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Production of a α-L-Rhamnosidase from *Aspergillus Terreus* Using Citrus Solid Waste as Inducer for Application in Juice Industry

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Naringinase is an enzyme complex consisting of a α-L-rhamnopyranosidase (Rha, EC. 3.2.1.40) and β-D-glucopyranosidase (βG, EC. 3.2.1.21). α-L-rhamnopyranosidase is an enzyme of considerable importance in food technology for increasing the aroma of fruit juices and alcoholic beverages. It is particularly used for debittering of citrus-juice by the hydrolysis of naringin and hesperidin, however this activity is low in commercial enzyme preparations. The aim of this work is the production of Rha from *Aspergillus terreus* fermentation using citrus solid waste (CSW), called “pastazzo”, as specific inducer. CSW is rich in fermentable materials such as sugars, in particular rhamnose, in pectins, and in flavanone glycoside as hesperidin and naringin. Rha and βG have been characterized from a culture of *A. terreus.*

1. Introduction

α-L-rhamnosidase (Rha) and β-D-glucosidase (βG) were produced and characterized. The Rha hydrolyses the linkage of rhamnose with other compounds, while βG hydrolyses the linkage of glucose, on several flavanone glycosides as hesperidin, naringin, rutin. This enzyme complex has considerable importance in food technology for increasing the aroma of wines, musts, fruit juices and other alcoholic beverages. In particular, it is used in citrus-juice debittering by naringin and hesperidin hydrolysis. Naringin is the major flavanone in grapefruit and it is responsible of its bitter taste. The presence of bitterness, generally, has been the major limitation for the commercial acceptance of juices. The naringin content of juices can be reduced by several chemical technologies, but these methods may alter the composition of juice, may affect organoleptic properties and the quality of the juice, for the removal of nutrients, flavour and colour components. Therefore, acid hydrolysis is not suitable to commercial processes.
Similarly, naringin can be completely removed from the solution, under selected conditions of pH and temperature, but a lot of the desirable flavoring components are also simultaneously removed.
The deglycosylation of the novel glycopeptide antibiotic, chloropolysporin from *Faenia interjecta*, was achieved successfully by the rhamnosidase activity of naringinase (Sankyo, 1988). The enzyme is used to produce L-rhamnose which is a chiral intermediate in organic synthesis and it is used as a

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pharmaceutical and plant protective agent (Daniels et al. 1990). The production and characterization of an *Aspergillus terrus* α-L-rhamnosidase activity of naringinase in combination with β-D-glucosidase is considered suitable for aroma enhancement in wine making (Caldini et al., 1994). Among the naringinase-producing fungi, the enzyme preparation from *Penicillium decumbens* (Young et al., 1989) is commercially available. Naringinase obtained from *P. decumbens* has been used for immobilization studies and for the transformation of flavonoids (Manjon et al., 1985; Romero et al., 1985; Puri et al., 2005). Debittering by enzymatic processing is a suitable method. The use of enzymes for debittering is increasing rapidly, because of minimal pollution during processing.

The aim of this work is the production of Rha from *Aspergillus terreus* (*A. terreus*) using citrus solid waste as specific inducer. In industrial citrus processing solid waste products are obtained, they are essentially constituted of peel and pulp, named "pastazzo". The citrus solid waste products are rich in fermentable materials as sugars and pectins, besides they contain high concentrations of flavanone glycosides, hesperidin and naringin, and lower amounts of many other flavonoids, which are important antioxidant compounds for human health.

A number of compounds of high interest for the chemical and pharmaceutical industry, can be extracted from pastazzo. Its composition is strictly related with the chemical composition of citrus.

Citruses contain (1-2 g/kg) of lipids (oleic, linoleic, linolenic, palmitic, stearic acids, glycerol, and a phytosterol), sugars (glucose, fructose, sucrose), acids (primarily citric and malic, but also tartaric, benzoic, oxalic, and succinic), insoluble carbohydrates (cellulose, pectin), enzymes (pectinesterase, phosphatase, peroxidase), flavonoids (hesperidin, naringin), bitter principles (limonin, isolimonin), peel oil (d-limonene), volatile constituents (alcohols, aldehydes, ketones, esters, hydrocarbons, acids), pigments (carotenes, xanthophylls), vitamins (ascorbic acid,Vitamin B complex, carotenoids), and minerals, primarily calcium and potassium. (Bampidis and Robinson, 2006)

Pastazzo is employed as energetic source for growing microorganisms due to its content in carbon and other nutrient components, and as specific inductor for synthesis of glycosidase thanks to the presence of flavonoids.

2. Material and methods

2.1 Chemicals

Yeast extract was obtained from OXOID. *p*-nitrophenyl-α-L-rhamnopyranoside (pNPR), *p*-nitrophenyl-β-D-glucopyranoside (pNPG), L-rhamnose, D-glucose, D-fructose, citric acid were purchased from Sigma. Bradford's reagent for proteins determination was from Bio-Rad Laboratories. All other chemicals and silica MS3030 for immobilization were reagent grade.

2.2 Fermentation process

The strains used for this study originated from the Culture Collection Fungi (CCF), of the Department of Botany, Charles University Prague, Czech Republic. A total of four fungal strains belonging to different genera were used. They are *Aspergillus terreus (A. terreus)*, *Emericella nidulans (E. nidulans)*, *Aspergillus aculeatus (A. aculeatus)*, *Aspergillus niger (A. niger)*. The strains were cultivated in breakwater flasks in aerobic conditions for 17 days at 30 °C; 120 rpm. The growth medium contained the following components: KCl 0.5 (g/L); KH_2PO_4, 15 (g/L); NH_4Cl, 4 (g/L); yeast extract, 5 (g/L); 1 mL of Vishniac solution (Monti et al. 2004); 5 mL 10 % (w/v) of $MgSO_4$ $7H_2O$; "pastazzo" 15 (g/L); pH was adjusted to 6.0. After sterilization the medium was inoculated with 5 ml of fungal spore suspension.

2.3 Enzyme assay

Rha and βG activities were measured by spectrophotometric method using, respectively, *p*-nitrophenyl-α-L-rhamnopyranoside and *p*-nitrophenyl-β-D-glucopyranoside as substrate. The assay mixture contained 200 µL of substrate solution (sodium phosphate buffer 0.15 M, pH 5.0) and 800 µL of enzyme solution, appropriately diluted. After incubation at 35 °C for 20 min, the reaction was stopped by addition of Na_2CO_3, 1 M. The absorbance at 400 nm due to release of *p*-nitrophenol in the mixture was measured. One unit of Rha and βG activity was defined as the amount of enzyme required to release 1 umol of *p*-nitrophenol for min in the solution. The protein concentrations were estimated by

Bio-Rad protein assay reagent for the Bradford dye-binding method (Bradford, 1976), with bovine serum albumin (BSA, Sigma) as protein standard.

2.4 Purification of α-L-rhamnosidase

An amount of lyophilized Rha was solubilised in $NaHCO_3$/NaOH 0.065 M buffer and incubated at pH 11.0 for 2 h at 50 °C. Silica MS3030 used for enzymatic immobilization. A sample of 30 mg of lyophilized enzyme was solubilised in 10 mL of buffer C-P pH 3.5 and 100 mg of silica MS3030 was added. Enzymatic immobilization was performed for 3 h at room temperature and under slow agitation.

3. Results and discussion

3.1 Screening of fungal strains

Four fungal strains were tested for the production of Rha and βG activities using both hesperidin and pastazzo as inducers. All strains showed extracellular activities of both enzymes in presence of only pastazzo as inducer, and no activity in its absence, except for *A. terreus* strain (Figure 1) that showed a low activity also without inducer. *A.terreus* is the strain that shows the higher Rha activity, in terms of relative activity (U/mL). It shows, also, a comparable activity with hesperidin and with pastazzo as inducer.

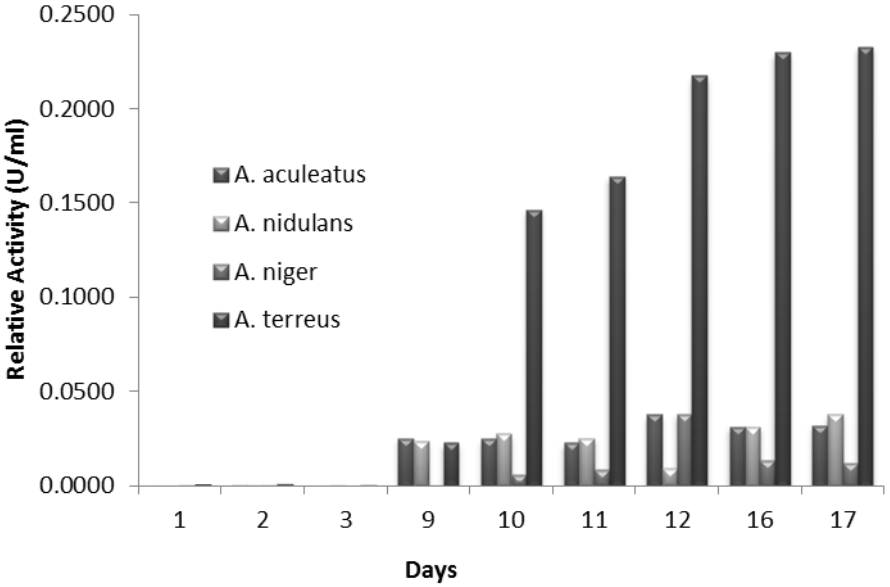

Figure 1: Relative activity of Rha for Aspergillus strains in medium containing pastazzo as inducer.

3.2 Effect of pH and temperature on enzyme activity

The optimum pH was at 4.0 for both enzymes (Figure 2). The effect of pH was tested in a range between 3 and 9. The Rha and βG have different behaviour, and they are stable in this range (Figure 2). Rha shown almost 80 % of activity between pH 5 and 9, while βG lost 50 % of activity at pH 6. The optimum temperature for both crude enzymes was found to be 50°C (Figure 3). Rha loses about 15 % of its activity at 60 °C, and both the enzymes lose 40 % of activity at 40 °C. At 70 °C both enzymes were inactivated. Stability studies of both enzymes in optimal conditions showed that βG activity decreased until to 50 % after 7 h of incubation (pH 4; 50 °C), while Rha lost 30 % of activity after 70 h.

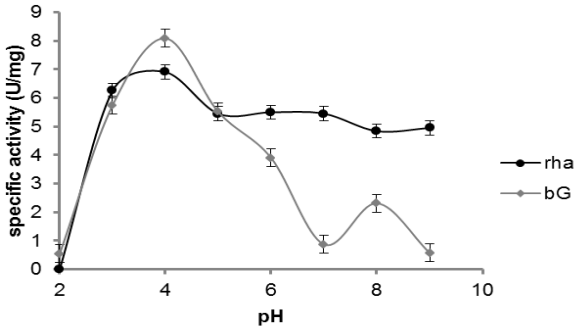

Figure 2: Effect of pH on βG and Rha

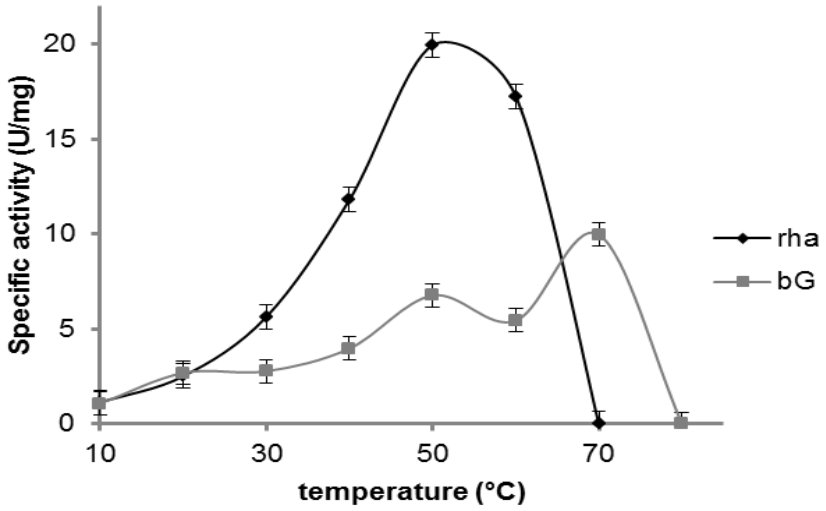

Figure 3: Effect of temperature on βG and Rha

3.3 Kinetics parameters characterization

Kinetic parameters, maximum reaction velocity (V_{max}) and Michaelis-Menten constant (K_m), were determined for both enzymes in according to Lineweaver-Burk plot. For Rha K_m was 0.52 mM and V_{max} was found to be 8.45 U/mg; while for βG K_m was 1.89 mM and V_{max} 9.41 U/mg.

3.4 Inhibition by sugars and ascorbic acid

Inhibition tests were conducted using *p*NPR and *p*NPG as substrates. Rha shows 50 % of its maximal activity in presence of 0.24 % of L-rhamnose; βG showed 50% inhibition by D-glucose at a concentration of 0.80 %. Both enzymes don't show any inhibition by D-fructose. Ascorbic acid at a concentration of 1.20 %, for both enzymes the activity is reduced to 50 % (Figure 4 a, b, c).

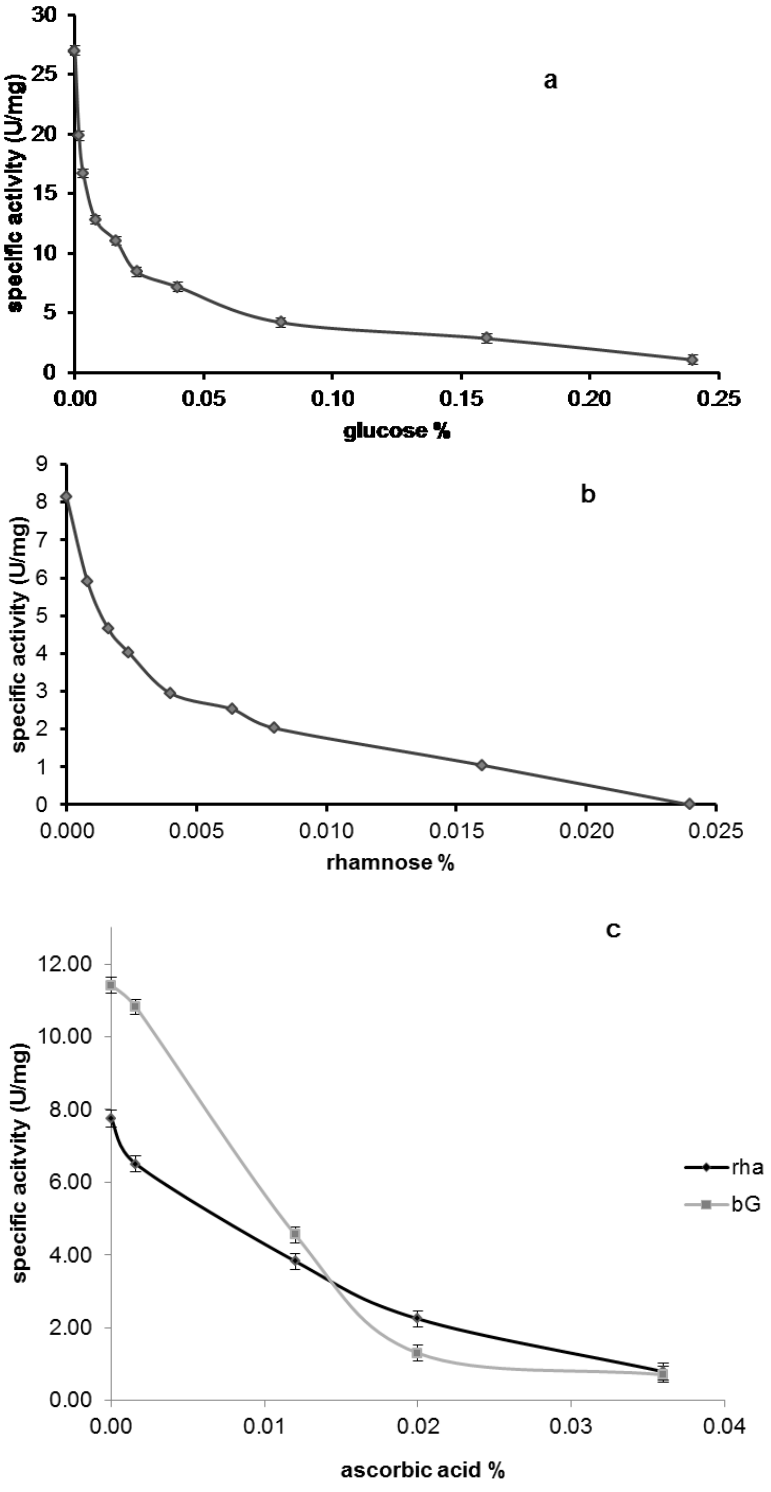

Figure 4 a, b,c: Effects of glucose concentration on the activity of βG (a), of rhamnose concentration on activity of Rha (b) and of ascorbic acid concentration (c) on the activitiy of βG and Rha.

3.5 Purification of a-L- rhamnosidase

A frequent difficulty in purification of Rha is the removal of βG. A lot of work reported separation of two enzymes with ion exchange, size exclusion or DEAE-sepharose chromatography. Due to the great stability of the Rha at high pH, the contaminant activity was easily reduced incubating at pH 11 at 50 °C for 2 hours. In this conditions βG activity was eliminated and α-L-rhamnosidase was preserved 80 % its activity.

Enzymes were also immobilized on silica MS3030 by means adsorption. Immobilized enzymes preserve 50 % their original activity and their activity decrease in the five following days (data not shown).

4. Conclusions

From our results we conclude that the best strain producing α-L-rhamnosidase is *A. terreus*. We obtained interesting results from characterization of Rha and βG for the optimal pH and temperature values and inhibitions suggest a possible use in industrial citrus process. By effect of temperature on βG activity and immobilization on silica MS 3030, it is possible obtain a purified Rha. The use of citrus waste is an advantage for the company because it decreases the expenses for waste disposal.

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