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Fungus β-glycosidases: immobilization and use in alkyl-β-glycoside synthesis

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Abstract

Production of β-glycosidases: β-xylosidase and β-glucosidase by the fungus *Sclerotinia sclerotiorum* was optimized in the presence of different carbon sources. Immobilization supports with different physico-chemical characteristics were evaluated for use in continuous reactors. Immobilization and activity yields were calculated. Among the adsorption on Duolite, Amberlite, Celite and DEAE-sepharose, and entrapment in polyacrylamide gel or reticulation using glutaraldehyde, highest yields were obtained when β-xylosidase was adsorbed on Duolite A 7 and when β-glucosidase was adsorbed on DEAE-sepharose.

Enzyme preparations from *S. sclerotiorum* cultures were used in a biphasic (alcohol/aqueous) medium for the synthesis of alkyl-glycosides by *trans*-glycosylation of sugars and long-chain alcohols. The synthesis was studied under different conditions with primary and secondary alcohols as substrates, in the presence of free or immobilized enzyme. Xylan and cellobiose were used for the synthesis of alkyl-xylosides and alkyl-glucosides, respectively. The majority of the immobilized preparations were unable to catalyze the synthesis of alkyl-glycosides.

Highest yields were obtained when using xylan and C_4–C_6-alcohols. The reaction produced alkyl-β-xyloside and alkyl-β-xylobioside, as confirmed by MS/MS. Up to 22 mM *iso*-amyl-xyloside and 14 mM *iso*-amyl-xylobioside were produced from *iso*-amyl alcohol and xylan.

Keywords: Alkyl-glycoside; Biosurfactant; β-glucosidase; β-xylosidase; Immobilization

1. Introduction

Xylan and cellulose form part of agricultural, forestry, and fruit and vegetable processing wastes. These renewable raw materials can be valorized by converting them into edible or non-edible products using different technologies. Biotechnology offers an interesting alternative for the manufacture of value-added natural products by using microorganisms and enzymes [5,8]. Using enzyme batteries, pathogenic fungi are able to efficiently degrade plant cell walls. The degradation of xylan and cellulose is performed by several enzyme activities, especially the xylanases, the glucanases and the β-glycosidases. These enzymes have also found applications in synthesis reactions. One of these applications is the synthesis of alkyl-glycosides from natural polysaccharides or their derivatives, and alcohols by reversed hydrolysis or *trans*-glycosylation [4,9,11,14]. Previously [12,13], we reported that the fungus *Sclerotinia sclerotiorum* produced different β-glucosidases when grown in a filter paper or in a xylose containing liquid medium. The purified enzymes were stable over a large pH range and at temperature up to 60 °C. They were characterized by studying the effect of some metallic cations and various reagents on their activities. One of the free β-glucosidases produced by *S. sclerotiorum* [13] catalyses glucooligosaccharide synthesis from cellobiose with strong *trans*-glucosylation activity in comparison to commercially available β-glucosidases. In another paper [1], we studied the production of xylanolytic enzymes by the same fungus in order to use them in soluble form in synthesis reaction of hexyl-β-xylosides in a biphasic medium. The purification of a β-xylosidase as well as its biochemical and catalytic properties were studied.

Alkyl-glycosides offer potential industrial applications as non-ionic surfactants [6] and are the topic of active research.

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Nevertheless, the application in industry remains limited to-date due to the high production cost and need for repeat enzyme purification. This could be addressed by the development of appropriate enzyme immobilization technologies. Enzyme immobilization provides many important advantages over the use of free enzymes, namely, enzyme reuse, continuous operation, controlled product formation and simple processing. The choice of an immobilization method is dependent on the enzyme, support, chemical reagent, and reactor. Although much is reported on the immobilization of β-galactosidase [2,16,17], few reports were found on the immobilization of β-xylosidase or β-glucosidase. Recently, Basso et al. [3] immobilized almond β-glucosidase by adsorption onto Celite, and used it for alkyl-glucoside synthesis. The authors reported that the amount of water added to the system as well as the presence of co-solvents have a great effect on the enzyme activity. In other works, β-glucosidase stability has been increased in biphasic media when immobilizing the enzyme in microcapsules [15] or on a modified polyacrylamide support [7].

The objective of this work was to investigate different immobilization methods for the β-xylosidase and β-glucosidase from the fungus: *S. sclerotiorum*, and to study their use in the synthesis of alkyl-β-glycosides.

2. Experimental

2.1. Microorganisms

S. sclerotiorum was isolated from sunflower (local isolate from Tunisia [12]).

Monosaccharides such as xylose and glucose, and polysaccharides such as xylan (from birch wood and oat spelt), cellulose and carboxymethylcellulose (CMC) were used as inducers and purchased from Sigma (Paris, France). Barley straw and oat spelt flour were purchased from the local market and ground before use.

Cultures were incubated on a shaker at 25 °C and 150 rpm for 12 days. Composition of the medium was as described previously [12]. The mycelia were then removed by filtration on GF/D paper and centrifugation at 9200 × *g* during 30 min at 4 °C. The supernatant containing the enzymes was then recovered and enzyme activities were measured.

Trichoderma reesei enzyme preparation (SP 431) was a gift from Novo-Nordisk (Bordeaux, France) and was used without further purification (32 U β-xylosidase/ml).

2.2. Enzyme preparation protocol

Two enzyme preparations were produced by growing *S. sclerotiorum* cultures in the presence of xylan (birch wood) or cellulose (filter paper) as sole carbon sources in 500 ml liquid media [12]. The first culture produced high amounts of xylanolytic enzymes and the second produced mainly β-glucosidase activity. After 12 days, extracellular proteins were recovered by filtration and centrifugation and the culture filtrate was treated twice with one volume acetone (pre-cooled to −20 °C) and left overnight at −20 °C, and then a second night at 4 °C. The precipitate was collected by centrifugation at 21 000 × *g* for 30 min and dissolved in 100 mM Tris–HCl pH 7. Finally proteins were concentrated by ultrafiltration on an amicon membrane (PM 10) at 3000 rpm during 20 min. Enzyme preparations obtained from xylan and cellulose cultures contained 5 U β-xylosidase/ml and 12.7 U β-glucosidase/ml, respectively.

Protein amounts were determined by Bradford's test (595 nm) using 1 mg/ml bovine serum albumin solution as a standard.

2.3. Enzyme assays

The β-xylosidase activity was measured in the presence of 0.5 mM *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX, Sigma, France) in 50 mM sodium acetate buffer pH 4, containing 200 mM NaCl. The reaction mixture (500 μl) was carried out at 60 °C and pH 4, for 30 min. The reaction was stopped by adding 600 μl 0.4 M glycine buffer pH 10.8 and the amount of free *p*-nitrophenol (*p*NP) was measured at 410 nm in a spectrophotometer (Beckman DU 530, Paris, France). One unit corresponds to the amount of enzyme producing 1 μmol *p*NP/min.

The β-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as a substrate using the same method and conditions described above at pH 5 but without the addition of NaCl.

Endo-xylanase and endo-glucanase activities were determined by measuring the amount of reducing sugars cleaved from xylan or cellulose, respectively. Substrate (1%, w/v) was incubated with the appropriate enzyme solution aliquot at 55 °C for 30 min. The concentration of reducing sugars was measured by the DNS method [10] and expressed as xylose or glucose equivalent. One unit corresponded to 1 μmol equivalent xylose or glucose liberated per minute under these conditions.

2.4. Enzyme immobilization

Enzymes prepared from *S. sclerotiorum* culture were immobilized on different supports. Immobilization steps and enzyme storage were carried out at 4 °C. Supernatants and washing volumes were pooled after each step and the non-immobilized activity was determined. The β-glucosidase produced by the fungus on cellulose (filter paper) was immobilized on DEAE-sepharose, in polyacrylamide gel and in serum albumin (Sigma, Paris, France).

The resin DEAE-sepharose (100 μl in equilibration buffer pH 7) was washed twice with 100 mM phosphate buffer pH 7 and then centrifuged for 2 min at 4600 × *g*. The resin was mixed with 30 μl of the enzyme preparation (32 U/ml) during 20 min under agitation. The mixture was then washed

twice with 25 mM phosphate buffer pH 7 and centrifuged for 2 min at 4600 × *g*.

Immobilization in polyacrylamide gel was achieved by mixing 3 ml of a solution of acrylamide and bis acrylamide (30:1), 4 ml water, 2 ml 100 mM Tris–HCl buffer pH 7 and 840 μl enzyme and polymerization by addition of 200 μl ammonium persulphate and 40 μl TEMED. The gel film was polymerized at 4 °C on a surface of 28 cm^2 and cut in pieces with area of 1 cm^2 corresponding to 30 μl of enzyme preparation. The gel pieces were washed twice with 25 mM phosphate buffer pH 7 before use.

The enzyme preparation was immobilized by cross-linking in the presence of serum albumin as follows: in a glass tube 1 ml of 1.5% glutaraldehyde was mixed with 0.5 ml of 20% BSA and 1 ml of 100 mM phosphate buffer, pH 6.8. After 5 min at room temperature, 30 μl of the enzyme preparation (32 U/ml) was added and the mixture was kept for 2 h at −20 °C. The hair gel obtained was defrosted progressively, washed twice with phosphate buffer pH 6.8 and used as covalently bound biocatalyst.

Amberlite IRA 67, Amberlite XAD 4, Amberlite XAD 7, Duolite A 7, Duolite A 568, Celite 545 and Alumine resins were used as supports for immobilization by adsorption of xylanolytic activities produced by *S. sclerotiorum*. After equilibration at pH 4 or 5.5, 0.5 g resin was separated from the buffer by filtration and mixed with 0.1 ml of enzyme preparation (32 U/ml). The mixture was stirred for 1 h and then dried for 24 h at 37 °C. Resins were washed twice with buffer at appropriate pH before activity testing.

Immobilization was estimated as follows:

$$\text{immobilization yield (\%)} = \frac{\text{total enzyme activity} - \text{non-immobilized activity}}{\text{total enzyme activity}} \times 100$$

$$\text{activity yield (\%)} = \frac{\text{immobilized activity}}{\text{total enzyme activity} - \text{non-immobilized activity}} \times 100$$

where total enzyme activity is the total number of units added to the support during the immobilization reaction; non-immobilized activity is the number of units found in filtrates and washing volumes after immobilization; immobilized activity is the number of units detected in the support after immobilization and washing.

2.5. Synthesis reaction

Alkyl-β-glycosides were prepared by reverse-hydrolysis or *trans*-glycosylation in biphasic medium (aqueous/alcohol) at 250 rpm. Alcohol aliquots (4 ml) were mixed with the aqueous phase containing the sugar substrate in 50 mM acetate buffer pH 4–5. The reaction was started by enzyme addition (0.25 U).

Thin layer chromatography on silica gel (TLC, Merck, Paris, France) with 1-propanol and water (85/15, v/v) as mobile phase was used for qualitative analysis of the synthesis of alkyl-glycosides. Alkyl-glycosides were visualized by spraying with 20% sulfuric acid in methanol followed by heating at 150 °C for 10 min. Quantitative analyses were by RP–HPLC (HP 1100, Paris, France) on a C18 reversed phase column Ultrasep 6 μm (Bischoff, Bordeaux, France, 4 mm × 250 mm), at 50 °C, followed by refractometry and spectrophotometry at 210 and 280 nm. The mobile phase (0.5 ml/min) was a mixture of methanol and ultra pure water and contained 0.2% acetic acid. The percentages of methanol and water used depended on the carbon chain length of the alcohol; varying from 40/60 (methanol/water) when butanol was used as a substrate, to 60/40 (methanol/water) when octanol was used as a substrate. Samples (20 μl) were injected in the column after homogenization with an equal volume of acetonitrile and centrifugation. Standards were octyl-β-D-glucopyranoside, heptyl-β-D-glucopyranoside, hexyl-β-D-glucopyranoside, pentyl-β-D-glucopyranoside, butyl-β-D-glucopyranoside and butyl-β-D-glucopyranosyl-galactopyranoside (Sigma, Paris, France) diluted in their corresponding alcohols and mixed with 1 v acetonitrile.

2.6. Product identification

The synthesis reaction between hexan-1-ol and xylan was conducted in the presence of free β-xylosidase obtained from the xylan culture as above. Products were identified by electrospray mass spectrometry (ES–MS triple quadripole, LRTL, INRA de Rennes, France), as hexyl-β-D-xyloside, and hexyl-β-D-xylobioside. Full scan mode (mass range: 150–700 by 0.3 amu, negative mode) showed synthesis of hexyl-β-D-xylotrioside and hexyl-β-D-xylotetraoside in small quantities (data not shown).

3. Results and discussion

A variety of carbon sources were tested for the production of different xylanolytic and cellulolytic enzymes by *S. sclerotiorum* in shaking flasks. Fig. 1 shows that β-xylosidase, endo-xylanase, β-glucosidase and endo-glucanase activities in *S. sclerotiorum* culture are induced by different carbon sources. The results showed that cultivation on xylan from oat spelt produced 2.1 U/ml β-xylosidase and 2.7 U/ml endo-xylanase activities with relatively low β-glucosidase and endo-glucanase activities. Such culture was used as a source of xylanolytic enzymes in subsequent experiments. In contrast, cultivation on filter paper (cellulose) was optimal for the production of β-glucosidase (4 U/ml). Enzyme production was low when glucose was used as the inducer. The capability of the fungus of growing on lignocellulotic residues (barley straw, oat meal and paper) with high production of enzyme activities could be considered as an

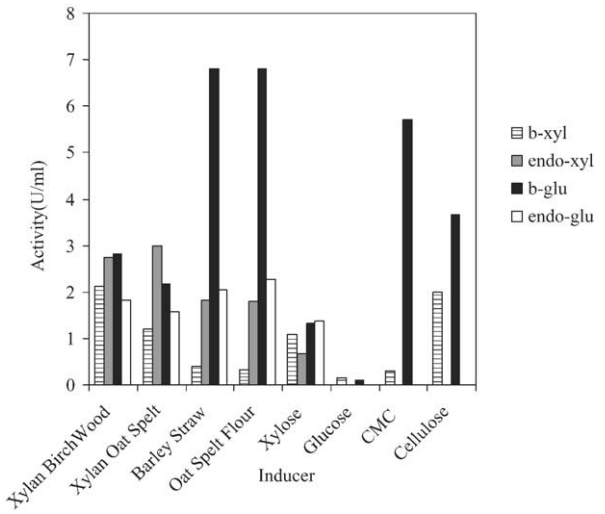

Fig. 1. Induction of β-xylosidase (b-xyl), endo-xylanase (endo-xyl), β-glucosidase (b-glu) and endo-glucanase (endo-glu) in the presence of different carbon sources (1% w/v) in liquid culture at 25 °C for 12 days.

advantage for the valorization of these low grade residues. These results confirmed that *S. sclerotiorum* offered an efficient source of cellulolytic and xylanolytic activities. Fig. 2 shows the kinetics of β-xylosidase and β-glucosidase production by *S. sclerotiorum* in the respective media. The fungus produced β-glucosidase at highest activities in comparison to that of other enzymes tested. The fungus was cultured for 12 days before recovery of enzyme activities.

The immobilization of enzyme preparations from *S. sclerotiorum* provides biocatalysts of potential interest for synthesis reactions. The advantage of using immobilized enzymes over free enzyme preparations is essentially due to better stability of the enzymes. Other advantages include the possibility of the repeated use of the biocatalyst and the feasibility of a continuous process. We produced, immobilized and evaluated β-glucosidase and β-xylosidase derived from the fungus *S. sclerotiorum*.

The enzyme preparation from cellulose cultures rich in β-glucosidase was immobilized on different supports (Table 1). The β-glucosidase half-life ($t_{1/2}$) was determined

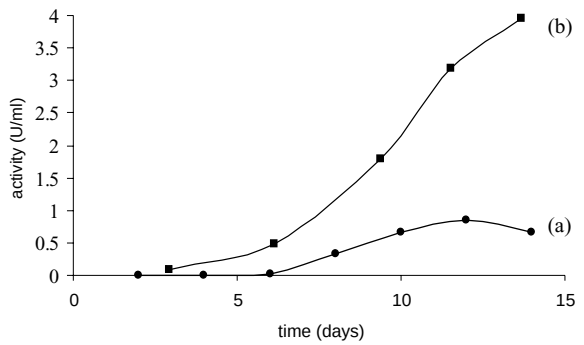

Fig. 2. Kinetics of the production of β-xylosidase (a) and β-glucosidase (b) by *S. sclerotiorum*, in the presence of cellulose (filter paper) and xylan (oat spelt) as carbon sources, respectively.

by measuring the residual activity in samples withdrawn at regular time intervals from an enzyme solution incubated under conditions described in Section 2 for β-glucosidase activity assay. Immobilization of the β-glucosidase entrapment in polyacrylamide gel in particular increased the enzyme half-life by 63–130%. In view of the low activity yield, the long half-life observed after polyacrylamide gel immobilization may have been partially due to an artifact arising from diffusion of reagents to and products from enzymes entrapped in the interior of the gel resulting in the measured prolonged activity. Highest activity yields were obtained when enzymes were adsorbed onto DEAE-sepharose (Table 1).

Seven resins were tested for the immobilization of xylanolytic activities by measuring the β-xylosidase activity. Table 2 shows that both immobilization and activity yields were higher for three of four resins tested when immobilization was at pH 5.5 than at 4. Among those tested, immobilization and activity yields were highest when xylanolytic activities were immobilized on Duolite A 7 resin at pH 4.

Free and immobilized enzyme preparations were evaluated for the synthesis of alkyl-glycosides by *trans*-glycosylation reaction with long-chain alcohols. The biphasic medium was composed of an alcohol and aqueous phase (4/1, v/v). Synthesis was studied under different conditions with primary and secondary alcohols as substrates and in the presence of free or immobilized enzyme preparations. We used cellobiose and xylan for the synthesis of alkyl-glucosides and alkyl-xylosides, respectively. Table 3 summarizes the results of the *trans*-glycosylation reactions catalyzed by the enzymes from *S. sclerotiorum* compared to those from other sources. Highest yields were obtained when using xylan and C_4 to C_6-alcohols in the presence of enzyme extract from *S. sclerotiorum*. The reaction produced alkyl-β-xyloside, alkyl-β-xylobioside as well as small amounts of alkyl-β-xylotrioside and alkyl-β-xylotetraoside as identified by ES–MS. HPLC analysis quantified the production of alkyl-β-xyloside and alkyl-β-xylobioside. Twenty-two millimole *iso*-amyl-xyloside and 14 mM *iso*-amyl-xylobioside were produced from *iso*-amyl alcohol and xylan. For the production of alkyl-xylosides from xylan, two enzymes in the preparation were involved. After hydrolysis of xylan with endo-xylanase, the β-xylosidase was able to catalyze *trans*-xylosylation between partially hydrolyzed xylan and hexan-1-ol [1]. Alkyl-xylosides were enzymatically produced from xylan and alcohols of different carbon chain lengths and primary or secondary alcohol groups. The enzyme easily accepts primary alcohols. Similar alkyl-glycoside production was observed in the presence of β-glucosidase activity prepared from *Tricoderma reesei* or *S. sclerotiorum*. The produced alkyl-xylosidases are biosurfactants with surface-active properties useful to the pharmaceutical and cosmetic industries were simple and cheap enzyme preparation limits commercial application. The enzyme preparation used in this reaction is a precipitate obtained easily from the culture medium in which *S.*

Table 1
β-Glucosidase half-life, immobilization and activity yields for the total enzyme and on the support after immobilization

Immobilization method	Total enzyme	DEAE-sepharose	Polyacrylamide	BSA
$t_{1/2}$ (min)	52	99	120	85
Immobilization yield (%)	–	91	95.2	80
Activity yield (%)	–	83.2	45.3	48.9

Table 2
Variation in immobilization and activity yields of β-xylosidase as function of the resine used

pH	Support	Amberlite (IRA 67)	Amberlite (XAD 4)	Amberlite (XAD 7)	Duolite (A 7)	Duolite (A 568)	Celite (545)	Alumine
5.5	Immobilization yield (%)	20.33	36.12	36.43	59.85	–	–	–
	Activity yield (%)	6.5	1.33	1.6	56.47	–	–	–
4	Immobilization yield (%)	16.1	21.89	26.45	70.56	67.3	27	5.2
	Activity yield (%)	4.02	0.6	8.52	67.31	0.7	10.6	40.6

Table 3
Results of the synthesis reactions: alkyl-xyloside and alkyl-glucoside production in biphasic media in the presence of different substrates and enzymes

Alcohol[a]	Butan-1-ol[b]	*Iso*-amyl alcohol[b]	Me-2-butan-2-ol[b]	Pentan-2-ol[b]	Pentan-1-ol[b]
Alkyl-β-glycoside	2.1	21	0.9	14.2	17
Alkyl-β-glycobioside	1	13.6	0.5	2.2	11
	Hexan-1-ol[b]	Hexan-1-ol[c]	Hexan-1-ol[d]	Octan-1-ol[b]	Octan-1-ol[e]
Alkyl-β-glycoside	9.7	1.1	12	2.8	2.5
Alkyl-β-glycobioside	3.8	nd	nd	2.1	nd

[a] Alkyl-β-xyloside or alkyl-β-glucoside in mM.
[b] Synthesis of alkyl-xyloside in presence of β-xylosidase from *S. sclerotiorum* in biphasic medium alcohol/aqueous (4/1) pH 4, 50 °C, 250 rpm, 10 mg/ml xylan oat spelt.
[c] Synthesis of alkyl-xyloside in presence of immobilized β-xylosidase from *S. sclerotiorum* on Celite 545 in biphasic medium alcohol/aqueous (4/1) pH 4, 50 °C, 250 rpm, 10 mg/ml xylan oat spelt.
[d] Synthesis of alkyl-glucoside in presence of β-glucosidase from *S. sclerotiorum* in biphasic medium alcohol/aqueous (4/1) pH 5, 50 °C, 250 rpm, 150 mg/ml cellobiose.
[e] Synthesis of alkyl-xyloside in presence of β-xylosidase from *T. reesei* in biphasic medium alcohol/aqueous (4/10) pH 4.5, 40 °C, 300 rpm, 14.3 mg/ml xylan oat spelt.

sclerotiorum is cultivated. In order to improve the efficiency of the process, the enzymes immobilized as described below were tested for synthesis. In spite of the success of the immobilized enzymes in the hydrolysis of *p*NPX or *p*NPG, the majority of the immobilized preparations were unable to catalyze the synthesis of alkyl-glycosides. β-xylosidase immobilized on Celite was the sole immobilized enzyme preparation tested that catalyzed hexyl-xyloside as confirmed by TLC.

4. Conclusions

The enzymes tested are produced by a plant pathogenic fungus *S. sclerotiorum*. The production of β-glucosidase and β-xylosidase was optimized in the presence of different carbon sources. Two enzyme preparations rich in xylanolytic or β-glucosidase activities were produced by the fungus on xylan and cellulose media, respectively.

The immobilized β-glycosidase systems using ion exchange resins were found to increase enzyme stabilities when tested for hydrolysis. Immobilization and activity yields calculated for each support allowed comparison of immobilization methods.

The enzyme preparations were tested for synthesis of alkyl-glycosides. *S. sclerotiorum* β-xylosidase exhibited great potential for the alkyl-β-xyloside synthesis by *trans*-glycosylation. Immobilization of enzymes, however, did not increase synthesis efficiency. The conformation of the enzyme when immobilized, may have been unfavorable for the synthesis reaction.

Research is continuing in our laboratory on the investigation of immobilization of other xylanolytic and cellulolytic enzymes from *S. sclerotiorum*, and on the identification of an efficient immobilized system for alkyl glycoside synthesis in a continuous reactor.

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