Ann Microbiol (2010) 60:763–766
DOI 10.1007/s13213-010-0115-3

SHORT COMMUNICATIONS

Pullulan production by *Aureobasidium pullulans* cells immobilized on ECTEOLA-cellulose

Thomas P. West

Received: 9 May 2010 / Accepted: 29 July 2010 / Published online: 13 August 2010

Abstract Cells of the fungus *Aureobasidium pullulans* ATCC 42023 were immobilized by adsorption on the ion-exchange resin ECTEOLA (epichlorohydrin triethanolamine)-cellulose and the immobilized cells were examined for their ability to produce the polysaccharide pullulan using batch fermentation. It was found that the cells immobilized on the ECTEOLA-cellulose at pH 2.0 produced higher pullulan levels than those cells immobilized at pH 3.0, 4.0, 5.0, 6.0 and 7.0 after 72 h at 30°C. The pH 2.0-immobilized cells were capable of producing pullulan for 2 cycles of 168 h. Pullulan production by the immobilized cells decreased slightly during the second production cycle but the difference in production was not statistically significant after 168 h.

Keywords Pullulan · Immobilization · Ion-exchanger · Adsorption · *Aureobasidium*

Introduction

The fungus *Aureobasidium pullulans* synthesizes the extracellular polysaccharide pullulan from various carbon sources (Bernier 1958; Ueda et al. 1963; Catley 1971). The structure of the polysaccharide involves cross-linked maltotriose and maltotetraose residues (Sowa et al. 1963; Catley 1970; Taguchi et al. 1973; Zajic and LeDuy 1973; Catley et al. 1986). A number of potential commercial applications exist for pullulan including use as a food additive, a flocculant, a packaging film, a blood plasma substitute, an adhesive and a dielectric material (Zajic and LeDuy 1977; Singh et al. 2008).

T. P. West (✉)
Department of Biology and Microbiology,
South Dakota State University,
Brookings, SD 57007, USA
e-mail: Thomas.West@sdstate.edu

Prior studies have examined the immobilization of *A. pullulans* cells by adsorption on solid supports as well as by entrapment. Sucrose-grown cells of *A. pullulans* strain 2552 have been immobilized on the solid support diatomaceous earth and were shown to be capable of producing pullulan (Mulchandani et al. 1989). Other studies have shown that corn syrup-grown cells of *A. pullulans* ATCC 42023 could be immobilized using diatomaceous earth, diethylaminoethyl-cellulose or sponge cubes (West and Strohfus 1996a, b). Cell immobilization using the sponge cubes appeared to be a particularly effective method for several cycles of polysaccharide production (West and Strohfus 1996b). Entrapment of *A. pullulans* cells in polyurethane foam or in a plastic composite support has been reported with the immobilized cells shown to produce pullulan (Mulchandani et al. 1989; Cheng et al. 2010). Although a composite agar layer-microporous membrane system devised to immobilize *A. pullulans* glucose-grown cells did not prove feasible for pullulan production (Lebrun et al. 1994), other studies have shown that agar-entrapped or alginate-entrapped *A. pullulans* cells could be used for polysaccharide production (West 2000; West and Strohfus 2001; Urkut et al. 2007).

In the present work, pullulan production by corn syrup-grown cells of *A. pullulans* ATCC 42023 immobilized on the ion-exchange resin ECTEOLA (epichlorohydrin triethanolamine)-cellulose was studied using batch fermentation. The reusability of these fungal cells immobilized on ECTEOLA-cellulose for batch pullulan production was also examined.

Materials and methods

Aureobasidium pullulans ATCC 42023 was the strain utilized in this work (Zajic and LeDuy 1973). The culture medium was prepared as described previously (West and

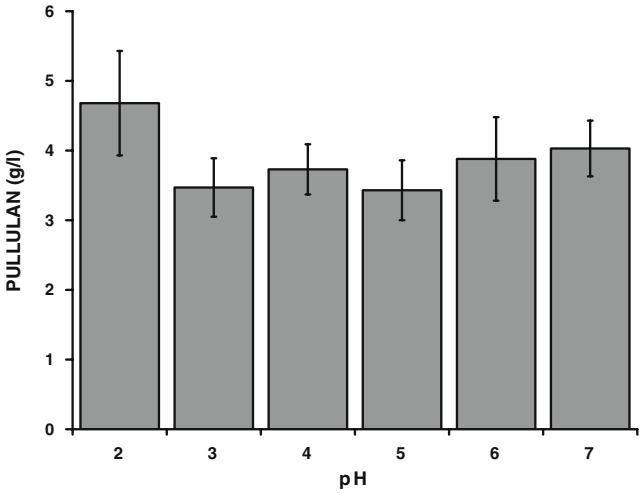

Fig. 1 Comparison of pullulan levels (g/l) produced by *Aureobasidium pullulans* ATCC 42023 cells adsorbed at pH 2-7 after 72 h at 30°C. Error bars indicate the standard deviations of mean data values

Reed-Hamer 1991). The medium contained corn syrup as the carbon source at a final concentration of 2.5% (w/v) while the nitrogen source was ammonium sulfate at a final concentration of 0.06% (w/v). Batch cultures (50 ml) were inoculated using overnight cultures (0.5 ml) grown in the same culture medium (pH 6.0). Each batch culture was shaken at 200 rpm for 48 h at 30°C.

ECTEOLA-cellulose (1 g) was initially pretreated by sequentially washing with 200 ml of 0.25 N HCl and 0.25 N NaOH in a sterile 250 ml Erlenmeyer flask. The ion-exchange resin was subsequently resuspended in 200 ml of 0.5 N HCl overnight to sterilize it (Bar et al. 1986, 1987). After the resin was washed at least three times with sterile water (200 ml) and the water drained, the resin was ready for use. The ECTEOLA-cellulose was suspended in 50 ml of culture medium (pH 2.0, 3.0, 4.0, 5.0, 6.0 or 7.0) containing 2.5% (w/v) corn syrup. The suspension was inoculated with approximately 10^5 fungal cells/ml from 48 h batch cultures and each culture was shaken at 100 rpm for 48 h at 30°C. The ion-exchange resin with the adsorbed fungal cells was collected by low speed centrifugation and washed twice with 0.85% NaCl. The resin was suspended in 50 ml of culture medium (pH 6.0) containing 2.5% (w/v) corn syrup. Pullulan production by these cultures was quantified following 72 h of shaking (125 rpm) at 30°C.

Pullulan production by fungal cells immobilized at pH 2.0 on ECTEOLA-cellulose was measured daily after the immobilized cells were suspended in 50 ml culture medium (pH 6.0) cultures containing 2.5% (w/v) corn syrup and then shaken for 168 h at 30°C. Following the initial cycle of 168 h, the solid support was collected by low-speed centrifugation, washed and again suspended in culture medium (pH 6.0) containing 2.5% (w/v) corn syrup. The cultures were shaken (125 rpm) for a second cycle of 168 h at 30°C during which the pullulan concentration was determined daily.

Samples of culture medium (2 ml) were removed and centrifuged at 14,600 × *g* for 30 min at 4°C. The resultant supernatant was used during the pullulan determinations. To quantitate pullulan levels, two volumes of 95% ethanol were added to one volume of the pullulan-containing supernatant to precipitate the pullulan in the supernatant. The precipitated pullulan was collected on 0.45 μm HVLP filters (25 mm diameter). All filters were dried to constant weight at 105°C and reweighed to determine pullulan concentrations (West and Reed-Hamer 1993).

Samples of the ion-exchange resin with the adsorbed cells were collected under aseptic conditions, resuspended in sterile 0.85% NaCl solution (5 ml) and agitated using a vortex mixer. The viable fungal cell concentration was determined by measuring the number of colony-forming units on potato dextrose agar plates. The samples of ion-exchange resin were collected by filtration on 0.45 μm HVLP filters (47 mm diameter). All filters were dried to constant weight at 105°C and reweighed to determine the dry weights of the supports.

Pullulan concentrations were expressed as g/l and represent the mean of three independent determinations. The Student's *t*-test was used during statistical analysis.

Results and discussion

The influence of culture medium pH on fungal cell adsorption onto the ion exchange resin ECTEOLA-cellulose was initially investigated. The culture medium pH used to adsorb the *A. pullulans* ATCC 42023 cells onto the resin ranged from 2.0 to 7.0. It was observed that cells adsorbed onto ECTEOLA-

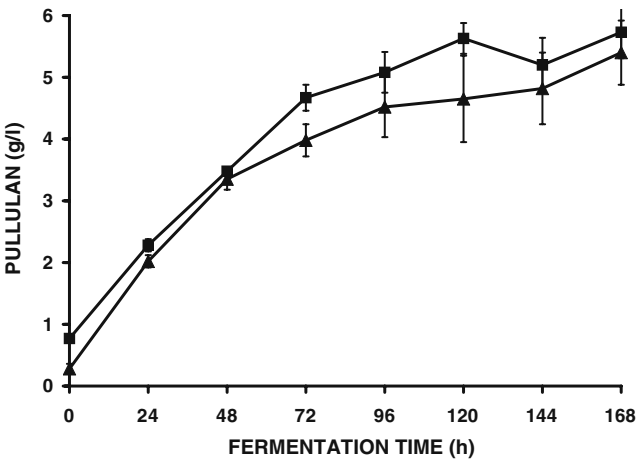

Fig. 2 Pullulan levels (g/l) produced by pH 2.0-adsorbed *Aureobasidium pullulans* ATCC 42023 cells on ECTEOLA-cellulose over a period of 168 h at 30°C during cycle 1 (■) or cycle 2 (▲). Error bars indicate the standard deviations of mean data values

cellulose at pH 2.0 produced the highest pullulan level after 72 h when incubated in the medium (pH 6.0) containing 2.5% corn syrup (Fig. 1). The pH 2.0-adsorbed ATCC 42023 cells produced a higher level of pullulan after 72 h than did cells adsorbed at pH 3.0, 4.0, 5.0, 6.0 or 7.0 (Fig. 1). A statistically significant ($P<0.05$) difference in pullulan production was only noted between the cells adsorbed at pH 2.0 and the cells adsorbed at pH 3.0, 4.0 or 5.0. Pullulan production by the pH 5.0-adsorbed cells was lowest after 72 h (Fig. 1). The concentration of ATCC 42023 cells adsorbed at pH 2.0 onto the ECTEOLA-cellulose was determined to be $1.6\times10^5\pm1.2\times10^5$ (mean ± standard deviation) colony-forming units/mg dry weight of resin. A statistically significant difference between the concentration of pH 2.0-adsorbed cells and the cell concentrations adsorbed at the other pH values tested was not observed.

With the highest pullulan level being produced by the pH 2.0-immobilized cells, it was of interest to learn whether the adsorbed cells incubated in medium (pH 6.0) containing 2.5% corn syrup could be used for two cycles of pullulan production for 168 h using batch fermentation. As can be seen in Fig. 2, pullulan production by the pH 2.0-adsorbed cells produced pullulan after 24 h and continued to produce it for 120 h during the initial cycle of production. Pullulan production by the immobilized cells appeared to be increased little after 120 h (Fig. 2). During the second cycle of production by the pH 2.0-adsorbed cells, pullulan production again occurred after 24 h of incubation in the medium (pH 6.0) containing 2.5% corn syrup and pullulan production continued during the entire cycle (Fig. 2). After 144 h of pullulan production during the second cycle, there was no statistical difference in pullulan production during the second production cycle compared to the initial production cycle. This indicated pullulan production by the immobilized cells was equally effective for two cycles of production for 168 h.

A prior study examined using the ion-exchange resin diethylaminoethyl-cellulose to immobilize *A. pullulans* ATCC 42023 cells and the ability of the immobilized cells to produce pullulan using batch fermentation (West and Strohfus 1996a). Similar to this study, it was reported that ATCC 42023 cells adsorbed onto diethylaminoethyl-cellulose at pH 2.0 produced the highest concentration of polysaccharide after 72 h of incubation in the medium (pH 6.0) containing 2.5% corn syrup (West and Strohfus 1996a). Unlike the findings of this study, pullulan production by the pH 7.0-adsorbed cells was lowest after 72 h (Fig. 1). When pullulan production by ATCC 42023 cells immobilized on diethylaminoethyl-cellulose at pH 2.0 was examined for a cycle of 168 h in a medium (pH 6.0) containing 2.5% corn syrup, it was found that the highest pullulan level occurred after 120 h (West and Strohfus 1996a). The second cycle of pullulan production for 168 h resulted in lower levels of polysaccharide compared to the first production cycle. During the second production cycle, the highest pullulan level also occurred after 120 h (West and Strohfus 1996a). Cells of a mutant strain of ATCC 42023 have been immobilized on the ion-exchange resin phosphocellulose and examined for their ability to produce pullulan using batch fermentation (West and Strohfus 1996c). It was found that these cells adsorbed onto phosphocellulose at pH 2.0 produced the highest concentration of polysaccharide after 72 h of incubation in the medium (pH 6.0) containing 2.5% corn syrup (West and Strohfus 1996c). Pullulan production by the phosphocellulose-adsorbed cells after the initial cycle of 168 h was lower than that noted for the ATCC 42023 cells adsorbed on ECTEOLA-cellulose or diethylaminoethyl-cellulose (West and Strohfus 1996a, c). The second cycle of pullulan production for 168 h by the phosphocellulose-adsorbed cells was lower than the level of production during the initial production cycle (West and Strohfus 1996c). The second 168 h cycle of pullulan production by the phosphocellulose-adsorbed cells was also lower compared to the level of pullulan production witnessed during the second production cycle of the ATCC 42023 cells adsorbed on ECTEOLA-cellulose or diethylaminoethyl-cellulose (West and Strohfus 1996a, c).

In conclusion, the ion-exchange resin ECTEOLA-cellulose is an effective adsorption agent to immobilize *A. pullulans* ATCC 42023 cells. The immobilized cells could be utilized to produce pullulan for two production cycles of 168 h using batch fermentation with only a slight decrease in pullulan production occurring during the second production cycle.

Acknowledgements Financial support of this work was provided by the South Dakota Agricultural Experiment Station and USDA Grant No. 94-37501-0884. The technical assistance of Beth Nemmers was greatly appreciated.

References

Bar R, Gainer JL, Kirwan DJ (1986) Immobilization of *Acetobacter aceti* on cellulose ion exchangers: adsorption isotherms. Biotechnol Bioeng 28:1166–1171

Bar R, Gainer JL, Kirwan DJ (1987) Ethanol fermentation by ionically adsorbed *Zymomonas mobilis*: environmental effects on cell immobilization. Biotechnol Bioeng 28:1166–1171

Bernier B (1958) The production of polysaccharides by fungi active in the decomposition of wood and forest litter. Can J Microbiol 4:195–204

Catley BJ (1970) Pullulan, a relationship between molecular weight and fine structure. FEBS Lett 10:190–193

Catley BJ (1971) Utilization of carbon sources by *Pullularia pullulans* for the elaboration of extracellular polysaccharides. Appl Microbiol 22:641–649

Catley BJ, Ramsay A, Servis C (1986) Observations on the structure of the fungal extracellular polysaccharide, pullulan. Carbohydr Res 153:79–86

Cheng KC, Demirci A, Catchmark JM (2010) Effects of plastic composite support and pH profiles on pullulan production. Appl Microbiol Biotechnol 86:853–861

Lebrun L, Junter G-A, Jouenne T, Mignot L (1994) Exopolysaccharide production by free and immobilized microbial cultures. Enzyme Microbiol Technol 16:1048–1054

Mulchandani A, Luong JHT, LeDuy A (1989) Biosynthesis of pullulan using immobilized *Aureobasidium pullulans*. Biotechnol Bioeng 33:306–312

Singh RS, Saini GK, Kennedy JF (2008) Pullulan: microbial sources, production and applications. Carbohydr Polym 73:515–531

Sowa W, Blackwood AC, Adams GA (1963) Neutral extracellular glucan of *Pullularia pullulans* (de Bary) Berkhout. Can J Chem 41:2314–2319

Taguchi R, Kikuchi Y, Sakano Y, Kobayashi T (1973) Structural uniformity of pullulan produced by several strains of *Pullularia pullulans*. Appl Microbiol 25:628–635

Ueda S, Fujita K, Komatsu K, Nakashima Z (1963) Polysaccharide produced by the genus *Pullularia*. I. Production of polysaccharide by growing cells. Appl Microbiol 11:211–215

Urkut Z, Dagbagli S, Goksungur Y (2007) Optimization of pullulan production using Ca-alginate-immobilized *Aureobasidium pullulans* by response surface methodology. J Chem Technol Biotechnol 82:837–846

West TP (2000) Exopolysaccharide production by entrapped cells of the fungus *Aureobasidium pullulans* ATCC 201253. J Basic Microbiol 40:397–401

West TP, Reed-Hamer B (1991) Ability of *Aureobasidium pullulans* to synthesize pullulan upon selected sources of carbon and nitrogen. Microbios 67:117–124

West TP, Reed-Hamer B (1993) Polysaccharide production by a reduced pigmentation mutant of the fungus *Aureobasidium pullulans*. FEMS Microbiol Lett 113:345–349

West TP, Strohfus BRH (1996a) Use of adsorption in immobilizing fungal cells for pullulan production. Microbios 85:117–125

West TP, Strohfus BRH (1996b) Polysaccharide production by sponge-immobilized cells of the fungus *Aureobasidium pullulans*. Lett Appl Microbiol 22:162–164

West TP, Strohfus B (1996c) Fungal cell immobilization on ion exchange resins for pullulan production. Microbios 88:177–187

West TP, Strohfus B (2001) Polysaccharide production by immobilized *Aureobasidium pullulans* cells in batch bioreactors. Microbiol Res 156:285–288

Zajic JE, LeDuy A (1973) Flocculant and chemical properties of a polysaccharide from *Pullularia pullulans*. Appl Microbiol 25:628–635