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Construction and optimization of a monophasic organic–water system for enzymatic synthesis of *p*-nitrobenzyl β-D-glucopyranosides by reverse hydrolysis

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Abstract

A monophasic organic–water system for efficient enzymatic synthesis of β-D-glucopyranoside by reverse hydrolysis was constructed and optimized. *p*-Nitrobenzyl alcohol (*p*NBA), selected as a model substrate alcohol, was readily glucosylated with D-glucose through reverse hydrolysis using almond β-D-glucosidase in a monophasic aqueous-organic medium, producing a new glucoside, *p*-nitrobenzyl β-D-glucopyranoside (pNBG). The effects of different buffers, organic solvents and water contents were investigated. Buffer type and pH affected the initial reaction rate but had little effect on the final yields. The ratio of organic solvent to water plays a crucial role in shifting the reaction equilibrium toward synthesis, but a minimum amount of water is necessary to maintain the enzyme activity. Dioxane, which was previously known as an unsuitable solvent for β-D-glucosidase-catalyzed reactions, was found to be the most appropriate solvent for this synthetic procedure. The reaction equilibrium and enzyme stability in the reaction medium were also investigated. Under the optimal reaction conditions, i.e. 90% dioxane (v/v) + 10% buffer (Na_2HPO_4–KH_2PO_4, 70 mM, pH 6.0) with alcohol-to-glucose molar ratio of 9:1, *p*-nitrobenzyl β-D-glucopyranoside was produced with a maximum yield (13.3%).

Keywords: Reversed hydrolysis; β-D-Glucopyranoside; Almond β-D-glucosidase; Organic medium; Dioxane–water system

1. Introduction

To prepare anomerically pure glycosides by traditional chemical methods is either to perform a circuitous and expensive procedure or to synthesize the glycosides as a mixture of anomers requiring subsequent resolution [1]. These problems existing in conventional chemical synthesis have stimulated the development of enzymatic approaches since various glycosidases or glycosyl tranferases can catalyze the synthesis of anomerically pure glycosides in one step. In organisms, glycosidases are generally involved in glycoside degradation/processing; for direction of biosynthesis, nature uses glycosyl transferases. A large number of enzymes involved in glycoside synthesis are well documented and some of them are commercially available. β-Glucosidases are widely used in enzyme-catalyzed glycoside hydrolysis or synthesis [2]. Owing to their high anomer-selectivity and mild reaction conditions, β-glucosidases can be a good alternative for glucosylation of alcohols through either transglucosidation or reverse hydrolysis (for reviews, see [1–5]). Many reports [6–13] describe the former procedure where an activated glucosyl donor such as *p*-nitrophenyl glucoside (pNPG) is used, which increases the cost of the enzymatic synthesis. Also it often makes the isolation of products difficult because different by-products (oligosaccharides), resulting from the transfer of the glucosyl donor onto itself, may be generated. The reversed hydrolysis of glucosides is a thermodynamically controlled reaction [14–29]. In an aqueous medium the equilibrium normally favors hydrolysis, but

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in an organic medium it can be shifted in the glucosylation direction. A water-immiscible solvent has been successfully introduced into an aqueous phase to reduce the water activity for the synthesis of alkyl glucosides in a two-phase system using the hydrophobic alcohol as both substrate and organic phase [15–26]. However, the need to use a very high concentration of the alcohol clearly limits the scope and application of the reaction especially when the alcohol is either solid under the planned reaction conditions, expensive or unavailable in large quantity. An alternative approach is to add a water-miscible solvent, which decreases the thermodynamic water activity and thereby favors glycoside synthesis. Vic et al. [4,6,14,15,22,29] had made great efforts towards establishing this approach. In their work, solvent was found to play a vital role among many factors affecting the product yield. Acetonitrile, *tert*-butanol and acetone were selected as appropriate solvents for enzymatic synthesis of glucosides. In this work, however, we found that dioxane–aqueous medium was more effective for our reaction than the traditional *tert*-butanol–water or acetonitrile–water system. In our previous work [30], several glucosides including a natural salidroside 2-(4-hydroxyphenyl)ethyl β-D-glucoside which was shown to possess some medicinal functions, were successfully synthesized in a monophasic aqueous-dioxane medium by using a crude meal of apple seed as a new catalyst. However, the product yields in this medium were still relatively low, which was a drawback for applying this system to the synthesis of other novel glycosidic compounds with biological activities. Therefore, a systematic optimization of the medium system is necessary and important for improving glycoside yields.

In this study, the enzymatic glucosylation of hydrophobic *p*-nitrobenzyl alcohol by reverse hydrolysis in the dioxane–buffer medium with almond β-glucosidase as a catalyst was established and optimized. Commercially available almond β-glucosidase, instead of the crude meal of apple seed, was selected as the catalyst to make the results comparable to other studies, since almond β-glucosidase has been one of the most widely used glycosidases and its features were clearer than those of the home-made crude meal of apple seeds. *p*-Nitrobenzyl alcohol (*p*NBA) was selected as a model alcohol (aglycone) substrate because both the substrate (*p*NBA) and the product (*p*-nitrobenzyl β-D-glucopyranoside, pNBG) have relatively strong ultraviolet absorbance at 254 nm which makes the TLC or HPLC assay of the substrate and/or the product more sensitive and convenient. The effects of aqueous buffer, organic solvent, thermodynamic equilibrium, and enzyme inactivation and alcohol concentration on the yield of glycoside were investigated in detail.

2. Materials and methods

2.1. Chemicals

Almond β-D-glucosidase (EC 3.2.1.21, 3.8 U mg^{-1}) was purchased from Sigma Co. (USA) and *p*-nitrobenzyl alcohol (*p*NBA) was from Acros Chemical Co. (Belgium). The enzyme was used without further purification. Glucose was purchased from Shanghai Renjie Chemical Reagent Co. (Shanghai, China) and 1,4-dioxane, acetonitrile, dimethylsulfoxide and *tert*-butanol were from Shanghai Chemical Reagent Co. Ltd.

2.2. HPLC and NMR analysis

HPLC was performed using a Shimadzu LC-10AT_{VP} pump with a UV detector. *p*-Nitrobenzyl β-D-glucopyranoside (pNBG) was analyzed on a Ø5 mm × 150 mm YGW C_{18} column using water–methanol (60:40, v/v) at a flow rate of 0.8 ml min^{-1}. Concentrations of *p*NBA and pNBG were calculated from peak areas using a calibration curve. ^{1}H NMR spectra were recorded in D_2O using a Bruker 500 Hz spectrometer.

2.3. The assay of enzyme activity

The synthetic activity of almond β-D-glucosidase was measured in 80% (v/v) dioxane–buffer (Na_2HPO_4–KH_2PO_4, 70 mM, pH 6.0). Glucose (0.25 mmol) was dissolved in 0.2 ml buffer and was added to 0.8 ml dioxane, giving a total volume of 1.0 ml. *p*NBA (1.0 mmol) was then dissolved in the medium and 5.0 mg of almond β-D-glucosidase was added to start the reaction.

The hydrolytic activity of almond β-glucosidase was measured spectrophotometrically using *p*-nitrophenyl β-D-glucopyranoside (pNPG) as a chromogenic substrate [31].

2.4. Enzyme stability in media with high content of dioxane

Almond β-D-glucosidase (5.0 mg) was suspended in 1.0 ml of dioxane–buffer (Na_2HPO_4–KH_2PO_4, 70 mM, pH 6.0) mixture containing different fractions of dioxane (100, 95, 90, or 80%, v/v). The enzyme suspension was preincubated at 50 °C and shaken at 160 rpm for 1, 3, 6, 12, or 24 h. Then the mixtures were adjusted to 80:20 (v/v) dioxane–buffer with buffer or dioxane, and the glucose and alcohol (*p*NBA) were added to start the reaction. The residual synthetic activity was assayed as described above by measuring the concentration of pNBG synthesized with HPLC.

2.5. Enzyme stability in media with low content of dioxane

To various dioxane–buffer (Na_2HPO_4–KH_2PO_4, 70 mM, pH 6.0) mixtures (1.0 ml) was added almond β-D-glucosidase (5.0 mg). The dioxane concentration was 0, 10, or 50%. The suspension was incubated at 50 °C and shaken at 160 rpm for 1, 3, 6, 12, 24 h, respectively. The residual activity was assayed spectrophotometrically as described above using pNPG as the substrate of hydrolysis.

2.6. Enzymatic synthesis of pNBG

Analytical-scale synthesis: To a test tube, was added 5 mg almond β-D-glucosidase powder, 0.1 ml buffer (Na_2HPO_4–KH_2PO_4, 70 mM, pH 6.0) containing 0.25 mmol glucose, 1.0 mmol *p*NBA and a certain amount of solvent/buffer, making a final volume of 1.0 ml. The mixture was shaken for 72 h at 160 rpm and 50 °C. Aliquots (50 μl) were removed at time intervals and quenched by the addition of 950 μl methanol. The samples were filtered through a micropore (0.45 μm) membrane and then subjected to HPLC analysis.

Preparative-scale synthesis: To 0.5 ml solution containing 1.25 mmol glucose and 70 mM Na_2HPO_4–KH_2PO_4 (pH 6.0), was added 50 mg of the enzyme. The reaction was started by the addition of 4.5 ml dioxane containing 11.25 mmol *p*NBA and the mixture was shaken for 72 h at 160 rpm and 50 °C. The reaction was quenched by addition of 10 ml methanol. Then the enzyme was filtered off and washed with methanol (5 ml × 2). The filtrate obtained was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by flash chromatography on Silica Gel 60 (100–200 mesh) with ethyl acetate/methanol (12:1) as eluent, giving 51 mg pNBG (isolation yield: 13.0%) as amorphous solid. ^{1}H NMR δ (D_2O, ppm): 3.32 (m, 4H), 3.69 (dd, 1H, J = 5.8, 12.3 Hz), 3.88 (dd, 1H, J = 2.0, 12.3 Hz), 4.51 (d, 1H, J = 7.9 Hz), 4.82 (d, 1H, J = 13.1Hz), 4.98 (d, 1H, J = 13.1 Hz), 7.57 (d, 2H, J = 8.6 Hz), 8.14 (d, 2H, J = 8.7 Hz).

3. Results and discussion

3.1. Effect of buffer type and pH

Effect of buffer types and pH on the yield of pNBG (6 h) under our synthetic conditions (dioxane: buffer, 9/1, v/v) was first examined. As shown in Fig. 1, phosphate buffer (Na_2HPO_4–KH_2PO_4, 70 mM, pH 6.0) showed the best results among the four buffers (acetate, citrate, phosphate, and citrate/phosphate) at pH 5.0–6.0. We further investigated the initial rate of enzymatic glycosidation in the phosphate buffer with the pH ranging from 5.0 to 7.0, and observed the highest rate at pH 6.0 (data not shown). Roode et al.'s research [16] also showed that almond β-D-glucosidase activity differed in different buffer types. The activity in acetate buffer was less than half of that in citrate/phosphate buffer. The result obtained in our dioxane–water system was similar. However, the composition and pH of the buffer had little effect on the final yields (data not shown), which is in accordance with the result in acetonitrile–water medium reported by Vic et al. [14]. Phosphate buffer (Na_2HPO_4–KH_2PO_4, 70 mM, pH 6.0) was thus used in the subsequent experiments.

3.2. Effect of organic solvents

The effects of different organic solvents and their contents in the reaction medium were investigated with respect

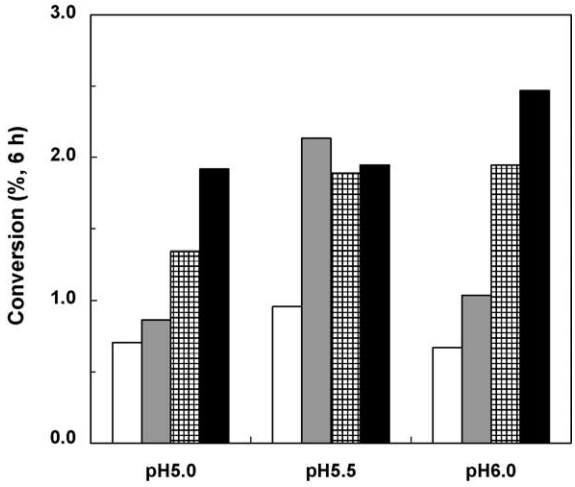

Fig. 1. Effect of different buffers on the synthesis of *para*-nitrobenzyl glucoside catalyzed by almond β-glucosidase in monophasic systems containing 10% (v/v) buffer and 90% dioxane. The reaction was carried out by shaking at 50 °C and 160 rpm a mixture of 5.0 mg enzyme powder, 0.1 ml buffer containing 0.25 mmol glucose, 0.15 g *p*NBA (1.0 mmol) and 0.9 ml dioxane. Symbols: open bars, NaAc–HAc buffer; grey bars, citrate/phosphate buffer; grid bars, citrate buffer; solid bars, phosphate buffer.

to both the initial rate and the final yield (at 72 h) in the β-D-glucosidase-catalyzed synthesis of pNBG. Since the optimal water content of the monophasic buffer–solvent system was not known in advance and might depend on the nature of the organic cosolvent of interest; the effect of three selected solvents (dioxane, acetonitrile, and *tert*-butanol) was investigated with different water contents (10, 20 and 40%, v/v). Another solvent, DMSO, was examined not only with low water contents (10 and 20%, v/v) but also with high water contents (80 and 90%, v/v) because the literature [6] indicated that less DMSO gave better results.

However, our results (Fig. 2a and b) clearly indicate that DMSO was not suitable at all as a cosolvent for enzymatic glycosidation no matter how much of water was used. A severe inactivating effect of DMSO on the enzyme is probably responsible for the very low activity and therefore the very low yield (<1%) of pNBG in the DMSO system. In contrast, the highest yield of pNBG was obtained in 90% (v/v) dioxane plus 10% buffer, although the enzyme activity in 90% dioxane was significantly lower than that in the system of dioxane or other solvents (except DMSO) with higher water contents (e.g., 20 or 40%). Similarly, in either *tert*-butanol or acetonitrile, the best yield was given again with a water content of 10% (v/v) even though the highest activity was observed with 20% water. These facts suggest that the final yield of glycoside in reverse hydrolysis is mainly controlled by the thermodynamic equilibrium as long as the enzyme is kept active.

Since both the enzyme activity and the position of the reaction equilibrium depend to a large extent on water content of the medium, the best medium (dioxane–buffer) was further optimized in the whole range of 0–100% (v/v) dioxane. As illustrated in Fig. 3, though the best activity was

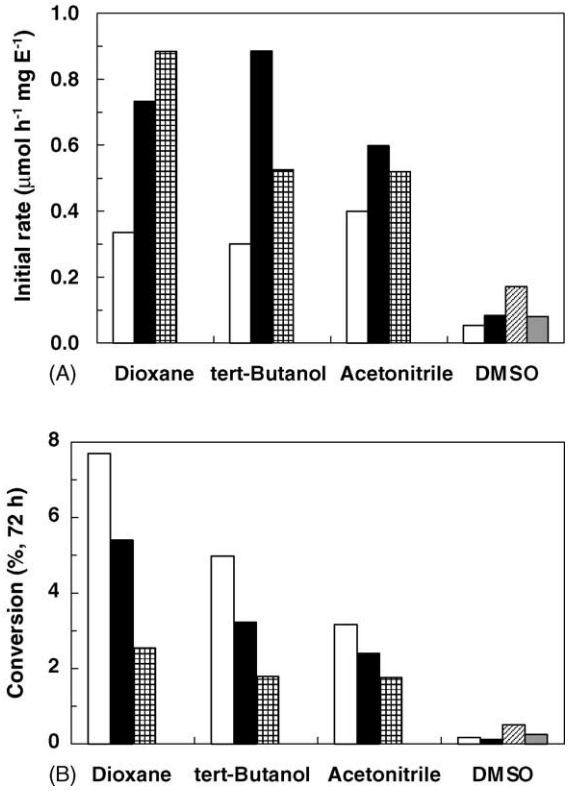

Fig. 2. Effect of organic solvent on the initial rate (A) and the final yield at 72 h (B). The reaction was carried out at 50 °C and 160 rpm/min by shaking a mixture of 0.25 mmol glucose, 1.0 mmol *p*NBA and 5.0 mg enzyme powder in 1.0 ml medium containing different volume fractions of a solvent and phosphate buffer (Na_2HPO_4–KH_2PO_4, 70 mM, pH 6.0). Symbols: open bars, 90% solvent; solid bars, 80% solvent; grid bars, 60% solvent; hatched bar, 20% DMSO; grey bar: 10% DMSO.

observed in the 50% dioxane medium, the highest yield (at 72 h) was obtained again in 90% dioxane (or 10% water). When the water content was further reduced to 5% (v/v), the final yield of pNBG dropped dramatically to below 1% because the enzyme activity in the 95% dioxane system was

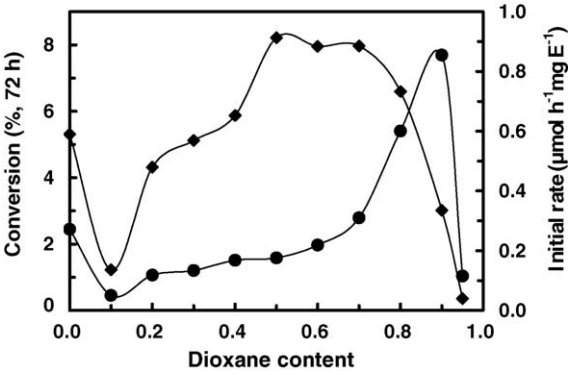

Fig. 3. Dependence of pNBG synthesis on volume fraction of the organic solvent in dioxane–water system. The reaction was performed in 1.0 ml medium of dioxane–buffer (Na_2HPO_4–KH_2PO_4, 70 mM, pH 6.0) by shaking a mixture of 0.25 mmol glucose, 1.0 mmol *p*NBA and 5 mg enzyme powder at 50 °C and 160 rpm. Symbols: (♦) initial rate; (●) final conversion.

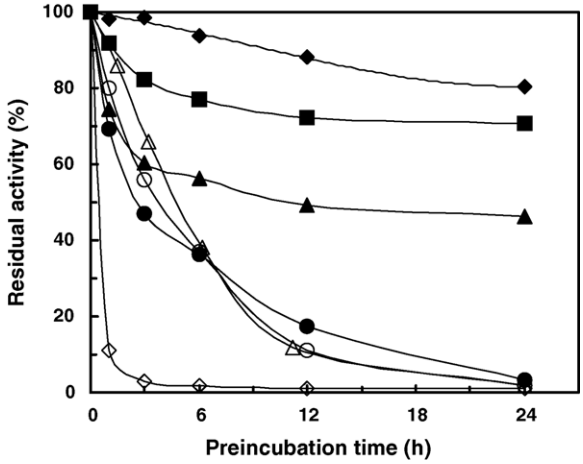

Fig. 4. The inactivation profiles of almond β-D-glucosidase at 50 °C in the organic–water system with different volume fractions of dioxane. The methods for the stability test were described in Sections 2.4 and 2.5. Symbols: (♦) 100%; (■) 95%; (▲) 90%; (●) 80%; (◊) 50%; (○) 10%; and (△) 0%.

almost completely inhibited owing to the lack of essential water. These results were in accordance to those reported in literature [6,9,14–16,19,20] where the best yield was also obtained in 90% (v/v) organic solvent, because a minimum of water was necessary to maintain enzyme activity. Therefore, the yield of glycoside in enzymatic reverse hydrolysis cannot be infinitely enhanced by further reduction of water content in the monophasic medium system.

In the work of Vic et al. [6,14,29] on solvent effects on the enzymatic synthesis of 2-hydroxybenzyl β-D-glucopyranoside, dioxane gave an unfavorable result while the best yield was obtained in 90% (v/v) acetonitrile or *tert*-butanol. This was explained in terms of enzyme stability in the organic solvents and it was suggested that enzyme stability would be optimum in solvents with a low δ_d (London dispersive forces) mostly close to that of water (δ_d $H_2O = 6$ cal cm^{-1}), such as *tert*-butanol, acetone and acetonitrile. However, in our study, dioxane (with a high δ_d value) seems to be the optimal solvent to obtain the best yield of *p*-nitrobenzyl β-D-glucopyranoside. Thus it is possible that the product yield is influenced not only by the nature of solvent but also by that of the substrate itself.

3.3. Enzyme stability in the dioxane–water systems

To further elucidate the disparity between the effect of water content on the initial rate and on the final yield, the stability of the almond β-glucosidase in the dioxane system was examined as a function of water content (0, 5, 10, 20, 50, 90, and 100%, v/v). As shown in Fig. 4, after 24 h of incubation in 90% (v/v) dioxane with 10% water, the enzyme retained 46% of its initial synthetic activity while the remaining activity in 80% (v/v) dioxane with 20% water was only 4%. Therefore, the higher yield in 90% (v/v) dioxane was not only owing to less water favoring the synthesis equilibrium but also to the higher stability of the enzyme in the medium with the lower

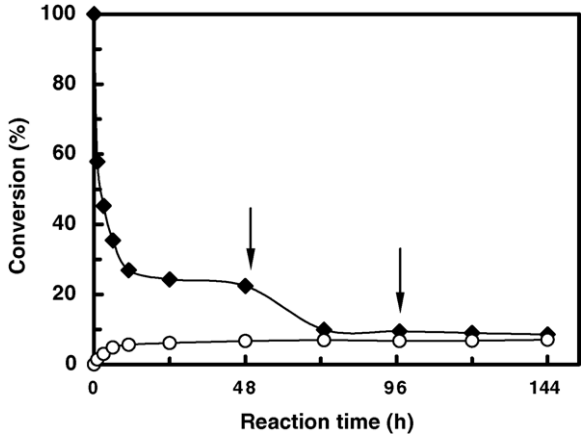

Fig. 5. The equilibrium between the enzymatic hydrolysis and synthesis of pNBG in the monophasic medium containing 90% (v/v) dioxane. Both the reactions were performed with 5 mg enzyme in 0.1 ml buffer and 0.9 ml dioxane at 50 °C and 160 rpm. Symbols: (◯) synthesis of pNBG: starting from 0.25 M glucose and 1.00 M *p*NBA; (♦) hydrolysis of pNBG: initiating from 0.25 M pNBG and 0.75 M *p*NBA; (↓) supplement of the enzyme (5 mg).

content of water. Although the enzyme stabilities in 100 and 95% (v/v) dioxane were even higher, the rates of synthesis in such media were much lower, which further indicates that the enzyme needs a minimum amount of water (about 10%) to maintain its synthetic activity. It should also be noticed that in 50% (v/v) dioxane medium the enzyme lost almost all activity in as little as 1 h although the initial rate in this medium was the highest (see Fig. 3). On the other hand, in the system of 100% buffer or 90% buffer with 10% dioxane, the time courses of enzyme inactivation were similar, which was in accordance with the phenomena commonly seen in enzymatic reactions in aqueous systems. Thus it can be concluded that in systems with a high content of the organic co-solvent (dioxane), enzyme stability increases with increase in dioxane content. However, the enzyme needs approximately 10% water to remain active.

3.4. Thermodynamic equilibrium

Since the final yields at 72 h in the media described above were still relatively low, we investigated whether the reaction had reached thermodynamic equilibrium. Both the hydrolysis and the synthesis reactions were performed under the same medium condition (dioxane–buffer, 90:10, v/v). In order to eliminate the effect of enzyme inactivation, another 10 mg of the enzyme was added every 48 h to each reaction system. As shown in Fig. 5, the rate of the hydrolysis reaction increased after the addition of further enzyme, indicating enzyme inactivation in the medium. But the synthesis reaction had reached or approached equilibrium before 48 h and therefore the enzyme supplement at 48 h has no effect on the conversion by reverse hydrolysis. After 72 h, the hydrolysis reaction also gradually reached equilibrium where the concentration of the remaining pNBG was closely approaching that of the pNBG synthesized in the reverse hydrolysis. Thus in the reverse hydrolysis reaction described above, thermodynamic equilibrium plays a major role in determining final yields, in spite of the fact that the enzyme was partially inactivated in the dioxane–water system during the reaction process.

Since the equilibrium yield of glycoside in the reverse hydrolysis was mainly controlled by the water content of the reaction system, it should be possible in principle to further enhance the final equilibrium yield by continually reducing water content of the medium to a level below the optimum point of 10% (v/v). We attempted to perform the reaction in a medium consisting of 95% (v/v) dioxane and 5% buffer. To partially release the severe inhibition of enzyme activity owing to water depletion in such a low-water medium, the solid glucose was added directly to the dioxane system without dissolution in advance in the buffer, as was done before. As a result, the β-D-glucosidase-mediated reverse hydrolysis in 95% (v/v) dioxane system did indeed give a significantly higher yield (ca. 11%) than in 90% dioxane (ca. 8%, see Figs. 3 and 5). However, the rate of the former reaction was much slower than the later. It took about 196 h for the former reaction to closely approach equilibrium, making this water-poor medium practically unfeasible for synthetic applications.

3.5. Effect of alcohol concentration

Shift of the thermodynamic equilibrium towards the direction of glucoside synthesis also depends on the initial concentration of substrates. When the glucose concentration was higher than 0.25 M, it became insoluble in the medium (90% dioxane + 10% buffer). Therefore, the extra glucose did not further increase the yield of pNBG. On the contrary, *p*-nitrobenzyl alcohol was completely soluble in the dioxane up to 2.25 M and so, as seen in Fig. 6, the increase of the alcohol concentration up to nine equivalents of glucose concentration (0.25 M) led to a corresponding increase in both the initial rate and the final yield. However, the yield at alcohol concentrations above 2.25 M increased only slightly because alcohol at more than 2.25 M (or nine equivalents of glucose concentration) became insoluble in the medium.

Under the optimal conditions as described above (e.g., 90% dioxane, 10% buffer, 2.25 M alcohol and 0.25 M glucose), the preparative scale (5 ml) synthesis of *p*-nitrobenzyl β-D-glucopyranoside was carried out and the time-course of glucoside (pNBG) synthesis was monitored by HPLC (the inserted curve of Fig. 6). The reaction reached 13.3% conversion at 72 h, forming 10.5 g/l of pNBG. The reaction was quenched and the crude product was purified by flash chromatography on Silica Gel 60 (100–200 mesh) with ethyl acetate/methanol (12:1) as eluent, affording 51 mg of *p*-nitrobenzyl-β-D-glucopyranoside as an amorphous powder in 13.0% isolated yield.

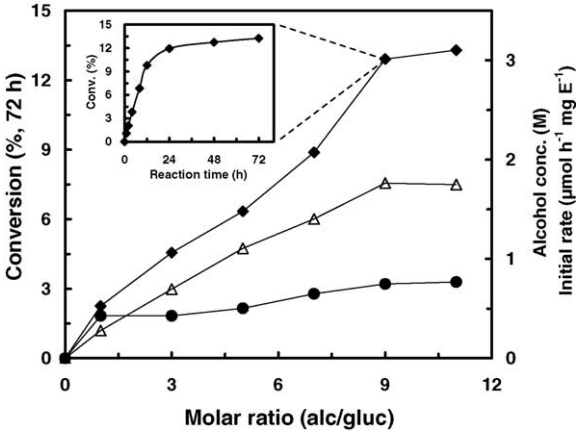

Fig. 6. Effect of alcohol/glucose molar ratio on the initial rate and final yield of pNBG synthesis in the medium containing 90% (v/v) dioxane. The reaction was carried out by shaking at 50 °C and 160 rpm a mixture of 5 mg enzyme powder, 0.1 ml buffer containing 0.25 mmol glucose, a certain amount of *p*NBA and 0.9 ml dioxane. Symbols: (●) initial rate; (♦) final yield; (△) *p*NBA concentration in the medium. The inserted curve was the time course of pNBG synthesis in the medium containing 90% dioxane with the *p*NBA/glucose molar ratio of 9:1 in a preparative scale (5 ml).

4. Conclusions

A monophasic organic–water medium system for efficient enzymatic synthesis of β-D-glucopyranoside by reversed hydrolysis has been constructed and optimized. *p*-Nitrobenzyl alcohol (*p*NBA), as a model hydrophobic alcohol, was glycosylated to produce *p*-nitrobenzyl β-D-glucopyranoside (pNBG) with a best yield of 13.3% (10.5 g/l) in 90% (v/v) dioxane–buffer (Na_2HPO_4–KH_2PO_4, 70 mM, pH 6.0). This dioxane–buffer system has been successfully applied to the synthesis of other hydrophobic glucosides such as salidroside, a physiologically active natural product [30]. The salidroside was produced in 12.9 g/l (15.8% yield), which was much higher than the result of plant cell cultures producing salidroside with the maximum concentration of 0.9 g/l [32]. It is expected that our approach could be applied to a broad range of alcohols (especially those in solid state) for preparation of novel glycoside compounds with biological activities.

Acknowledgments

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