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β-Glucosidase catalyzed synthesis of octyl-β-D-glucopyranoside using whole cells of *Pichia etchellsii* in micro aqueous media

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ABSTRACT

Octyl-β-D-glucopyranoside was synthesized by transglucosylation between *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) and octanol as an acceptor using whole cells of thermo tolerant yeast *Pichia etchellsii* displaying cell wall bound β-glucosidase. Effect of several parameters such as glucosyl donor concentration, enzyme units and initial water activity was studied to optimize product yield. An initial water activity interval of 0.33–0.64 was favorable and increase in total enzyme units had marginal effect on conversion yield. An empirical model was developed to describe the relationship between various parameters and octyl glucoside yield. These factors were combined in a batch replacement strategy whereby octyl-β-D-glucopyranoside was synthesized in 4 h to a concentration of 30 mM (9.25 mg/ml) with a conversion yield of nearly 70% with *p*NPG as a glucosyl donor. Quantitative analysis was done by a highly reproducible reverse-phase high-performance liquid chromatography (RP-HPLC) method and detection was achieved using refractive index detector. The structure of the product was confirmed by ^{13}C and ^{1}H NMR spectroscopy. Additional products like octyl diglucoside were also formed, the structure of which was confirmed by mass spectrometry.

1. Introduction

Alkyl glycosides are well-characterized non-ionic surfactants and find important applications in pharmaceutical and detergent industry (Bhatia et al., 2002). Keeping in view the ecotoxicity issues with non ionic surfactants, such as alkyl phenol ethoxylates, interest in ecofriendly biodegradable biosurfactants has emerged. These are prepared from naturally occurring renewable sources, sugars and fatty alcohols (Akita et al., 2004). Alkyl glycosides are used in biomembrane investigation for solubilization of water insoluble membrane proteins without denaturation and have also been used in reconstitution of biological membranes (Ronzon et al., 2004). Octyl-glucoside (OG) is an interesting non-ionic, dialyzable surfactant used to purify membrane proteins. Favorable properties such as rapid decomposition and easy removal from final protein extracts (Ronzon et al., 2004) makes it a detergent of choice.

Synthesis of alkyl glycosides by enzymatic means is an area of active research. This synthesis can be achieved through reversed hydrolysis or transglycosylation. Enzymatic synthesis of alkyl glycosides from alcohol and carbohydrate (reversed hydrolysis) works fairly well when both substrates are of moderate size (Svensson et al., 2009). Miscibility problems prevent efficient synthesis as the size of the glycosyl acceptors (longer alcohols) and glycosyl donors (longer carbohydrate part) increases. This presents a challenging problem, since the potential products of such reactions are attractive surfactants and are also very difficult to synthesize by chemical methods (Svensson et al., 2009).

Whole cells are potentially attractive biocatalysts for synthesis of alkyl glycosides. The advantage over the use of purified enzymes is that the process can be economical and exhibit less sensitivity to denaturation conditions (Das-Bradoo et al., 2004). Yeasts provide an excellent example of the same as these can be cultivated on inexpensive substrates and stored for a long time. Another recent concept has been to use whole cell biocatalysts that display recombinant enzymes on the cell surface as a source of immobilized enzyme by fusion with cell-wall-anchored proteins. (Junji et al., 2007).

The synthesis and stability of alkyl glycosides is dependent upon low water activity, hence enzymatic reactions need to be carried out in low water environments. This has been achieved by controlling water activity (Ismail et al., 1999), use of bi-phasic system (Ismail et al., 1999; Kwon et al., 2007) and organic one phase liquid systems using co-solvents (Ismail et al., 1999; Ducret et al., 2002; Basso et al., 2002; Gargouri et al., 2004). Reverse micelles for solubilization of enzymes and sugars to improve yields has also been attempted with success (Kouptsova et al., 2001). Few reports appear on the use of whole cell biocatalysts for synthesis of alkyl glucosides (Das-Bradoo et al., 2004; Junji et al., 2007). In this paper, we report the synthesis

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of OG by transglucosylation using whole cells of thermo-tolerant yeast *Pichia etchellsii* that displays cell wall bound β-glucosidase activity.

2. Materials and methods

2.1. Cultivation and lyophilization of P. etchellsii

The whole cells of yeast strain *P. etchellsii* JFG-2201 (Deutsche Sammlung Von Mikroorganismen, DSM: Germany) were used in synthetic reactions. The yeast was maintained at 4 °C on YEPD slants containing (per liter), yeast extract: 5 g, peptone: 2.5 g, glucose: 10 g, and agar: 20 g. The pH was adjusted to 5.5. For enzyme production, a single colony was inoculated in 50 ml of phosphate succinate minimal (PS) medium in 250 ml Erlenmeyer flask, which contained (per liter) yeast extract: 2.5 g, peptone: 5 g, succinic acid: 6 g, $CaCl_2$: 0.3 g, K_2HPO_4: 8.7 g, $(NH_4)_2SO_4$: 4 g, $MgSO_4$: 0.5 g, pH 4.7 (self adjusted). The cells were allowed to grow at 40 °C with shaking at 220 rpm in an orbital shaker (Orbitek, Scigenics) for 8–10 h. This initial inoculum (2%, v/v) was transferred to 200 ml PS medium contained in 1000 ml Erlenmeyer flasks and allowed to grow for 14 h. Induction of β-glucosidase was carried out at this time by adding filter sterilized cellobiose (to a final concentration of 10 mM) as described (Wallecha and Mishra, 2003). After induction, cells were allowed to grow for another 6–8 h. Cells were harvested by centrifugation at 2300 × *g* (Sigma) for 10 min at 4 °C. The pellet was washed twice with 50 mM phosphate citrate buffer (PC), pH 6. The cells were lyophilized without adding any lyoprotectant and tested for β-glucosidase activity by *p*NPG assay.

2.2. Temperature stability of whole cell biocatalyst

One milliliter of cell suspension in PC buffer (equivalent to 18 I.U. of β-glucosidase activity) was taken in a micro centrifuge tube in triplicates and incubated at different temperatures (35–50 °C) up to 6 h. Aliquots were removed at regular intervals to measure residual β-glucosidase activity.

2.3. Initial water activity adjustment

Reaction contents were equilibrated separately via the gas phase in three vials containing glucosyl donor (cellobiose or *p*NPG), acceptor (octanol) and whole cell biocatalyst and placed in a sealed chamber with a small volume of saturated salt solution containing some salt crystals. The equilibration was continued for 16–40 h at room temperature to achieve the desired value corresponding to water activity of saturated salt solutions (Wehtje et al., 1997). The following salts were used to preset the water activity (a_W) in the reaction system, $MgCl_2$ (0.33), KI (0.69), NaCl (0.74), KNO_3 (0.94); a_W value for a saturated salt solution at room temperature is given in the parentheses.

2.4. Synthesis of OG by transglucosylation using cellobiose as glucosyl donor

OG synthesis was carried out at 40 °C in 5 ml screw capped vials equipped with teflon tape containing total reaction volume of 2 ml, comprising 0.2 ml cellobiose (0.3 M), 1.8 ml octanol and 20 mg of lyophilized cells equivalent to 8 I.U. of β-glucosidase activity. The reaction vials were kept horizontally in reciprocating shaker (SI-300R Jeo Tech) maintained at 300 rpm. Reactions were set at different water activities, that ranged from 0.33 to 0.94, to study the effect of the same on OG synthesis. The effect of cellobiose concentration, which was varied from 0.1 to 0.7 M, was studied with the initial water activity set at 0.33. The reactions were also studied at 45 °C with initial water activity and substrate concentration of 0.33 a_W and 0.3 M, respectively.

2.5. Synthesis of OG using pNPG as glucosyl donor

2.5.1. Effect of water activity and substrate concentration

Synthesis of OG was carried out at 5 ml scale using *p*NPG as glucosyl donor at different water activities set from 0.33 to 0.94 (a_W) as described in Section 2.3. The reaction mixture contained 4.5 ml octanol (7 M) as glucosyl acceptor, 60 mg of *p*NPG and 40 mg of whole cells equivalent to 16 I.U. of β-glucosidase activity. The effect of glucosyl donor (*p*NPG) concentration was investigated by using 50, 60, 90, 120, 150, 180 and 210 mg at water activity of 0.33 (a_W). Cells and substrates were equilibrated separately. Reactions were initiated by mixing the cells and substrate in 10 ml screw capped vials and kept horizontally at 40 °C in reciprocating shaker maintained at 300 rpm. Aliquots were removed every 2 h from the upper phase and amount of OG synthesized determined.

2.5.2. Effect of enzyme units on OG yield

The effect of number of enzyme units on transglucosylation reaction and stability of the synthesized product was studied at 120 mg of *p*NPG as donor and 4.5 ml of octanol as glucosyl acceptor. Whole cell biocatalyst (10, 20, 40, 50, 60 mg) equivalent to approximately 4, 8, 16, 20 and 24 I.U. of β-glucosidase activity were used.

2.6. Experimental design

Design-Expert® software version 8.0.3.1 (Stat-Ease Inc. MN, USA) was used to generate the Box–Behnken Design matrices and also for the analyses of the data. Variables like water activity, glucosyl donor and enzyme concentration, which significantly affected the conversion yield, were optimized using a response surface Box–Behnken design (Box and Behnken, 1960).

2.7. Batch replacement synthesis and solid phase extraction of the product

Based on optimization of the glucosyl donor concentration, enzyme units, reaction temperature, incubation time and initial water activity (a_W) pilot scale experiments were carried out in a total reaction volume of 20 ml. It contained 18 ml octanol, 480 mg of *p*NPG and 40 mg of whole cell biocatalyst equivalent to 16 I.U. of β-glucosidase activity pre equilibrated at initial water activity of 0.33. After 4 h of reaction time, upper organic phase was replaced carefully without removing the whole cell biocatalyst and glucosyl donor from micro aqueous phase and 18 ml fresh octanol was added without further addition of either the enzyme or *p*NPG. This was repeated twice after 4 h. Silica gel (60–120) mesh size pre equilibrated by passing n-hexane was chosen as an adsorbent for the product OG. Organic phase containing the product was passed through adsorption column, (10 cm × 2.5 cm) at flow rate of 0.3 ml/min. Octanol was flushed out from adsorption column by passing hexane containing 20% ethyl acetate and the bound OG was eluted by passing 100% methanol and finally column was flushed by methanol containing 10% water. All collected fractions were concentrated in Speed Vac (Savant) and analyzed on TLC. The fractions containing pure OG were pooled.

2.8. Analytical methods

2.8.1. Qualitative and quantitative estimation of octyl glucoside

After a particular incubation time, reaction vials were removed and allowed to stand very briefly to let the whole cells settle down. Upper organic phase (100 μl) was collected carefully. The collected