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Secretion of α-L- rhamnosidases by some *Fusarium* strains using agro waste as inducer

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Abstract

Naringin is the main bitter flavanoid in citrus fruit juices. The few fungal stains were screened for the production of α-L-rhamnosidase in order to hydrolyzed the naringin from removal of the bitter taste from juices. The present study was conducted to isolate and characterize the α-L-rhamnosidase enzyme from few fungal strains belonging to genera Fusarium. We initiated the easy procedure to isolate and characterize the α-L-rhamnosidase producing microorganism by using solid support as inducers which is easily available agro industrial residues such as corn cob, sugarcane baggase, and citrus peel. Among these, sugar cane Bagasse in combination with naringin and sucrose were found to be the best inducer. α-L-rhamnosidase production was highest after 4th day to 8th day of incubation at 30ºC caused maximum production of enzyme. The temperature optima and pH optima of α-L-rhamnosidases were determine in the range of 50-65ºC and 4.5-11.0 respectively. α-L-rhamnosidases secreted from above fungal strain suitable for the debittering of orange fruit juice, derhamnosylation of natural glycosides, and aroma enhancement of wine. The paper concludes that fusariumgenera can be source of α-L-rhamnosidase enzyme for industrial purposes.

Keywords: α-L rhamnosidase, Naringin, *Fusarium,* Orange fruit juice, Glycosides.

Introduction

α-l-rhamnosidase [EC.3.2.1.40] is biotechnologically important enzyme having applications in debittering of citrus fruit juice[1-3], in the preparation of l-rhamnose and other pharmaceutically important bioactive compounds by derhamnosylation of natural rhamnosides containing terminal α-l-rhamnose[4,5]. It is cleaves terminal α-l-rhamnose specifically from a number of rhamnosides and is widely distributed in nature[6]. The research work on α-l-rhamnosidase has been reviewed recently by Yadav et al.[7]. It has also been used for enhancement of wine aromas by enzymatic hydrolysis of terpenyl glycosides containing L-rhamnose[8,9], elimination of hesperidin crystals from orange juices[10,11], conversion of chloropolysporin B to chloropolysporin C[11], the derhamnosylation of many L-rhamnose containing steroids for example, diosgene, desglucoruscin, ginsenosides-Rg2, etc. whose derhamnosylated products have their clinical importances[5,12,13].

α-L-Rhamnosidases with different physicochemical properties are suitable for different applications. α-L-Rhamnosidase selectively derhamnosylates natural glycosides containing terminal α-L-rhamnose, the derhmnosylated products are rare compounds of pharmaceutical importance. α-L-Rhamnosidase transforms naringin toprunin which has enhanced bioavailability compare to naringin and its aglyconnaringenin while maintaining its bioactivity. The enzymatic hydrolysis method has been applied industrial production of icariside I[14].

The derhamnosylation of hesperidin by α-L-rhamnosidase gives hesperetin-7-O-glucoside, which is a rare pharmaceutically important compound. α-L-Rhamnosidase transforms rutin to isoquercitrin which has been reported as a drug for a number of diseases due to its non- oxidisable, anti-inflammatory, anti-mutagenetic, antiviral properties and other pharmacological effects. Moreover, isoquercitrin is a precursor for the enzymatic biosynthesis of enzymatically modified isoquercitrin (EMIQ) which has been approved as a multiple food additive[10-11]. These biotechnological applications of α-L-rhamnosidases have prompted the authors to purify and characterize α-L- rhamnosidases from new sources with novel properties and to demonstrate their applications for different biotransformation. Keeping in view the biotechnological importance of α-l-rhamnosidase, we initiated research on these enzymes with the objective of finding these enzymes with properties suitable for different biotechnological applications[7]. In this communication, authors report purification and characterization of an α-l-rhamnosidase from the culture filtrates of few microorganism belonging to *Fusarium* genera and have assessed their properties.

Materials and Methods

Chemicals: p-Nitrophenyl-α-L-rhamnopyranoside, naringin, L-rhamnose, 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.

Microorganism: *Fusarium acuminatum MTCC-1983, F. compactum MTCC-2014, F. culmorum MTCC-349, F. culmorum MTCC- 2090, F. decemcellulose MTCC-2079, F. ventricosum MTCC-720, F. tumidum MTCC-2463, F. solani MTCC-3004, F. solani MTCC-2082, F. sambucerum MTCC-2085, F. oxysporum MTCC-3075, F. pollidoroseum MTCC-2083, F. graminearum MTCC-1893, F. moniliforum MTCC-2088, F. poe MTCC-2086, F. crookwellense MTCC-2084, F. graminearum MTCC-2089, F. moniliforme MTCC-2015,* 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 4ºC.

Secretion of α-L-rhamnosidase: The secretion of α-L-rhamnosidases by the fungal strains wares studied in the reported liquid culture medium which consisted of $CaCl_2$ 1.0g, $MgSO_4.7H_2O$ 3.0g, KH_2PO_4 20.0g, $N(CH_2COONa)_3$ 1.5g, $MnSO_4$ 1.0g, $ZnSO_4.7H_2O$ 0.1g, $CuSO_4.5H_2O$ 0.1g, $FeSO_4.7H_2O$ 0.1g, H_3BO_3 10.0mg, sucrose 40.0g, ammonium tartarate 8.0g, water (MilliQ)1000mL. The solid substrates and liquid culture medium were separately autoclaved. After cooling and mixing in the proportion of 1:2 w/v in Erlenmeyer flasks (100ml) the fungal strains were inoculated with $5x10^6$ spores /mL from the agar slants and incubated at 30ºC, with 70-80% relative humidity under stationary conditions. Aliquots of one ml of the growing liquid culture medium were withdrawn at regular intervals of 24 hrs. Filtered through Millex syringe filters (0.22µm) and was analyzed for the presence of α-L-rhamnosidase activity by reported method[16]. Three sets of α-L-rhamnosidase secretion of experiment were performed. In the first sets, the effects of the presence of sucrose, glucose and rhamnose in the liquid culture medium on the secretion of α-L-rhamnosidase were studied. Keeping in view the medium with no carbohydrate was as in the control. In the second sets, the effects of the presence of hesperidin, naringinand rutin in the liquid culture growth medium containing 4.0% sucrose on the secretion of α-L-rhamnosidase were studies. In the third sets of experiment the effect of solid support moist with the liquid culture growth medium contains sucrose and naringin on the secretion of α-L-rhamnosidase were studied. The solid support were extracted in 30 to 50 ml of sodium acetate / acetic acid buffer solution pH 4.5 and by agitation (200 rpm) at 4ºC. The slurry was filtered and centrifuge at 2000 g for 5 min and 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%.

Enzyme assay: The activity of α-L-rhamnosidase was determined using p- nitrophenyl-α- L- rhamnopyranoside as the substrate following the reported method[16]. 1.0 ml reaction solution consisted 0.4mM p-nitrophenyl-α- L-rhamnopyranoside in 0.2M sodium acetate / acetic acid buffer pH 4.5, maintained at 50°C. 0.1ml of the enzyme extract was added to the above solution. 0.1mL aliquot was withdrawn immediately and was diluted 3.0mL of 0.5M NaOH. Then, 0.1mL aliquot withdrawn at regular intervals of 2.0min and were treated in the same manner as mentioned above.

The sample was maintained at the ambient temperature at least for 30 minutes and the absorbance was measured spectrophotometrically at 400nm 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.

Effect of different carbon sources on enzyme production: The carbon sources of glucose and rhamnose (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 1% and 4% 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 same as the basal medium.

Effect of inducer on the enzyme production: Naringin, rutin and hesperidin were used as inducer in this experiment. The concentration of inducers was only changed in liquid broth of control medium and the concentration ranges 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.

Results and Discussion

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. The effect of naringin, rutin and hesperidine as inducers and carbon source on the secretion of α-L-rhamnosidase by *Fusarium* strains. It can be seen that naringin was the better inducer than the other inducers.

The effect of carbon source is important in order to produce higher amount of enzyme. Carbon source will act as a source for energy and carbon. The glucose is widely utilized as carbon source but other carbon sources like sucrose, maltose, lactose, rhamnose etc. have been used. The sucrose gave prominent α-L-rhamnosidase enzyme production than glucose and rhamnose[18]. Another study indicates that sucrose and molasses can exhibit maximum α-L-rhamnosidase production but maltose and lactose produce low level of α-L-rhamnosidase production[15]. The study of α-L-rhamnosidase production by *Micrococcus* gave the result as when increasing concentration of sucrose from 0.25% to 0.5%, the α-L-rhamnosidase enzyme was increased and then after declined[16]. Sucrose was best carbon source for α-L-rhamnosidase production by *Streptomycetes* than glucose[17]. The present study indicates that sucrose at 4.0% of concentration is the best carbon source than glucose.

Inducers increased the α-L-rhamnosidase secretion and this studies indicates 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.

Effect of concentration of naringin was studied concluded that stepwise addition of smaller concentration of naringin to the medium was more effective than the addition of higher amount of naringin at initial[18]. Hence the concentration of naringin should be considered during α-L-rhamnosidase production from 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[15]. 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 labor. The solid support might be a good solution for this than the submerged fermentation system[19]. The solid support should be optimum surface area for oxygen diffusion, nutrient absorption and assimilation. The paddy husk functions as a good support for solid state fermentation by the bacteria[20-21]. The filamentous fungi were used to produce α-L-rhamnosidase in SSF using grape fruit rind and orange rind as support but the grape fruit rind functioned as a best support[22]. The possibilities are found to produce α-L-rhamnosidase enzyme by fungal strains using sugar cane bagasse, corn cob and orange peel which are capable of producing α-L-rhamnosidase enzyme. Table-1 shows the production of α-L-rhamnosidase using sugar cane Bagasse as solid support is best inducer. In order to compare the results, the activities of α-L-rhamnosidases produced on different solid materials were expressed as units per ml of nutrient solution. It can be seen that sugar cane bagasse obtained as best inducer.

The enzymatic characteristics of α-L-rhamnosidases like Michaelis–Menten behaviour of the enzyme using p-nitrophenyl-α-L-rhamnosidase as the substrate, the variation of the pH of the reaction solution and variation of the activity of the α-L-rhamnosidases with temperature of the reaction solution ware determined. The results are shown in Table-2. The α-L-rhamnosidases was active in the acidic pH range 4.5-11.5. The α-L-rhamnosidase in this pH range is suitable for the embittering of orange fruit juice and de-rhamnosylation of natural glycosides support[23]. The temperature optimum of the α-L-rhamnosidases ware 50°- 65°C. The temperature optima of the α-L-rhamnosidases reported in the literature[23] were in the range 40–80°C.

Table-1: Culture conditions for the production of α-L-rhamnosidase.

Microorganism	Growth Temp. (ºC)	Enzyme Secretion	Inducers Glycosides	Carbon sources carbohydrates	Solid Support Agro waste	Peak value U/mL
Fusariumacuminatum MTCC-1983	27	-	Rutin	Sucrose	---	00
F. compactum MTCC-2014	27	+	Naringin	Sucrose	---	0.569
F. culmorum MTCC -349	25	-	Rutin	Sucrose	---	00
F. culmorum MTCC- 2090	25	-	Naringin	Sucrose	---	00
F. decemcellulose MTCC- 2079	30	-	Naringin	Sucrose	---	00
F. ventricosum MTCC-720	30	+	Hesperidin	Sucrose	Corncob	0.215
F. tumidum MTCC-2463	25	-	Naringin	Sucrose	---	00
F. solani MTCC-3004	27	+	Naringin	Glucose	Orange peel	0.521
F. solani MTCC-2082	25	+	Naringin Rhamnose	Sugarcane baggase	0.320	
F. sambucerum MTCC- 2085	30	-	Rutin	Sucrose	---	00
F. oxysporum MTCC-3075	30	+	Rutin	Sucrose	Sugarcane baggase	0.362
F. pollidoroseum MTCC-2083	27	+	Naringin	Glucose	Corncob	0.652
F. graminearum MTCC- 1893	27	+	Hesperidin	Sucrose	Corncob	0.635
F. moniliforum MTCC-2088	30	+	Naringin	Sucrose	Sugarcane baggase	0.914

F. poe MTCC-2086		+	Hesperidin	Sucrose	Sugarcane baggase	0.535
F. crookwellense MTCC-2084	25	+	Rutin	Sucrose	Corn cob	0.621
F. graminearum MTCC-2089	30	-	Naringinrhamnose	---	00	
F. moniliforme MTCC-2015	30	-	Naringinrhamnose	---	00	

Table-2: Enzymatic Characteristics on the production of α-L-rhamnosidase.

Microorganism mM	K_m (°C)	pH stability	Temperature stability (°C)	pH specificity	Temperature	Substrate
F. compactum MTCC-2014	0.45	11.0	55	8.5	10	Rutin
F. ventricosum MTCC-720	1.89	8.5	57	8.0	10	Naringin
F. solani MTCC-3004	1.25	10.5	50	7.0	10	Naringin
F. solani MTCC-2082, MTCC- 2085	*0.27*	9.5	55	8.0	10	Naringin
F. oxysporum MTCC-3075	0.98	6.5	60	6.0	10	Naringin
F. pollidoroseum MTCC-2083	1.96	6.0	57.5	6.0	10	Hesperidin
F. graminearum MTCC- 1893	*0 .65*	4.5	55	5.0	10	Naringin
F. moniliforum MTCC-2088	0.35	10.5	50	8.0	10	Naringin
F. poe MTCC-2086	*0.45*	10.0	55	8.0	10	Hesperidin
F. crookwellense MTCC-2084	*0.34*	8.0	65	7.0	10	Rutin

Conclusion

In conclusion, this communication reports the production of α-L- rhamnosidases by few *Fussarium* strains using agro wastes as solid support reported in the literature. The secretion of α-L-rhamnosidase by different *Fussarium* strains are not very high, with the development in the area of molecular biology it would be possible to isolate the gene of the above enzymes and over express 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.

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