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Bioconversion of sucralose-6-acetate to sucralose using immobilized microbial cells☆

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ABSTRACT

Bioconversion of sucralose-6-acetate to sucralose, an artificial sweetener has been carried out using *Arthrobacter* sp. (ABL) and *Bacillus subtilis* (RRL-1789) strains isolated at IIIM, Jammu, India. Biotransformation of sucralose-6-acetate to sucralose involves use of microbial whole cells, immobilized whole cells and immobilized whole cell bioreactor. Immobilized whole cells packed bed reactor has shown much superior biotransformation process in aqueous system using green technology, where purification of the final product is not required. The final sucralose bioproduct was directly concentrated under vacuum to get white crystalline powder. The immobilized whole cell bioreactor was used for more than three cycles continuously, thus provided much cheaper, less time consuming and easy down streaming process. Moreover, the method does not require any purification steps, which is otherwise requisite for presently available methods for sucralose production, resulting in even lower cost of overall process.

1. Introduction

Sucralose, trichlorogalactosucrose or 4,1′,6′-trichlorogalactosucrose is known as an artificial sweetener having 600 times sweetening ability than sucrose. It is used in place of sugar to eliminate or reduce calories in a wide variety of products, including beverages, baked goods, desserts, dairy products, canned fruits and syrups. Sucralose is chemically known as 1,6-dichloro-1,6-dideoxy-beta-D-fructofuranosyl-4-chloro-4-deoxy-alpha-D-galactopyranoside and has been derived from sucrose by replacing the hydroxyl groups in the 4,1′, and 6′ positions with chlorine.

First report on sucralose appeared in 1987 by Queens Elizabeth College in London and Tate & Lyle, a private company where, chemical modification of sucrose by selective replacement of three hydroxy groups with chlorine atoms was reported. Canada was the first country to approve use of sucralose in foods in 1991 followed by the United States in 1998. It is now being used in at least 28 countries as sweetening agent. Sucralose is being sold under the brand name Splenda by McNeil Specialty Products Company, New Brunswick, New Jersey. About 120 products using sucralose as a sweetener are in the U.S. market.

Sucralose is generally synthesized by chemical methods involving five-step process by selective substitution of three chlorine atoms for three hydroxyl groups in the sucrose molecule. These methods for synthesis of sucralose (4,1′,6′-trichlorogalactosucrose; TGS) involve the chlorination through multistep protection and deprotection strategy to obtain final sucralose. The reagents used in the reaction are hazardous and involve the formation of different byproducts, which need to be removed [1–7]. Involvement of several purification steps, reduce the overall yield of the process for production of sucralose.

The enzymatic methods of sucralose synthesis involve removal of 6-chloro-6-deoxygalactosyl moiety from the 6-position of chlorinated sugar tetrachlororaffinose (TCR) [8]. Luo et al. [9] described improved synthesis of sucralose from sucrose by chemical synthesis, which again needs purification to obtain pure product. Ratnam et al. [10] reported enzymatic process for sucralose production wherein they could achieve maximum 95% deacetylation using free or immobilized enzyme. Such process essentially requires several downstream processing and product purification steps. Thus, the process is quite expensive and time consuming. Alternative methods involve deacetylation of sucrose-6-ester followed by chlorination and purification to get pure sucralose [11]. To the best of our knowledge, this is only one report of enzymatic deacetylation of sucralose-6-acetate, however *Arthrobacter* sp. or *Bacillus sublilis* strains were not used. We have therefore, used these two indigenous strains for a single step hydrolysis of sucralose-6-acetate to obtain the sucralose (TGS) with 100% purity without any purification. The method is green and does not involve any hazardous reagents.

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Present report demonstrates the enzymatic hydrolysis of sucralose-6-acetate to sucralose in a single step using acyl hydrolase enzyme from IIIM Jammu, India microbial isolates; *Arthrobacter* sp. (ABL) and *Bacillus subtilis* (RRL 1789). The bioconversion of sucralose-6-ester has been carried out in aqueous system without pH adjustment or use of any buffering salts using free enzyme/whole cells/immobilized enzyme/immobilized whole cells. The final bioproduct does not require any purification and can be directly concentrated to get crystalline powder.

2. Experimental

2.1. Materials and general experimental conditions

The reagents and solvents used in the present study were mostly LR grade. Substrate sucralose-6-acetate was a kind gift from Dr. V. Arosker, Mumbai. Silica gel coated aluminum plates from M/s Merck were used for TLC. Commercial enzymes were purchased from Sigma. 1H NMR spectra in CD_3OD were recorded on Bruker 500 MHz spectrometers with TMS as the internal standard. Chemical shifts were expressed in parts per million (δ ppm). MS were recorded on LC MS Agilent 1100 series. IR was recorded on a FT-IR Hitachi (270-30) spectrophotometer. Optical rotations were measured on Perkin-Elmer 241 polarimeter at 25 °C using sodium D light.

2.2. Enzymes

Commercial enzymes such as *Candida rugosa* lipase (CRL), *Candida cylindracea* lipase (CCL), *Candida antarctica* lipase (CAL), *Pseudomonas* sp. lipase (PSL), *Mucor miehei* lipase (MM), *Porcine pancreas* lipase (PPL), *Mucor javanicus* lipase (MJL) were procured from Sigma. Indigenous lipase such as *Arthrobacter* sp. lipase (ABL), *B. subtilis* lipase (RRL-1789) and *Trichosporon beigelii* lipase (Y-15) were grown in specified media and conditions followed by enzyme isolation by ultrasonication as described previously [12–14].

2.3. Production of biomass

Biomass from *Arthrobacter* sp. was grown in 1% peptone, 0.5% beef extract and 0.5% NaCl, pH 7.0 as described previously [12]. *B. subtilis* biomass was grown in 1% peptone, 0.1% yeast extract, 0.5% NaCl, and 0.5% sucrose at pH 7.2 for 30–36 h [13].

2.4. Immobilization of microbial cells

2.4.1. Entrapment in sol–gel supports

Arthrobacter sp./*B. subtilis* microbial cells were immobilized in sol–gel supports prepared from tetraethylorthosilicate precursor as previously reported [15]. Entrapment was carried out by adding ABL cells suspension/free enzyme to the homogenized sol during polymerization process. Gelation was carried out further for 24 h. The gel was filtered and washed several times with buffer to remove any adhered whole cells/free enzyme. The entrapped ABL was then further used for biotransformation studies.

2.4.2. Entrapment of whole cells in calcium alginate

Freshly harvested *B. subtilis*/*Arthrobacter* sp. cells were mixed with sodium alginate (sodium alginate:cell biomass used was 1:1, 1:2, 1:3) and passed through syringe into 2% calcium chloride solution as previously described [16]. The entrapped cells thus obtained were washed with water and further used for hydrolysis of sucralose-6-acetate at different substrate concentrations.

Table 1
Screening of various indigenous and commercial enzymes for bioconversion of sucralose-6-acetate to sucralose.

Enzyme	Time (h)	Conversion (%)
Arthrobacter sp. lipase (ABL)	96	100
Bacillus subtilis (RRL-1789)	48	100
Trichosporon beigelii (Y-15)	24	5
Pig Liver Esterase (PLE)	50	100
Candida antarctica (CAL-B)	96	85
Pseudomonas fluorescence (PSF)	24	ND
Pseudomonas sp. lipase (PSL)	24	5
Mucor miehei lipase (MM)	24	ND
Mucor javanicus (MJ)	24	5
Amano AS	5	90% + 10% side product
Porcine pancreas lipase (PPL)	24	20
Candida rugosa lipase (CRL)	24	30
Candida cylindracea (CCL)	24	25

2.5. Biotransformation of sucralose-6-ester to sucralose using free isolated enzyme

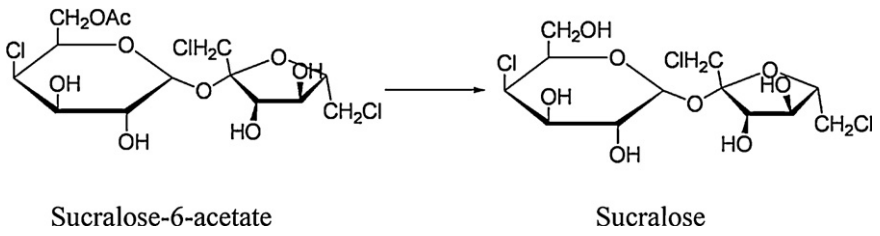
Biotransformation of sucralose-6-ester to sucralose was performed using several commercial lipases as shown in Table 1 and indigenous lipase/esterase enzymes *B. subtilis* (RRL-1789)/*Arthrobacter* sp. (ABL), *T. beigelii* (Y-15) for comparative performance (Table 1). Enzymatic reaction was performed by suspending sucralose-6-ester and enzymes (purified lyophilized powder) in 1:1 ratio in water/buffer under shaking. Biotransformation reaction was monitored by TLC at different time intervals till complete biotransformation is achieved. On completion of biotransformation, reaction was terminated by centrifugation to remove insoluble impurities and the product was extracted with chloroform, dried and purified by column chromatography in 5:95%, methanol:DCM solvent system. Bioproduct was finally analyzed by HPLC, optical rotation and NMR for its purity.

2.6. Biotransformation of sucralose-6-ester to sucralose using microbial cells

Biotransformation of sucralose-6-ester to sucralose (Scheme 1) was carried out using freshly harvested microbial cells from indigenous strains *B. subtilis*/*Arthrobacter* sp. Reaction mixture was prepared by suspending sucralose-6-ester in requisite amount of water to obtain 10–50 g/L substrate concentration. Enzymatic reaction was initiated by addition of enzyme/cell biomass/immobilized enzyme in equal amount of substrate. Biotransformation was monitored on TLC at different time intervals. On completion of biotransformation, the reaction was terminated by centrifugation and concentrated under vacuum, purified by column chromatography followed by crystallization. The product purity was tested by HPLC, optical rotation and NMR.

2.7. Biotransformation of sucralose-6-acetate to sucralose in immobilized whole cells/immobilized whole cell reactor

Reaction mixture was prepared by dissolving 1.0 g of sucralose-6-ester in water to get 10–50 g/L concentration. The reaction was initiated by addition of immobilized whole cells (beads) equivalent to requisite amount of immobilized/entrapped whole cells beads so as to make S:E ratio 1:1. To carry out reaction with 1.0 g of substrate, 30.0 g of entrapped whole cell beads (equivalent to 1.0 g of whole cells) were used under shaking at 50 rpm. Biotransformation was monitored on TLC at different time intervals till complete biotransformation was achieved. Aqueous product was separated by filtration, beads were washed with water and concentrated under vacuum to obtain white crystalline powder.



Scheme 1. Bioconversion of sucralose-6-acetate to sucralose.

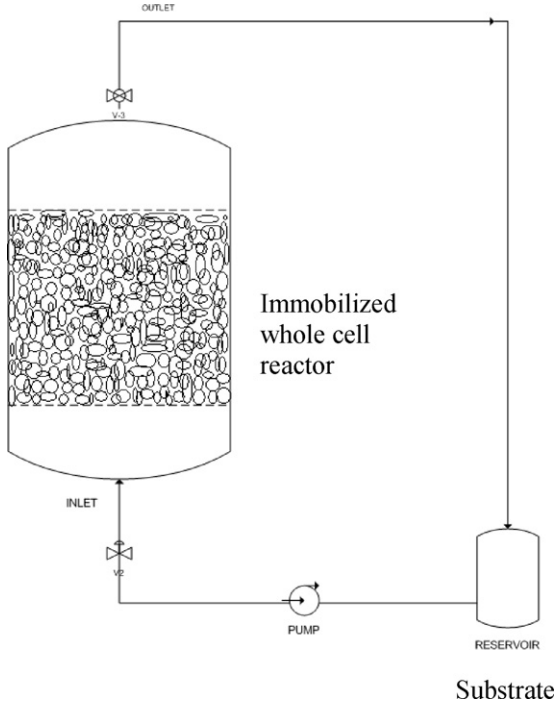
Immobilized whole cell bioreactor was fabricated by packing 150 g of immobilized whole cell calcium alginate beads in a reactor (1.5′ × 15′). 5 g substrate was suspended in water to prepare 10 g/L concentration and circulated through the reactor continuously at a flow rate of 10 mL/min until 100% bioconversion was obtained. The design of the reactor has been shown in Scheme 2.

The aqueous product was separated from the reactor, the reactor was washed well with water and bioproduct was concentrated under vacuum providing a cost effective and easy downstream processing. There is no need to separate soluble impurities or proteins which is essential in case of soluble enzymes. The reactor was used continuously three times for the biotransformation process.

2.8. Characterization of product

Biotransformation process was monitored initially by TLC and HPLC until 100% conversion is achieved. The purified crystallized product obtained was tested for its purity by TLC, HPLC, NMR and optical rotation. Conversion and purity profile of the reaction product was determined by Perkin-Elmer HPLC on C-8 column using RI (refractive index) detector. Mobile phase for HPLC was ACN:Water, 17:83; flow rate 1 mL/min and column temperature 40 °C. Elemental analysis of purified product was carried out using Elementar Vario EL III.

6-O-acyl-4,1′,6′-trichloro-4,1′,6′-trideoxygalactosucrose: HPLC Purity >99%; $[\alpha]_D^{25}$ +74.0 (*c* 1, MeOH) 1H NMR, *δ*: 2.01 (s, 3H), 3.71–3.75 (m, 2H), 3.96–3.85 (m, 2H), 3.87–3.92 (m, 2H), 3.99–4.07 (m, 1H), 4.08 (dd, *J* = 3.5 Hz and 10.2 Hz, 1H), 4.11–4.19 (m, 1H), 4.24 (dd, *J* = 3.83 Hz and 6.59 Hz, 1H), 4.31 (d, *J* = 8.39 Hz, 1H), 4.35–4.41 (m, 1H), 4.52–4.60 (m, 1H), 5.38 (d, *J* = 3.9 Hz, 1H); 13 C NMR, *δ*: 20.9, 44.8, 46.2, 65.0, 66.1, 69.1, 69.5, 69.6, 77.3, 77.6, 83.6, 93.9, 104.8, 172.4. ESI-MS (*m*/*z*): 438. Anal. Calc. for $C_{14}H_{21}Cl_3O_9$: C, 38.24; H, 4.81. Found C, 38.73; H, 5.24.



Scheme 2. Design of immobilized whole cell packed bed reactor for bioconversion of sucralose-6-acetate to sucralose.

4,1′,6′-trichloro-4,1′,6′-trideoxygalactosucrose: HPLC Purity >99%; $[\alpha]_D^{25}$ +71.5 (*c* 1, MeOH) 1H NMR, *δ*: 3.63–3.68 (m, 2H), 3.71 (s, 2H), 3.79 (dd, *J* = 11.1 Hz and 3.0 Hz, 1H), 3.82 (dd, *J* = 3.9 Hz and 10.1 Hz, 1H), 3.85–3.93 (m, 2H), 4.02–4.09 (m, 2H), 4.25–4.30 (m, 1H), 4.34 (dt, *J* = 1.0 Hz and 6.7 Hz, 1H), 4.38–4.40 (m, 1H), 5.37 (d, *J* = 3.91 Hz, 1H); 13C NMR, *δ*: 44.9, 46.3, 63.1, 65.1, 69.4, 69.8, 71.8, 77.3, 77.6, 83.8, 93.9, 104.8. ESI-MS (*m*/*z*): 396. Anal. Calc. for $C_{12}H_{19}Cl_3O_8$: C, 36.25; H, 4.82. Found C, 36.01; H, 5.00.

3. Results and discussion

3.1. Enzymatic biotransformation of sucralose-6-ester to sucralose

Various lipases/esterases from commercial and indigenous source were screened and tested for biocatalytic efficiency for the production of sucralose from sucralose-6-ester. As shown in Table 1, most of the commercial enzymes presented significant hydrolysis of the substrate. Amano AS hydrolyzed the ester very fast in (5 h), but the product contained side products/impurities. However, enzymes from indigenous source *Arthrobacter* sp. lipase, *B. subtilis* and commercial PLE enzymes demonstrated complete bioconversion of sucralose-6-ester at a low speed without any side products. Since these biotransformation reactions were carried out using soluble enzymes, the protein impurities were present in the reaction mixture. The required purification steps resulted in an expensive and time consuming process. The product obtained from the experiments using whole cell biomass from indigenous strains was fairly good but still some purification was required.

3.2. Biotransformation using microbial whole cells of B. subtilis and Arthrobacter sp. at various substrate concentrations

In order to avoid the purification of product while using enzymatic biotransformations, freshly prepared biomass from lipase/esterase producing indigenous strains *Arthrobacter* sp. and *B. subtilis* were used for biotransformation reactions. Fig. 1(a) shows *B. subtilis* whole cells catalyzed biotransformation at different substrate concentrations (10–300 g/L). Best results were obtained with 10–20 g/L concentration where 100% product was observed in 48 h whereas using 50 g/L concentration, complete conversion was observed in 72 h. On further increasing the substrate concentration (100 g/L) complete conversion was observed in 96 h.

Arthrobacter sp. Lipase could result in complete biotransformation in 72 h, whereas increase in substrate concentration slowed down the reaction rates due to which, complete

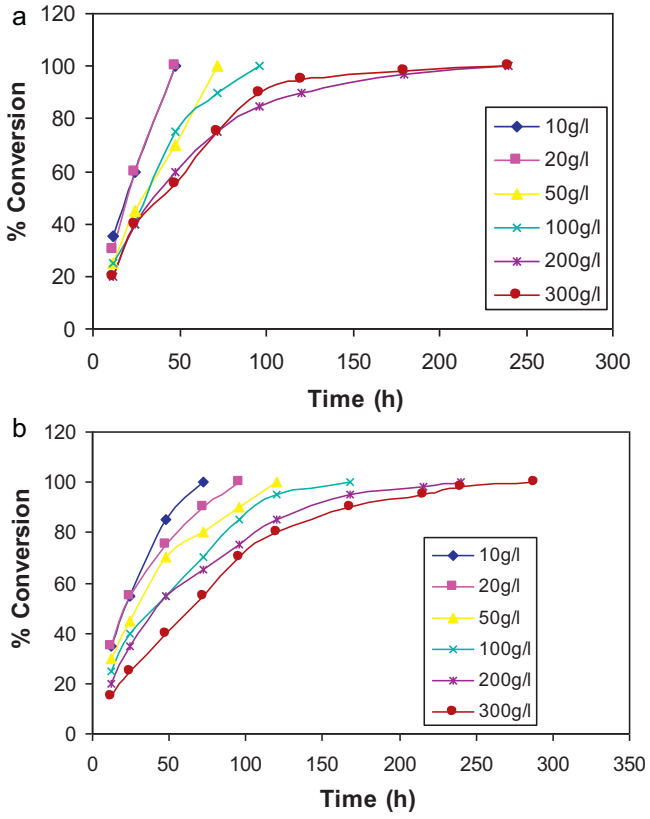


Fig. 1. (a) Biotransformation using microbial whole cells of *Bacillus subtilis* at various substrate concentrations. (b) Biotransformation using microbial whole cells of *Arthrobacter* sp. at various substrate concentrations.

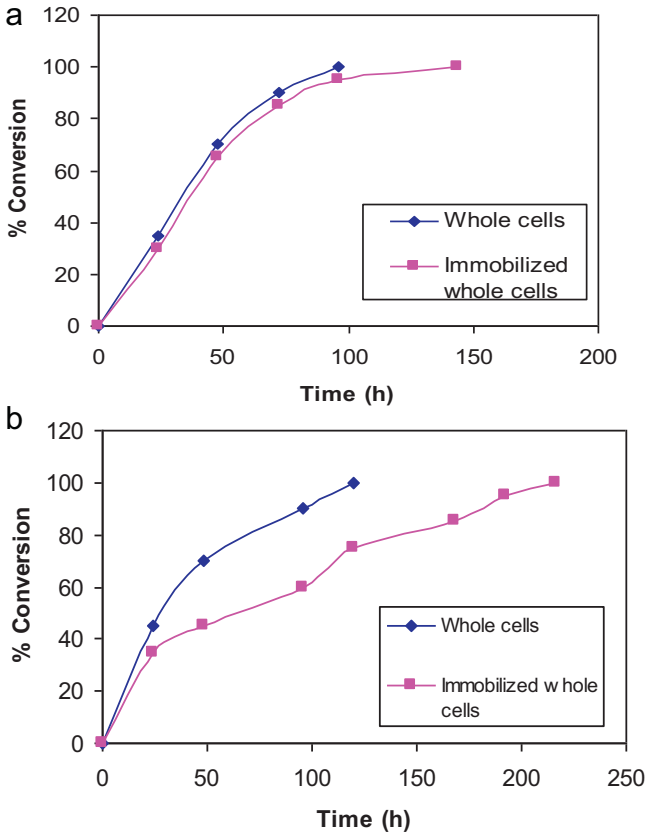


Fig. 2. (a) Biotransformation using immobilized whole cells of *Bacillus subtilis*. (b) Biotransformation using immobilized whole cells of *Arthrobacter* sp.

hydrolysis of sucralose-6-acetate to sucralose was obtained in 120–168 h (Fig. 1b).

3.3. Biotransformation using B. subtilis and Arthrobacter sp. biomass with different substrate to enzyme ratio

Bioconversion of sucralose-6-acetate to sucralose at 10–100 g/L concentration was carried out at different substrate:enzyme ratio, i.e. 1:0.25, 1:0.5, 1:0.75, 1:1, 1:2, 1:3 using *Arthrobacter* sp. and *B. subtilis* cell biomass. The results showed that 1:1 ratio of substrate to enzyme ratio was suitable for biotransformation of sucralose-6-ester at 10–100 g/L substrate concentrations using whole cells//immobilized whole cells of *B. subtilis* (Fig. 2a) and *Arthrobacter* sp. (Fig. 2b). Therefore, S:E ratio of 1:1 was used for all biotransformation studies.

3.4. Biotransformation of sucralose-6-ester to sucralose using calcium alginate entrapped microbial cells

Biotransformation of sucralose-6-acetate (1.0 g) was carried out under shaking conditions. It was observed that calcium alginate entrapped *B. subtilis* cells resulted in complete bioconversion in 72–96 h at 10–20 g/L substrate concentration (Fig. 3a). On further increasing the substrate concentration up to 50 g/L, rate of reaction was much slower resulting in 100% biotransformation in 7 days (168 h). Calcium alginate entrapped microbial cells from *Arthrobacter* sp. (Fig. 3b) showed slower reaction rates with production of pure product in 9 days (216 h). The reaction was terminated by filtration and concentrated under vacuum for crystallization of product followed by purity testing.

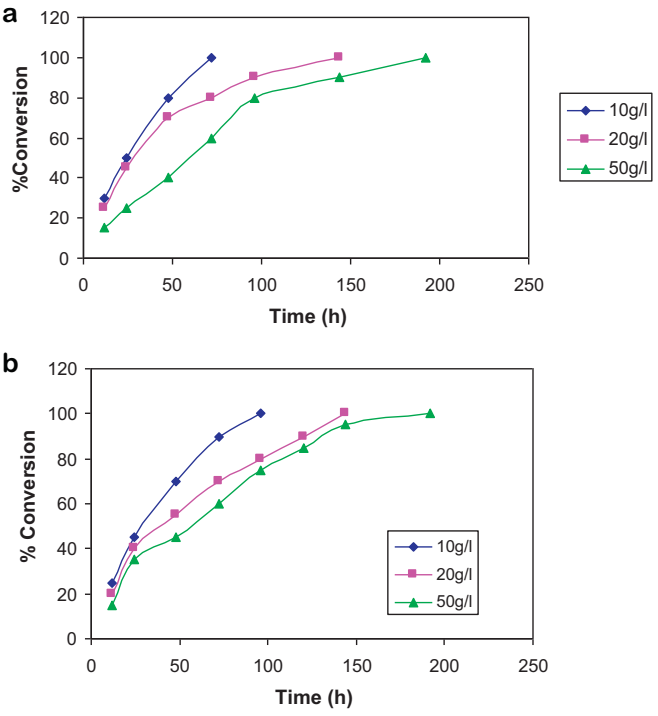


Fig. 3. (a) Biotransformation of sucralose-6-acetate to sucralose in immobilized whole cells reactor of *Bacillus subtilis*. (b) Biotransformation of sucralose-6-acetate to sucralose in immobilized whole cell reactor of *Arthrobacter* sp.

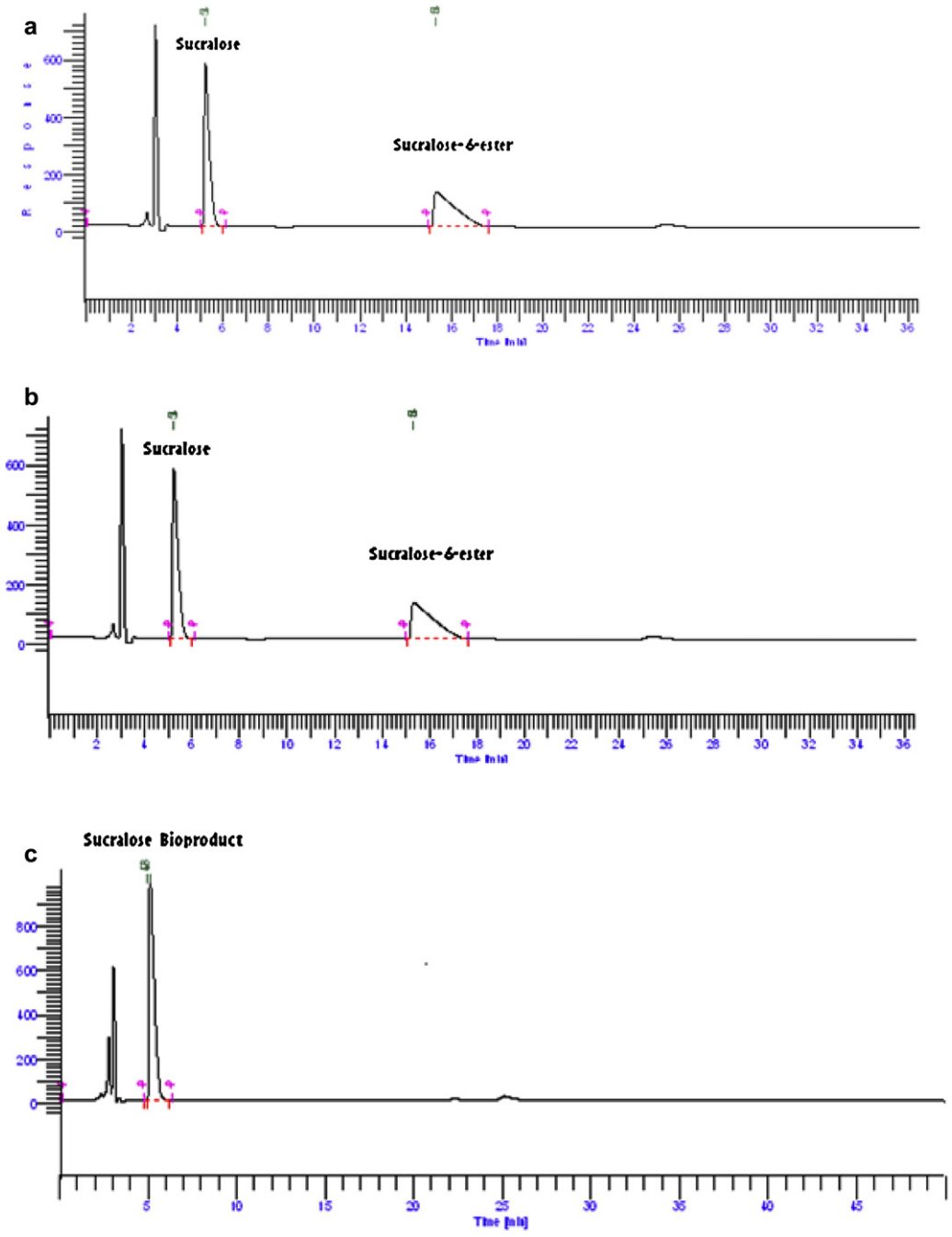


Fig. 4. (a) HPLC chromatograph of standard mixture sucralose and sucralose-6-acetate. (b) HPLC chromatograph reaction mixture during biotransformation. (c) HPLC chromatograph of bioproduct from immobilized whole cell bioreactor.

3.5. Biotransformation of sucralose-6-acetate to sucralose in immobilized whole cell bioreactor

Immobilized *B. subtilis* whole cells bioreactor was fabricated by packing of calcium alginate entrapped whole cell beads in a reactor. Biotransformation was carried out with 10–50 g/L concentration at a flow rate of 10 mL/min (Scheme 2). Biotransformation was monitored on TLC/HPLC at different time intervals. Fig. 4 shows HPLC chromatograms of the standard substrate/product mixtures (Fig. 4a), during bioconversion process (Fig. 4b) and the bioproduct (Fig. 4c). On achieving complete conversion, the reaction was terminated by taking out the aqueous product from the reactor. The reactor was thoroughly washed with water to remove adhered bioproduct. The product was concentrated under vacuum for crystallization of product. This process for biotransformation was found to be the best among all, as there was no requirement of filtration or removal of biocatalyst. Thus downstream process is much easier and less time consuming when immobilized whole cell bioreactor was used.

3.6. Reuse of immobilized whole cell reactor for biotransformation

The immobilized whole cell bioreactor of *B. subtilis* and *Arthrobacter* sp. was reused for biotransformation of sucralose-6-ester at 5.0 g batch scale. Fig. 5 shows the biotransformation results for *B. subtilis* (Fig. 5a) and *Arthrobacter* sp. (Fig. 5b) immobilized whole cell reactor. The continuous flow reactor was continuously

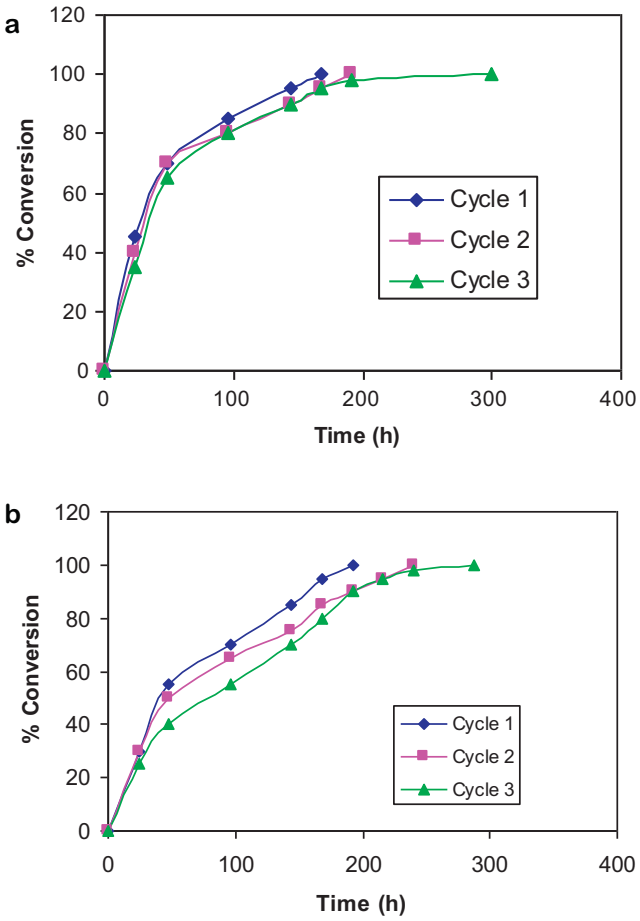


Fig. 5. (a) Reuse of immobilized whole cell reactor of *Bacillus subtilis* for biotransformation of sucralose-6-acetate to sucralose. (b) Reuse of immobilized whole cell reactor of *Arthrobacter* sp. for biotransformation of sucralose-6-acetate to sucralose.

used for at least three consecutive cycles for three batch biotransformations. On reusing the same batch of immobilized *B. subtilis*, 100% hydrolysis of substrate was obtained in 7–8 days whereas on third time reuse complete bioconversion was observed in 12 days. Reuse of immobilized *Arthrobacter* sp. reactor in batch process could provide 100% conversion in 8, 10 and 12 days respectively in consecutive cycles.

4. Conclusions

Biotransformation process for the conversion of sucralose-6-acetate to sucralose has been successfully developed using immobilized whole cell reactor of *Arthrobacter* sp. and *B. subtilis*. *B. subtilis* strain proved to be better as it took less time for complete bioconversion of substrate as compared to *Arthrobacter* sp. strain. The immobilized whole cell packed bed reactor has shown easy and inexpensive batch biotransformation process for sucralose production in aqueous system. The immobilized whole cell packed bed reactor was used more than three cycles with equal quality of product. Thus, a single step hydrolysis provides pure sucralose (TGS) with 100% purity without any purification.

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