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ASPERGILLUS ORYZAE β-GALACTOSIDASE – AN EFFICIENT CATALYST FOR ALKYL-β-GALACTOSIDE SYNTHESIS IN ORGANIC MONO-PHASE SYSTEM

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Reverse hydrolysis and alcoholysis reactions were performed enzymatically in mono-phased water-saturated hexanol system. Galactose, lactose, and *p*-nitrophenyl-β-galactoside were used as substrates for hexyl-β-galactoside production by *Aspergillus oryzae* β-galactosidase. When hexyl-β-glycoside was obtained via reverse hydrolysis using galactose as a glycosyl donor, the initial rate of synthesis and the maximal product concentration were 19.6 μmol/mg·min and 1.52 mM, respectively. Alcoholysis of lactose proceeded with higher initial rate of synthesis (45 μmol/mg·min) and maximal product concentration of 2 mM. In both cases the limiting factor was the poor solubility of the substrates in alcohol.

The substrate *p*-nitrophenyl-β-galactoside was completely soluble in hexanol. Its conversion resulted in product yield of 38% and initial rate of synthesis 64 μmol/mg·min. Maximal product concentration achieved was 3,8 mM, which was the highest value obtained of all three substrates examined. In both alcoholysis of lactose and *p*-nitrophenyl-β-galacoside, the rate of synthesis and the yield increased with increase of the water activity, having highest values for the water activity close to the saturation level.

Addition of the surfactant sodium dodecyl sulphate (SDS) increased both the total initial enzyme activity (1.28 times) and the hexyl-β-galactoside yield (1.18 times), while the immobilization onto the carrier Amberlite IRC (50)-H resulted in an increased initial enzyme activity (1.11 times), but not in an increased product yield.

Key words: *Aspergillus oryzae* β-galactosidase; hexyl-β-galactoside; enzymatic synthesis; organic mono-phase system

СИНТЕЗА НА АЛКИЛ-β-ГАЛАКТОЗИДИ ВО МОНОФАЗНА ОРГАНСКА СРЕДИНА СО КОРИСТЕЊЕ НА ГАЛАКТОЗИДАЗА ОД *ASPERGILLUS ORYZAE*

Ензимската синтеза на хексил-β-галактозид во монофазна органска средина на хексанол беше спроведена преку користење на реакциите на реверзна хидролиза и алкохолиза. Како супстрати за продукција на овој алкилгликозид со помош на β-галактозидазата од *Aspergillus oryzae* беа користени неколку гликозилни донори, и тоа: галактозата, лактозата и *p*-нитрофенил-β-галактозидот. При синтезата на хексил-β-галактозидот со употреба на галактозата како гликозилен донор, почетната брзина на синтеза и максималната концентација на продуктот беа 19,6 μmol/mg·min и 1,52 mM, соодветно. Алкохолиза на лактозата се одвиваше со почетната брзина на синтеза од 45 μmol/mg·min, а максималната концентрација на хексил-β-галактозид во овој случај беше 2 mM. Користењето и на двата споменати супстрата е проследено со заеднички ограничувачки фактор, а тоа е нивната слаба растворливост во средината на хексанолот. Супстратот *p*-нитрофенил-β-галактозид покажа целосна растворливост во хексанол. Неговата употреба како гликозилен донор резултираше со принос на продукт од 38% и со почетна брзина на синтеза од 0,64 μmol/mg·min. Максималната концентрација на продукт при конверзија на овој супстрат изнесуваше 3,8 mM, што е наедно и најголема добиена вредност меѓу испитаните супстрати. При алкохолизата и на лактозата, а и на *p*-нитрофенил-β-галактозидот, забележано е зголемување на концентрацијата и на приносот на продукт со зголемувањето на вредноста на активноста на водата, а најголеми вредности се постигнати при активност блиска до нивото на заситување.

Додавањето на натриумдодецилсулфатот (SDS) како адитив на реакционата смеса резултираше со истовремено зголемување на вкупната ензимска активност и на приносот на хексил-β-галактозид,

со вредности 1,28 и 1,18 пати поголеми од добиените при користење на нативниот ензим како катализатор. Имобилизацијата, пак, на ензимот врз носачот Amberlite IRC (50)-H ја зголеми почетната брзина на синтеза на хексил-β-галактозид (1,11 пати), но не и приносот на продукт.

Клучни зборови: β-галактозидаза на *Aspergillus oryzae*; хексил-β-галактозид; синтеза; монофазна органска средина

INTRODUCTION

Alkyl-glycosides are known as compounds that, due to their high surface activity and biodegradability, have potential application in pharmaceutical, cosmetic, food, chemical and detergent industries [1, 2]. They have traditionally been produced via chemical route, the process that has many disadvantages, among which are low selectivity and aggressive operating conditions [3].

Unlike chemical synthesis, enzymatic alkyl-glycoside synthesis is characterized by mild reaction conditions and very strict regio- and stereoselectivity, properties that enable production of compounds with precisely determined structure [3, 4]. Two methodologies are used in enzymatic synthesis of alkyl-glycosides: thermodynamically controlled reversed hydrolysis, and kinetically controlled transglycosylation [5–9]. Because the transglycosylation reaction is kinetically controlled, it becomes possible to overshoot the equilibrium conversion of the reactant into product, which is not possible when using the reverse hydrolysis process [10–14].

In contrast to the traditional utilization of hydrolases for catalyzing the hydrolytic reactions, non-conventional biocatalysis utilizes them in synthesis reactions, by exchanging their water surrounding with an organic medium [15, 16]. Alkyl-glycoside synthesis in organic mono-phase system has been thought to be impossible because of the low solubility of the sugar substrate, until recently when successful condensation of glucose with buffer-saturated C_6-C_8 alcohols at 50°C in a mono-phase system has been performed [4]. Later on, heptanol mono-phase system has been applied as a reaction medium for both reverse hydrolysis and alcoholysis alkyl-glycoside production [8].

Very few studies have been reported concerning the influence of the water activity on the total glycosidase activity as a sum of hydrolysis and transglycosylation [5, 7]. Hansson *et al.* [5], while investigating the ratio of synthesis rate to hydrolysis rate as a function of water activity in transglycosylation reactions catalysed by several glycosidases, found out that in the majority of cases the r_S/r_H ratio increased with the water activity increase. The same researchers investigated pentyl-β-glucoside as a sugar donor. There are also very few studies that include both reverse hydrolysis and transglycosylation reaction concepts, investigating several substrates as sugar donors [8].

To the best of our knowledge, there are no published results on detailed investigation of water activity influence on the r_S/r_H ratio in alkyl-β-galactoside production by *A. oryzae* β-galactosidase, as well as on the comparison of the reaction concepts of reversed hydrolysis and transglycosylation, using the approach described in this work.

In the present work hexanol mono-phase system was used as a medium for hexyl-β-galactoside synthesis by *A. oryzae* galactosidase using several sugar substrates, applying both reverse hydrolysis and transglycosylation process concepts.

EXPERIMENTAL

Materials

Lyophilized *Aspergillus oryzae* β-galactosidase, hexanol, D-galactose, lactose, *p*-nitrophenyl-β-D-galactoside, hexyl-β-D-glucoside, molecular sieves (UOP TYP 3A) for drying solvents (0.3 nm diameter) and sodium dodecyl sulphate (SDS) were all obtained from Sigma Chemicals (St. Louis, USA). Acetonitrile was obtained from Merck (Darmstadt, Germany). The matrix for immobilization, Amberlite IRC (50)-H, was obtained from BDH Chemicals Ltd (Poole, England).

Enzymatic conversions

Saturated solutions of galactose and lactose in hexanol were used as substrate solutions for the enzyme. *p*-nitrophenyl-β-D-glucopyranoside was dissolved in hexanol (dried with 0.3 nm molecule sieves) to a concentration of 10 mM. Different amounts (40–120 μl) of aqueous buffer (50 mM

citrate buffer pH 5.0) containing the desired amount of enzyme were added. The final reaction volume was 2 ml. The reactions were carried out in closed glass vials on an orbital shaker at 50 °C with vigorous shaking. The water content of the reaction medium was measured by the Karl-Fisher titration method [9]. The corresponding water activity (a_w) was calculated by using UNIFAC Activity Coefficient Calculator [6]. At different reaction times, samples were withdrawn from the reaction mixture, diluted with mobile phase, and analyzed by HPLC.

HPLC analysis

The HPLC system used was equipped with Knauer K-1001 pump and RID differential refractive index detector Shodex RI-71. The column used was LiChrospher 100 RP-18 (4 × 250 mm, 5 μm) obtained from Merck, Germany. The analyses proceeded under the following conditions: temperature 25 °C, flow rate 1 ml/min, and the composition of the mobile phase 25:75 acetonitrile/water.

Spectrophotometric analysis

A spectrophotometric method for measuring the total enzyme activity (hydrolytic + transglycosylation) in the reactions where *p*-nitrophenyl-β-D-galactoside (pnpg) was used as a substrate was developed. The quantity of depleted pnpg was calculated from the amount of *p*-nitrophenol liberated. The reaction was followed by measuring the increase of absorbance at 405 nm. The spectrophotometric system used was Varian Cary 50 Scan UV-Visible. The extinction coefficient for the *p*-nitrophenol at 405 nm is $\varepsilon_{405;pH\ 5.0;50\ °C}$ 0.204 mM·cm^{-1}.

Enzyme immobilization

Immobilization on Amberlite IRC (50)-H was performed according to Hansson and Adlercreutz [9]. The immobilization carrier was first washed with distilled water and then with 50 mM citrate buffer, pH 5.0. The carrier was dried under vacuum overnight. One ml of the enzyme solution with a concentration of 1.8 mg/ml was mixed with 1 g Amberlite. The mixture was dried under vacuum and stored in the refrigerator.

Addition of the surfactant sodium dodecyl sulphate, known as SDS ($C_{12}H_{25}O_4SNa$), was added to the reaction mixture before water activity calibration to a final concentration of 1% (w/v).

RESULTS AND DISCUSSION

Reverse hydrolysis reaction with A. oryzae β-galactosidase

Condensation between galactose and *n*-hexanol was performed in a mono-phase system using alcohol both as a substrate and as a solvent. The highest product concentration obtained was 1.52 mM (Fig. 1), and the maximum product yield, calculated on the base of galactose dissolved in hexanol, was 60%. The maximum product concentration was achieved at the 60th minute, after which it reached a plateau and started decreasing. This indicates that after 60 minutes the secondary hydrolysis started dominating over the synthesis, and that, in this later stage of the reaction, galactosidase started hydrolyzing the produced alkyl-β-galactoside. The substrate was not completely consumed, and at the end of the reaction 20% of the substrate was present in the reaction mixture.

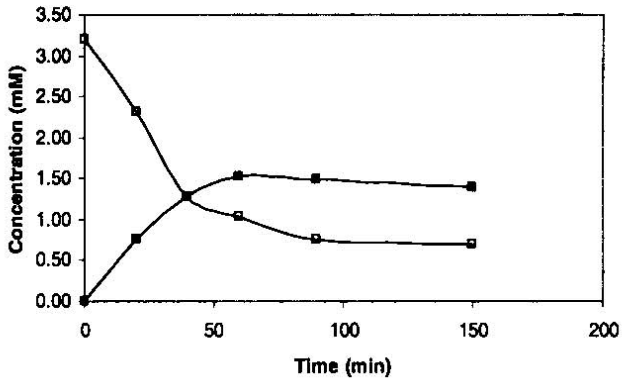


Fig. 1. Reverse hydrolysis with the *Aspergillus oryzae* β-galactosidase, using galactose (□) as a sugar substrate, followed by hexyl-β-galactoside production (■) at water activity of 0.92

It can be concluded that the value for the yield of the hexyl-β-galactoside obtained from galactose in this work is relatively high, if compared to the values reported in the literature for alkyl-glycoside synthesis using the reverse hydrolysis process concept [4, 8]. The value for the product yield corresponds well with the results reported, suggesting the possibility of getting high yields of alkyl-glycosides when, instead of using two-phase system, an organic mono-phase system is exploited

[7, 11]. Garica-Garibay *et al.* [8], while working with heptanol mono-phase system, obtained heptyl-β-glycoside yield as high as 35%. In contrast to high yields obtained in an alcohol mono-phase system, utilization of water-alcohol two phase system resulted in maximal hexyl-glucoside yield of 18% [4]. The main limitation for getting high alkyl-glycoside yields in a two-phase system is the domination of the hydrolysis reaction [5].

Many authors consider that the main bottleneck of enzymatic alkyl-glycoside synthesis in both one-phase or two-phase reaction systems is the extremely poor solubility of the sugars in the organic media [7–9]. Other factors, such as product hydrolysis, denaturation of the enzyme by the solvent, and the mass transfer limitations, are also reported to influence both the yield and the reaction rate of the alkyl-glycoside synthesis [2, 3].

Transglycosylation reaction with A. oryzae β-galactosidase using lactose as a glycon donor

In order to investigate the enzyme efficiency in converting substrates other than galactose, and to determine the effect of water activity on the yield and the initial reaction rate, lactose was used as a sugar substrate in mono-phase hexanol system at different water activities. *Aspergillus oryzae* β-galactosidase, due to its strict specificity, converted the lactose into hexyl-β-galactoside, while glucose was released as a side reaction product. The presence of hexyl-β-glucoside was not detected in the reaction mixture.

Hexyl-β-galactoside yield as high as 53% was observed when the reaction was carried out in the system with water activity of 0.92, in contrast to 16.7% maximal product yield when the water activity of the reaction medium was 0.65 (Fig. 2). The maximal product concentration of 2 mM was obtained in the system with water activity 0.92. This value is 1.32 times higher than the value for the concentration obtained in the reverse hydrolysis reaction with galactose, at the same water activity. The produced hexyl-β-galactoside, started hydrolysing in the secondary hydrolytic step of the reaction (after the 60th minute).

It is important to point out that both the yield and the initial reaction rate increased when the hexyl-β-galactoside synthesis was performed at increased water activity. The value of 0.01 mM/mg·min for the water activity of 0.65 increased 4.5 times, reaching the value of 0.045 mM/mg·min, when the water activity in hexanol was 0.92. Similar results have been reported in the literature. Hansson *et al.* [9], when using *P. furiosus* CelB galactosidase for lactose conversion into hexyl-β-glycosides, observed 13 times higher initial reaction rate at water activity close to 1 then at water activity of 0.6.

Regarding the solubility, similarly to galactose, lactose was not completely soluble in hexanol, and at the temperature of 50°C its solubility was 3.82 mM.

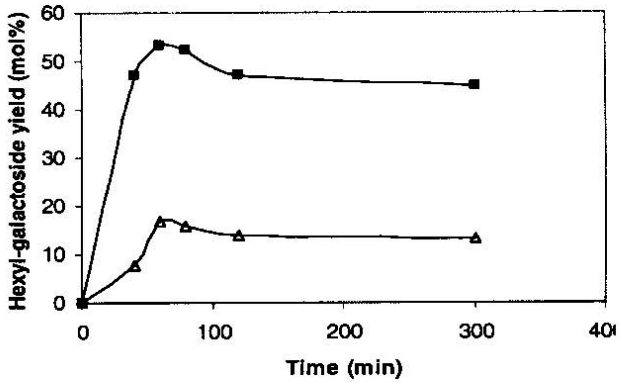


Fig. 2. Hexyl-β-galactoside yield as a function of time at water activities 0.65 (△) and 0.92 (■), in alcoholysis reaction using lactose as a glycosyl donor

The results for the maximum hexyl-galactoside yield obtained from lactose in this work correspond well with the literature data for the synthetic ability of *A. oryzae* β-galactosidase [2, 3]. Ismail *et al.* [2], using this enzyme for butyl-β-galactoside synthesis from 10g/l lactose in butanol, achieved 79.5% yield of hexyl-galactoside. Having in mind that the solubility of lactose in butanol was higher than in hexanol, and that the butanol was much better alcohol nucleophyle than hexanol [6], the much higher yields achieved by Ismail *et al.* [2] in butanol system are not unexpected. Therefore, the value of 53% for the yield obtained in our case seems very reasonable. In general, the more hydrophobic the alcohol is, the lower the expected alkyl-glycoside yield is. Garica-Garibay *et al.* [8] used hyperthermostable β-glycosidase, (CloneZyme Gly-001-02) for heptyl-β-galactoside synthesis from lactose at a temperature of 90 °C. The yield obtained was 39.5% and was 1.34 times lower than the yield obtained in our work. This suggests that using hexanol as a nucleophyle for the glycosyl-enzyme complex, higher yields can be obtained than with the more hydrophobic alcohol heptanol.

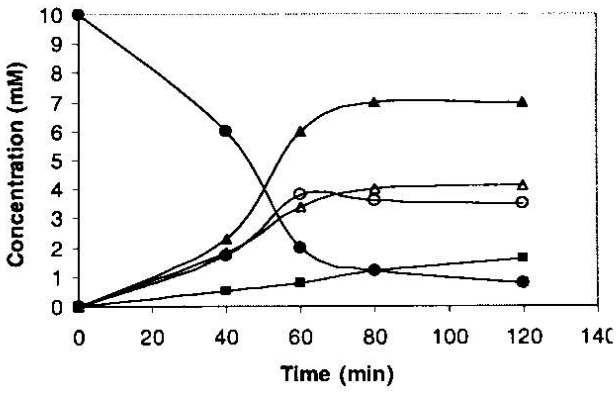
Transglycosylation reaction with A. oryzae β-galactosidase using p-nitrophenyl-β-galactoside as a glycon donor

p-nitrophenyl-β-galactoside, unlike the two other glycosyl donors used, has readily been solubilised in hexanol. It was completely converted to a mixture of hexyl-β-galactoside (formed in the transglycosylation reaction, also called alcoholysis) and galactose (formed by hydrolysis). The ratio between these two reactions determines the yield of the transglycosylation product. As already mentioned, galactose is poorly soluble in hexanol and a large part of it precipitated in the reaction mixture after a short reaction time (Fig. 3).

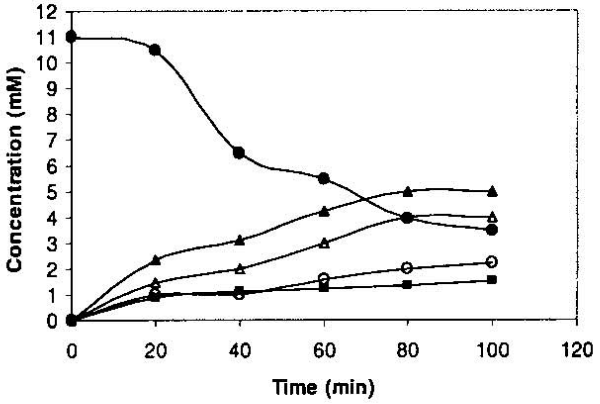
The substrate, *p*-nitrophenyl-β-galactoside, in the system with water activity of 0.65, was not consumed completely, and 30% of its initial amount was present in the reaction mixture after the 120th minute. Contrary to this, in the system with a_w of 0.92, the substrate was almost completely depleted at the same reaction time (Fig 3).

It can be noticed that the water activity also affects the selectivity of the enzyme. The ratio of the rates of synthesis and hydrolysis (r_S/r_H) in the system with water activity of 0.65 was 0.25, while in the system with water activity of 0.92, r_S/r_H was as high as 0.75 (Fig.3a).

This means that by increasing the water activity in the system both the rate of synthesis and the rate of hydrolysis increase, but the rate of synthesis increases more than the hydrolysis does. It is possible that the water activity influences the enzyme flexibility and therefore the approach of the big alcohol molecule to the enzyme active site is facilitated at higher water activities. Both the initial synthetic activity and the product yield increased when the water activity in the system increased. Having the value of 0.02 mM/mg·min for the water activity of 0.65, the initial synthetic activity increased 3.25 times and reached value of 0.06 mM/mg·min, when the water activity of the system was increased to a_w of 0.92 (Fig. 3). The yield obtained at a_w 0.65 equals 10%. When the water activity of the system increased to 0.92, the yield increased almost 4 times and reached a value of 38 %. The enzyme activity below a_w of 0.6 was very low. This means that the selectivity of the enzyme towards alcohol nucleophyles also increases with the water activity increase. Other researchers also suggest that the water is a factor that influences the enzyme flexibility and thereby the enzyme selectivity towards the alcohol molecules [5, 9]. That glycosidase activity is very low at water activity values below 0.6, as it has been already reported [6, 11].



a)



b)

Fig. 3. Time course of the transglycosylation reaction in *n*-hexanol with a water activity of a) 0.92 and b) 0.65. The substrate *p*-nitrophenyl-β-D-galactoside (●) was converted to hexyl-β-galactoside (○) and galactose (total amount of galactose: ▲). Some of the galactose dissolved in the reaction medium (■) and some of it precipitated (△))

The comparison of the different glycosyl donors, for the hexyl-β-galactoside synthesis with *A. oryzae* β-galactosidase, was performed by plotting the hexyl-β-galactoside concentration versus the reaction time for the three substrates used (Fig. 4).

It can be seen that when using *p*-nitrophenyl-β-galactoside as a substrate the highest product concentration of 3.8 mM was achieved, compared to that obtained with lactose (2.03 mM), and the galactose (1.52 mM). The initial rate of synthesis was also highest when pnpg was converted into hexyl-β-galactoside, having a value of 0.06 mM/mg·min, while when converting lactose and galactose into the product it had a value of 0.045

and 0.019 mM/mg·min, respectively. There are other data that also confirm the suitability of the *p*-nitrophenyl-*β*-galactoside as a glycosyl donor for the transglycosylation reactions with glycosidases [5, 6].

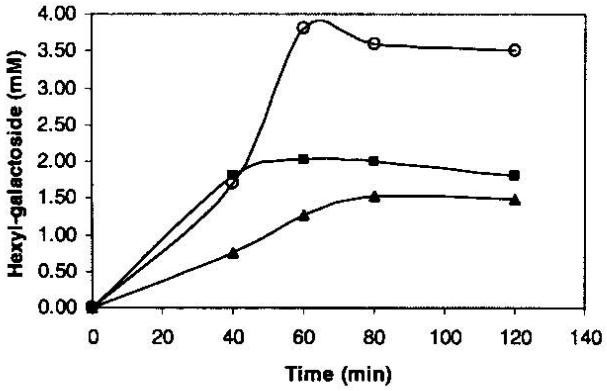


Fig. 4. Transglycosylation and reverse hydrolysis production of hexyl-*β*-galactoside using galactose (▲), lactose (■) and *p*-nitrophenyl-*β*-D-galactoside (○) as substrates

Working with a saturated solution of galactose in hexanol, we found out that its solubility was lower than when it was released from the activated substrate *p*-nitrophenyl-galactoside. Therefore the solubility of galactose in a medium with and without *p*-nitrophenyl-*β*-galactoside, or hexyl-*β*-galactoside, was investigated. The results showed that the solubility of the galactose in the presence of these compounds was several times higher compared with the solubility of the galactose when no other compounds were presented in the medium. These results suggest that the product, alkyl-glycoside or the activated substrate, *p*-nitrophenyl-*β*-galactoside, might act as surfactants and therefore enhance the solubility of galactose/glucose in this very hydrophobic alcohol.

Effect of enzyme immobilization or addition of additives on the enzyme activity

In this experiment the total enzyme activity was measured using the spectrophotometric method described above. This method is a modification of the enzyme activity assay originally introd uced by Hansson *et al.* [17]. It is based on measuring the rate of hydrolysis of *p*-nitrophenyl-*β*-galactoside and the consequent formation of the glycosyl-enzyme complex that can react with either water (hydrolysis) or alcohol (transglycosylation reaction) (Fig. 5). The sum of the rates of these two reactions gives the total enzyme activity. One unit of enzyme activity is defined as the amount of enzyme catalyzing the liberation of 1.0 μmol of *para*-nitrophenol per minute.

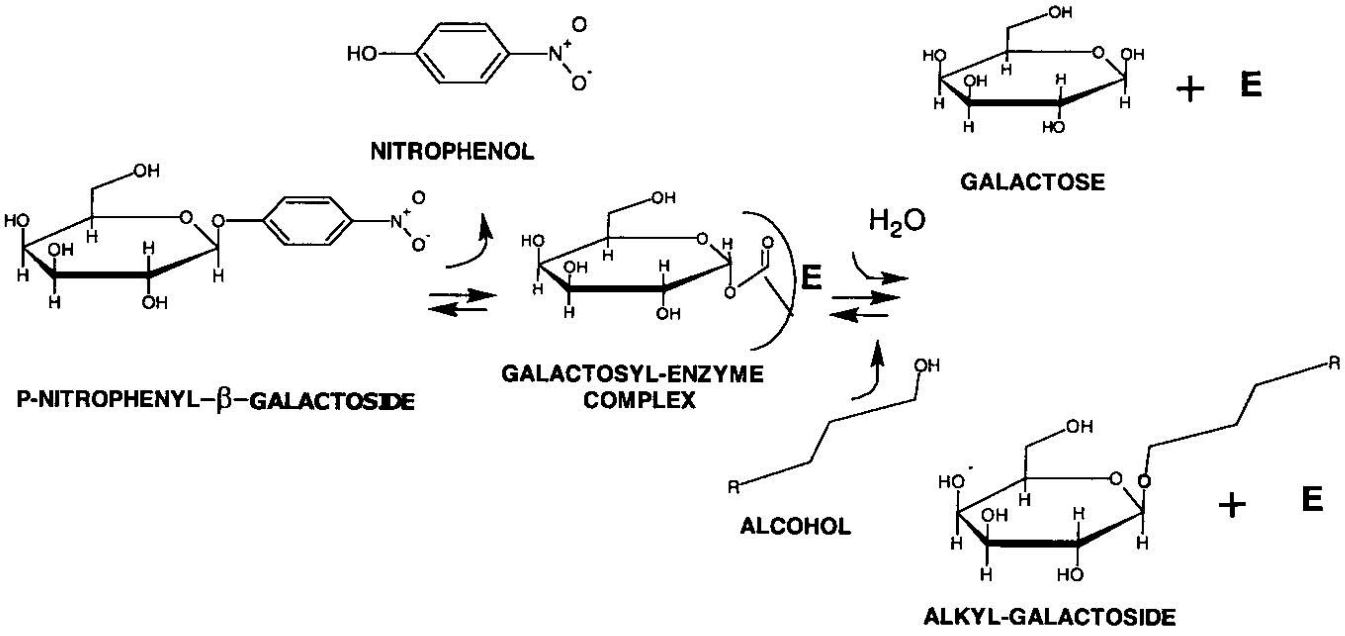


Fig. 5. Scheme of the conversion of *p*-nitrophenyl-*β*-galactoside into galactose (hydrolysis) or alkyl-*β*-galactoside (transglycosylation reaction) by *β*-galactosidase activity

In order to see if the total enzyme activity (transglycosylation + hydrolysis) might be influenced by immobilization or by the presence of some additives such as surfactants, enzyme immobilization on Amberlite (the acryl amide type resin) and the addition of the surfactant SDS were performed. While the liberation of the *p*-nitrophenol (for calculating the total enzyme activity) was measured spectrophotometricaly, the determination of the hexyl-β-galactoside concentration (for calculating the specific enzyme activity, i.e. the rate of synthesis) was performed using the HPLC method. The results are plotted at Fig. 6 and Fig. 7.

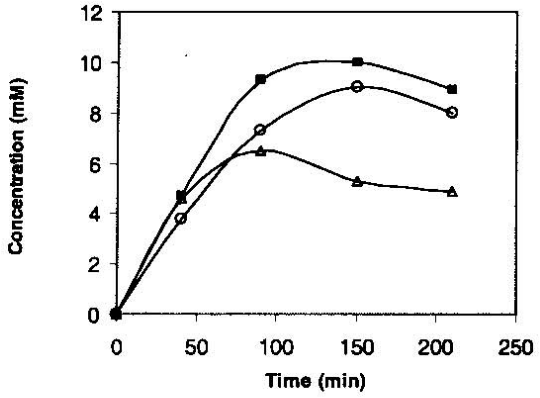


Fig. 6. Liberation of *para*-nitrophenol during the transglycosylation reaction in *n*-hexanol using *Aspergillus oryzae* β-galactosidase in native form (○), with addition of SDS (■) and immobilized onto Amberlite resin (△)

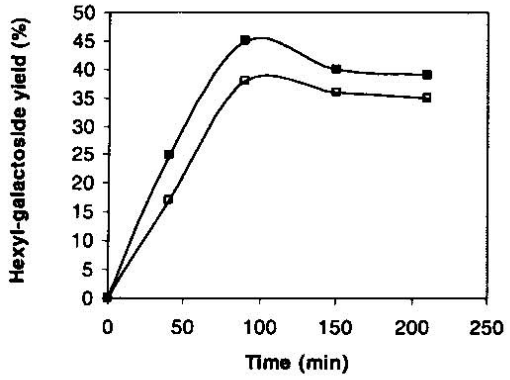


Fig. 7. Hexyl-β-galactoside yield as a function of time using *Aspergillus oryzae* β-galactosidase for the conversion of *p*-nitrophenyl-β-D-galactoside with addition of SDS (■) and without addition of SDS (□)

When compared with the system where native enzyme was used, in the system containing the additive SDS, both the total initial enzyme activity and the hexyl-β-galactoside yield were increased. The total initial activity of the enzyme in the system with SDS, having value of 1.43 mM/min·mg enzyme, was 1.28 times higher than the activity of the native enzyme, which in this case was 1.13 mM/min·mg enzyme. The immobilization on Amberlite increased the enzyme activity (1.11 times at Fig. 6), although the hexyl-β-galactoside yield was 1.35 times lower compared to that obtained with the lyophilized enzyme. It can be concluded that both modification techniques have a positive effect on the initial reaction rate. In an attempt to explain those effects, it can be suggested that the addition of SDS as a surfactant or immobilization of the enzyme onto a carrier, might offer some protection against organic solvent induced inactivation.

CONCLUSIONS

Aspergillus oryzae β-galactosidase proved to be an efficient biocatalyst for alkyl-β-glycoside production, applied in both reverse hydrolytic and transglycosylation reaction route. Poor solubility of the sugar substrates galactose and lactose was the main limitation factor that influenced the conversion of the substrate. Utilization of those substrates in the presence of the product could result in increase of sugar solubility in the alcohol medium. Utilization of *p*-nitrophenyl-β-galactoside as a completely soluble glycosyl donor resulted in the highest obtained product concentration among the three substrates used. Addition of the surfactant SDS increased both the total enzyme activity and the product yield, while immobilization of the enzyme onto the Amberlite resin increased the total enzyme activity, but did not increase the product yield.

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