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Short communication

Simultaneous production of exopolysaccharide and lipase from extremophylic *Pseudomonas aeruginosa* san-ai strain: A novel approach for lipase immobilization and purification

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ABSTRACT

In this study, exopolysaccharide (EPS) produced by an extremophylic strain of *Pseudomonas aeruginosa* san-ai was used as a support for immobilization of lipase produced together with EPS, using new and simple, single step procedure. The highest EPS production (36.5 mg L^{-1}) was observed in the Luria Bertani (LB) medium supplemented with sunflower oil and Tween 80. The EPS structure was analyzed by FT-IR and ^{1}H NMR, and it was found that EPS from *P. aeruginosa* is of the alginate type. Lipase, produced by the same strain was entrapped in Ca–EPS beads, formed by dripping a concentrated culture supernatant in $CaCl_2$ solutions. Effects of immobilization conditions on loading efficiency and immobilization yield were investigated, and the potential for reuse of immobilized lipase was monitored. It was shown that the immobilized enzyme could be used for up to three reaction cycles, after which 77% of activity was retained.

1. Introduction

Microbial EPSs produced by various genera of bacteria and yeasts have found a wide range of applications in biotechnology and biopharmaceutical industries (Sutherland, 1998). Some examples of industrially important exopolysaccharides are dextran, xanthan, gellan, pullulan, yeast glucans and bacterial alginates. Bacterial alginates were secreted by *Pseudomonas* species, *Azotobacter vinelandi* and *Azotobacter chrococcum* (Celik, Aslim, & Beyatli, 2008). Alginates are very important, due to their ability to form gel with variety of cross-linking agents. These gels are used in various fields like food industry, medicine and biotechnological applications including cell and enzyme encapsulation and drug delivery. Gelation of alginate is possible via interaction of carboxylate groups with divalent ions. Calcium cations are commonly used cross-linkers. They have been reported to bind preferentially to the poly-guluronic acid units (GG) of alginate in a planar two-dimensional manner, producing the so-called "egg-box" structure (Pathak, Yun, Lee, Baek, & Paeng, 2009). Numerous data exists on the specific interaction of alginate and lipase from *Pseudomonas* (Mondal, Mehta, Mehta, Varandani, & Gupta, 2006; Sharma & Gupta, 2001). It is likely that the interaction of alginate with lipase enhances lipase activity. Considering this, we assumed that this specific interaction can be used for isolation, purification, stabilization and immobilization of lipase at the same time.

In this study, submerse production of EPS from *Pseudomonas aeruginosa* san-ai was monitored in media with various carbon sources. The EPS was isolated and its structure was analyzed by FT-IR and ^{1}H NMR. We also used the EPS as a matrix for simple, single step isolation, purification and immobilization of lipase produced together with EPS by the same strain (Karadzic, Masui, Izrael-Zivkovic, & Fujiwara, 2006). To the authors' best knowledge, it is the first time that a lipase is immobilized through this unique procedure.

2. Materials and methods

2.1. Culture conditions

2.1.1. Optimization of EPS production

Three different media were used for investigation of EPS production: LB medium, LB medium supplemented with 0.7% (w/v)

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sunflower oil and 0.1% (w/v) Tween 80 and LB medium supplemented with 0.7% (w/v) sunflower oil and 0.1% (w/v) Trition X-100. Cultivation was carried out for nine days at 30 °C with shaking (250 cycles/min). Each day aliquots were withdrawn, biomass was removed by centrifugation at 10,000 rpm for 10 min and supernatant was used for EPS quantification.

2.1.2. Time course of cultivation in optimal medium for EPS production

Cultivation was carried out in LB medium supplemented with 0.7% (w/v) sunflower oil and 0.1% (w/v) Tween 80 for nine days. Aliquots were withdrawn each day, biomass was removed by centrifugation and a supernatant was used for pH, O.D. (590 nm), protein, EPS and lipase determination.

2.2. Quantification of EPS

The concentration of EPS was measured by the phenol–sulphuric method (Dubois, Gilles, Hamilton, Peters, & Smith, 1956) using starch as a standard. The main values were calculated from the data obtained with pentaplicate trials.

2.3. Isolation and spectroscopic determination of EPS

EPS isolation was carried out as demonstrated (Kılıça & Dönmez, 2008). A dried exopolysaccharides sample (1 mg) was dispersed in 100 mg of anhydrous KBr and pressed in a palette. The IR spectrum was recorded at room temperature in the wave number range of 400–4000 cm^{-1} on a Nicolet 6700 FT-IR device. ^1H NMR spectrum was recorded in D_2O at room temperature by a Gemini 200 spectrometer of 200 MHz.

2.4. Lipase activity determination

Lipase activity was measured spectrophotometrically using an assay based on the hydrolysis of *p*-nitro phenyl palmitate (*p*NPP) (Pencreach & Baratti, 1996). One unit (1 U) was defined as the amount of enzyme that liberated 1 μmol of *para*-nitrophenol (*p*NP) per minute (ε = 1500 l/mol cm) under the test conditions.

2.5. Protein determination

The amount of protein was estimated by the dye-binding method (Bradford, 1976), using bovine serum albumin (BSA) as a standard.

2.6. Immobilization of lipase in Ca–EPS beads

After five days of cultivation in optimal medium, biomass was removed and the pH of the culture supernatant was adjusted to 4.5 by 1 M HCl solution. The solution was centrifuged for 10 min on 10,000 rpm at room temperature. The precipitate (containing both EPS and lipase) was dissolved in 1.5 M Tris buffer pH 8.8. Protein concentration of the concentrated culture supernatant was 1.37 mg mL^{-1}, lipase activity was 28 IU mL^{-1} and the EPS concentration was 1400.32 mg L^{-1}. The concentrated culture supernatant (V = 0.5 mL) was dripped with a syringe into 10 mL $CaCl_2$ solutions with different concentrations (100 mM to 1 M). After 12 h of hardening, the beads were removed from $CaCl_2$ solutions by filtration and washed four times with 5 mL of 50 mM Tris–HCl buffer, pH 8.0. The average bead size was measured by an optical microscope.

2.7. Loading efficiency determination

$CaCl_2$ solutions, obtained after beads removal, and the washings were collected and used for loading efficiency determination via the following equation:

$$\text{Loading efficiency}(\%) = \left(\frac{CiVi - CfVf}{CiVi}\right) \times 100 \quad (1)$$

where *Ci* is the initial lipase activity (lipase activity of the concentrated culture supernatant) (IU mL^{-1}), *Vi* is the initial volume of enzyme solution, *Cf* is the lipase activity in the total filtrate volume ($CaCl_2$ solution after immobilization + washings), and *Vf* is the total volume of the filtrate.

2.8. Determination of lipase activity of Ca–EPS beads

Hydrolytic activity of lipase was determined using a modified standard assay (Sorensen's phosphate buffer used in the standard assay was replaced by a Tris buffer pH 8.0). The reaction was started by adding about 10 mg of lipase-entrapped beads per 10 mL of substrate and carried out for 5 min. Specific activity of immobilized lipase was expressed as number of IU mg^{-1} of immobilized proteins. The immobilization yield was calculated via the following equation:

$$\text{Immobilization yield} = \left(\frac{a_{imm}}{a_{free}}\right) \times 100$$

where a_{imm} is the specific activity of immobilized lipase and a_{free} is specific activity of free lipase (Won, Kim, Kim, Park, & Moon, 2005).

2.9. Reuse of lipase immobilized in EPS beads

The immobilized lipase was exposed to the substrate, and the hydrolysis reaction lasted 30 min (end of the reaction) after which the beads were washed with 50 mM Tris–HCl buffer pH 8.0, followed by addition of a new portion of fresh substrate. The activity of freshly prepared beads in the first run was defined as 100%.

3. Results and discussion

3.1. EPS production and time course of cultivation

The *P. aeruginosa* san-ai strain naturally grows in mineral cutting oil, a mixture of mineral oils and surfactants (Karadzic, Masui, & Fujiwara, 2004), thus EPS production was monitored by the time course of submerged growth of the san-ai strain in three different media (with and without oil and detergents), as described in Section 2.1.1. It was shown that EPS production was increased by supplementation of sunflower oil and detergents into the medium as described by other authors (Kumar, Mody, & Jha, 2007; Park, Kim, Hwang, Cho, & Yun, 2002). The highest EPS production was reached in LB medium supplemented with sunflower oil and Tween 80 (36.5 mg L^{-1}) after five days of cultivation (Fig. 1). The mechanism by which the surfactants improve the EPS production may be that they interact with the cell membrane in a way that would enhance the polymerization process and/or by facilitating release of the polymer from the membrane (Arockiasamy & Banik, 2008).

Since the EPS concentration was the critical parameter for lipase entrapment (Bhushan, Parshad, Qazi, & Gupta, 2008), lipase production was monitored by the time course of submerged growth of san-ai in the medium in which the highest EPS production was achieved (Fig. 3), as described in Section 2.1.2. This medium is also commonly used for *Pseudomonas* lipases production (Gupta, Gupta, & Rathi, 2004). From the results shown in Fig. 3, it is obvious that the highest EPS concentration was reached in the stationary phase of bacterial growth (Freitas et al., 2010) in the fifth day of cultivation. From then on, the EPS concentration decreased. Lipase activity significantly increased after five days of cultivation. Since optical density significantly decreased after five days of cultivation, the lipase activity increase was the result of bacterial cell dying and

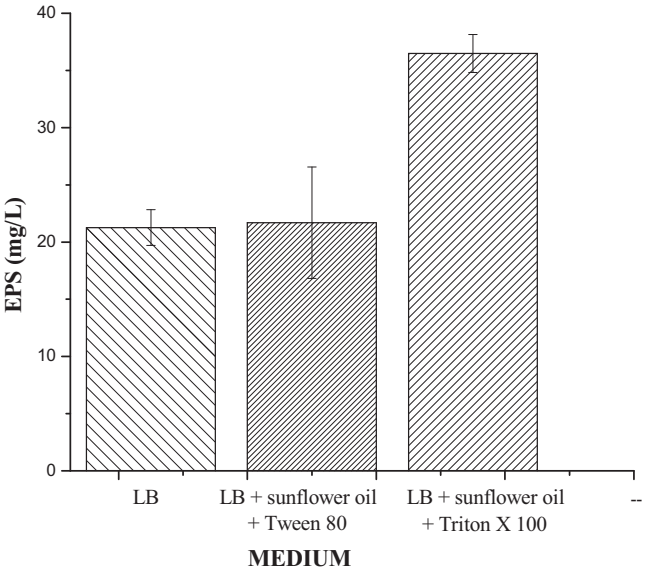

Fig. 1. Effect of different carbon sources on EPS production.

release of lipase molecules from the periplasm (Rosenau & Jaeger, 2000).

3.2. Structural consideration of EPS

The IR spectrum of EPS showed absorption bands at: 3388 cm^{-1} (OH stretching), 1657 cm^{-1} (COO^- asymmetric stretching), 1424 cm^{-1} (C–OH deformation vibration with contribution of the O–C–O symmetric stretching vibration of the carboxyl group), 1339, 1187 and 1046 cm^{-1} (C–C–H and O–C–H deformation, to C–O stretching vibrations and C–O and C–C stretching vibrations of pyranose rings, respectively), 958 cm^{-1} (uronic acid presence by the C–O stretching vibration), 860 and 805 cm^{-1} (α-L-gulopyranuronic asymmetric ring vibration and mannuronic acid residues, respectively). Thus, the FT-IR spectrum clearly indicated the presence of pyranose rings (α-L-gulopyranuronic) and mannuronic acid residues (Chandía, Matsuhiro, & Vásquez, 2001; Sakugawa, Ikeda, Takemura, & Ono, 2004). The FT-IR spectrum is shown in Fig. 2.

The signals in the anomeric region of the ^1H NMR spectra (A (4.95 ppm), B (4.80 ppm) and C (4.65 ppm)) contained specific information about the alginate composition and were assigned to the anomeric proton of G (L-gulopyranuronic acid residue) (A), the anomeric proton of M (mannuronic acid residue) and H-5 of G-units

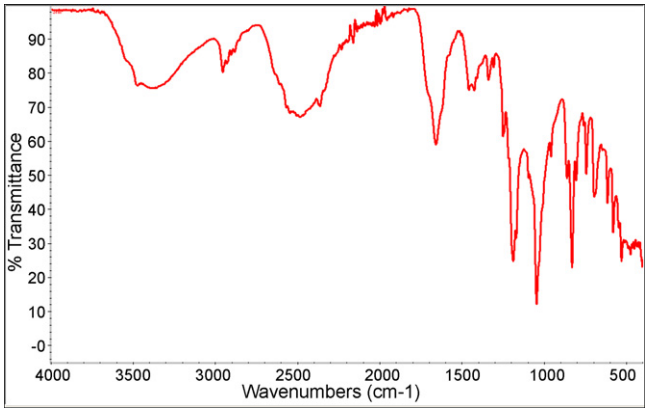

Fig. 2. FT-IR spectrum of *Pseudomonas aeruginosa* san-ai EPS.

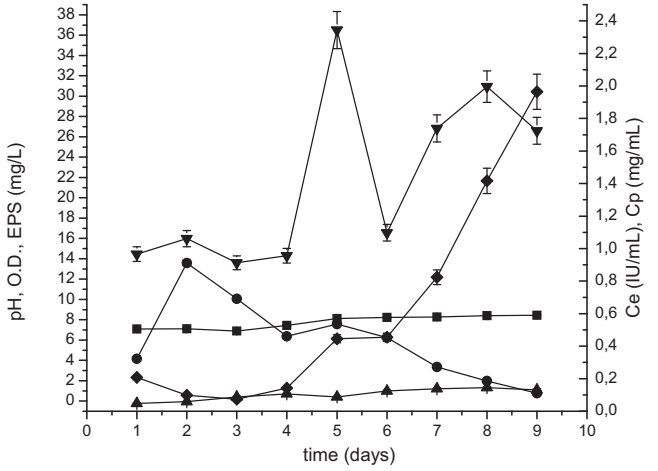

Fig. 3. Time course of *P. aeruginosa* san-ai cultivation in LB medium supplemented with sunflower oil and Tween 80. pH (■); O.D. (●); protein concentration Cp (▲); lipase concentration Ce (♦); EPS (▼).

adjacent to M (B), and H-5 of G-units adjacent to G (C) (results not shown).

From this spectrometric data it was concluded that EPS produced by *P. aeruginosa* san ai is of the alginate type EPS (Chen & Park, 2003; Franklin & Ohman, 1996; Salomonsen, Jensen, Stenbæk, & Engelsen, 2008).

3.3. Effect of $CaCl_2$ concentration on loading efficiency and immobilization yield

Loading efficiency and immobilization yield are defined as described in Sections 2.7 and 2.8, respectively. The specific activity of free lipase was 20.44 IU mg^{-1}. When the $CaCl_2$ concentration is increased from 100 mM to 1 M, the loading efficiency rises, while further increase of the $CaCl_2$ concentration over 1 M does not exhibit any significant effect on the loading efficiency (Fig. 4). This could be explained by saturation of the formed gel network with Ca^{2+} ions when an excess of Ca^{2+} ions is reached above the concentration of 1 M (Won et al., 2005).

The immobilization yield increases, even above 100% (Fig. 4), when the $CaCl_2$ concentration rises from 100 mM to 1 M, which

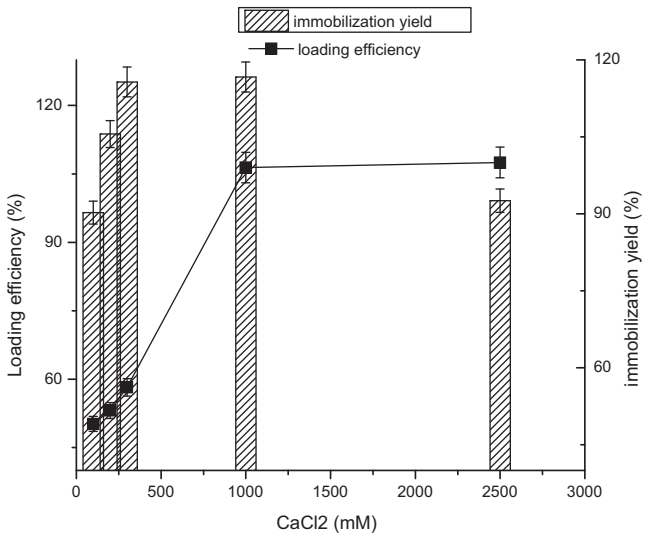

Fig. 4. Effects of $CaCl_2$ concentration on loading efficiency (■) and immobilization yield (bar).

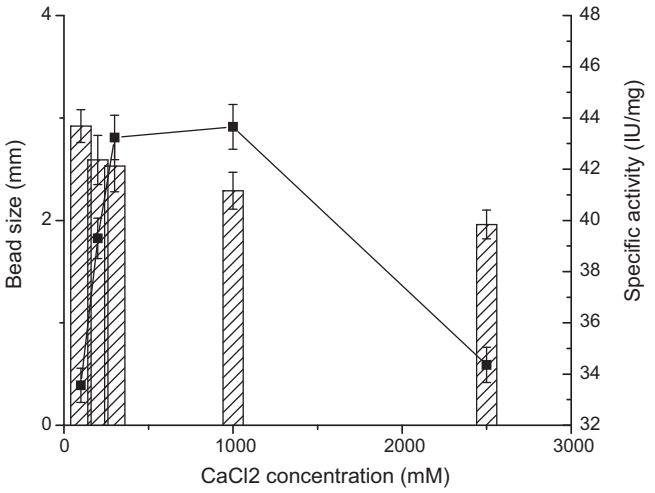

Fig. 5. Effect of bead size. Bead size (bars) and specific activity of immobilized enzyme, IU/mg of immobilized proteins (■).

was opposite from the results reported by other authors (Won et al., 2005). The unexpected rise of the immobilization yield was the consequence of enzyme purification that takes place simultaneously with enzyme immobilization, owing to specific interactions of lipase with EPS. Further increase in $CaCl_2$ concentration, above 1 M, causes saturation of lipase EPS beads, leading to entrapment of other proteins besides lipase in Ca–EPS beads, decrease of lipase specific activity in Ca–EPS beads, and immobilization yield.

3.4. Effect of bead size

In order to generate Ca–EPS beads of different size, a concentrated culture supernatant was dripped in solutions with different concentration of Ca^{2+} ions. Other workers proposed that the activity of immobilized enzyme decreases with increasing bead size due to mass transfer resistance (Fadnavis, Sheelu, Kumar, Bhalerao, & Deshpande, 2003; Knezevic et al., 2002). In this study it was shown that the average bead size (about 2.5 mm) did not vary considerably with changes of $CaCl_2$ concentration (Fig. 5). Therefore, changes of lipase specific activity (Fig. 5) are rather the consequence of enzyme purification, as in the case of immobilization yield.

3.5. Repeated use of lipase-entrapped beads

This study has shown that immobilized lipase could be used for up to three cycles with little loss of activity (77% of the initial activity was retained (results not shown)) and without the need for further stabilization. Significant activity loss (35% of retained activity after three cycles and complete loss of activity after four cycles) was reported by other authors (Anwar, Qader, Raiz, Iqbal, & Azhar, 2009). Also further stabilization of the beads was necessary in previous reports (Fadnavis et al., 2003). However, after three reaction cycles, loss of activity became pronounced. Activity loss might be explained by enzyme leakage from the exopolysaccharide gel beads or by damaging of the beads during repeated use as described by other authors (Knezevic et al., 2002). In order to overcome these problems, the bead strength can be efficiently enhanced by coating of Ca–EPS beads with chitosan or silicate (Won et al., 2005) or by treatment with glutaraldehyde with various concentrations and treatment times (Bandhyopadhyay, Das, & Maiti, 1999).

4. Conclusions

For the first time EPS produced by *P. aeruginosa* san-ai, found to be of the alginate type, was used as a support for immobilization of lipase produced by the same strain at the same time. This novel approach in lipase immobilization is cost-effective as well as time-saving. Since they naturally occur together, EPS and lipase have a stability that enables repeated use of entrapped lipase without further stabilization of the preparation. This procedure, based on the use of naturally occurring polymers, can be a good example of how to simplify and economize the application of immobilized lipases in future research.

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