rhamnosidases produced on different solid materials were expressed as units per ml of the nutrient solution. It can be seen that sugarcane bagasse was obtained as the best inducer as those from rice grain, corn cob, wheat bran and orange peel.

3.3 pH and Temperature Optima

The results of the dependence of the activity of α-L-rhamnosidases on the variation of the pH of the reaction solution are shown in Fig. 1. The α-L-rhamnosidases was active in the acidic pH range 3.5-5.0, but the maximum activity of the enzyme was at *pH* 4.0 and 4.5. The α-L-rhamnosidase in this pH range is suitable for the debittering of orange fruit juice and removal of hesperidin crystals from fruit juice [31].

The variation of the activity of the α-L-rhamnosidases with the temperature of the reaction solution is shown in Fig. 2. The temperature optimum of the α-L-rhamnosidases ware 50°-60°C. The temperature optima of the α-L-rhamnosidases reported in the literature [2] were in the range of 40–80°C.

Table 1. Effect of carbohydrates on the production of α-L-rhamnosidase from *Aspergillus ochraceous* MTCC -1810, A. wentii MTCC- 1901, A. sydowii MTCC- 635, A. foetidus MTCC-508

Carbon Source	α-L-rhamnosidase activity (Total U mL^{-1})			
	A. ochraceous	*A. wentii*	*A. sydowii*	*A. foetidus*
rhamnose	231.6	138.2	356.4	396.0
glucose	303.0	286.6	510.2	693.1
sucrose	363.6	397.0	596.3	738.0
fructose	236.1	138.0	239.9	436.0

Table 2. Effect of different inducers on the production of α-L-rhamnosidase from *Aspergillus ochraceous* MTCC-1810, A. wentii MTCC- 1901, A. sydowii MTCC- 635, A. foetidus MTCC-508

Carbon Source	α-L-rhamnosidase activity (Total U mL^{-1})			
	A. ochraceous	*A. wentii*	*A. sydowii*	*A. foetidus*
Naringin	235.3	202.6	328.4	639.0
Rutin	103.4	145.7	203.4	238.0
Hesperidin	195.9	179.3	300.3	396.0
Naringenin	70.0	83.0	138.3	356.0
quercetin	89.0	75.2	143.0	421.0

Table 3. Effect of different solid support on the production of α-L-rhamnosidase from *Aspergillus ochraceous* MTCC -1810, A. wentii MTCC- 1901, A. sydowii MTCC- 635, A. foetidus MTCC-508

Carbon Source	α-L-rhamnosidase activity (Total U mL^{-1})			
	A. ochraceous	*A. wentii*	*A. sydowii*	*A. foetidus*
Corn cob	69.0	73.0	142.3	39.0
Rice bran	34.0	43.1	73.1	115.9
Sugar Cane Bagasse	186.4	153.1	198.3	309.1
Wheat bran	52.1	60.3	39.0	201.0
Orange peel	69.3	39.1	105.0	92.5

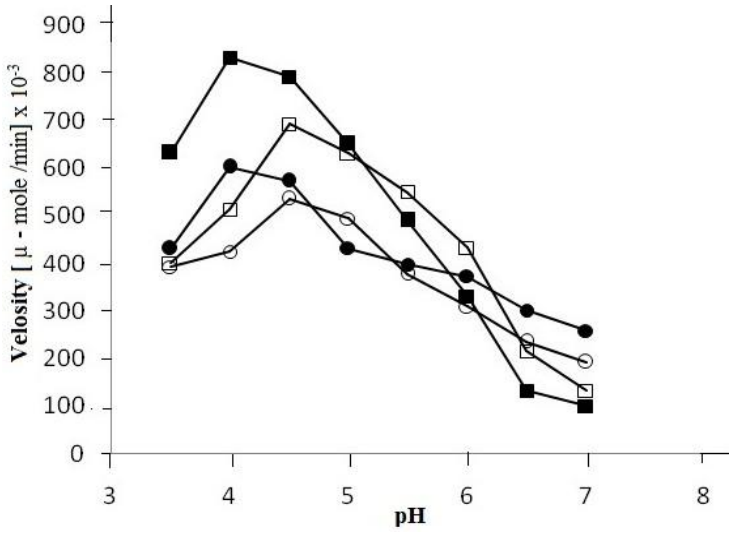

Fig. 1. Effect of pH on the activity of the enzyme
Aspergillus ochraceous MTCC -1810 (□), A. wentii MTCC- 1901 (■), A. sydowii MTCC- 635 (○), A. foetidus MTCC-508 (●)
The assay solution 1.0 mL contained 0.4 mM substrate, 1.50 µg of the crud enzyme in sodium acetate/ acetic acid buffer of varying in the range 3.5-7.0 at 50°C

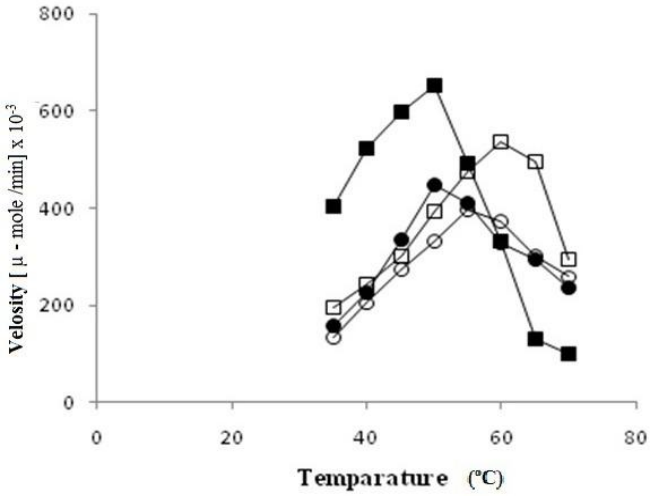

Fig. 2. Effect of temperature on the activity of enzyme

Aspergillus ochraceous MTCC -1810 (□), A. wentii MTCC- 1901 (○), A. sydowii MTCC- 635 (■), A. foetidus MTCC-508 (●)

The assay solution 1.0 mL contined 0.4 mM substrate, 1.50 µg of crud enzyme in 0.5 M sodium phosphate buffer pH 10.5 at varying temperature (30-70°C)

4. CONCLUSION

In conclusion, this communication reports the production of α- L- rhamnosidases from solid-state fermentation of few *Aspergillus* strains using agro wastes as solid support reported in the literature. The secretion of α-L-rhamnosidase by different *Aspergillus* strains are not very high, but with the development in the area of molecular biology it would be possible to isolate the gene of the above enzymes and overexpress them in suitable vector so that the amount of enzyme needed for commercial applications could be produced. The reported studies in this communication will be useful in achieving the above objective.

DISCLAIMER

This paper is based on the preliminary dataset. Readers are requested to consider this paper as the preliminary research article. Authors are aware that detailed statistical analysis is required to get a scientifically established conclusion. Readers are requested to use the conclusion of this paper judiciously as statistical analysis is absent. Authors also recommend detailed statistical analysis for similar future studies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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