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Pullulan production from deproteinized whey by *Aureobasidium pullulans*

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The production of pullulan from deproteinized whey by *Aureobasidium pullulans* P56 was investigated using an adaptation technique and a mixed culture system. The adaptation of *A. pullulans* and the mixed cultures of *A. pullulans* and/or *Lactobacillus brevis* X20, *Debaryomyces hansenii* 194 and *Aspergillus niger* did not increase the production of polysaccharide. Enzymic hydrolysis of lactose in deproteinized whey gave a higher polysaccharide concentration and polysaccharide yield than acidic hydrolysed lactose. Maximum polysaccharide concentration (11.0 ± 0.5 g L^{-1}), biomass dry weight (10.5 ± 0.4 g L^{-1}), polysaccharide yield (47.2 ± 1.8%) and sugar utilization (93.2 ± 2.8%) were achieved using enzyme-hydrolysed whey (pH 6.5) containing 25 g L^{-1} lactose and supplemented with K_2HPO_4 0.5%, L-glutamic acid 1%, olive oil 2.5%, and Tween 80 0.5%. In this case the pullulan content of the crude polysaccharide was 40%.

Keywords: pullulan; deproteinized whey; *Aureobasidium pullulans*; mixed culture; shake flask culture

Introduction

Cheese whey is a clean, wholesome, abundant food-grade material and a potential environmental pollutant. It is the product separated from milk during cheese making and consists of water, lactose (4–5%), proteins, vitamins, and mineral salts [14]. Pullulan is an extracellular water-soluble microbial polysaccharide produced by strains of *Aureobasidium pullulans.* It is a linear mixed linkage α-**d**-glucan consisting mainly of maltotriose units interconnected via α-(1 → 6) linkages. A number of potential applications have been reported for this biopolymer as a result of its good film-forming properties; pullulan can form thin films which are transparent, oil resistant and impermeable to oxygen. Pullulan may be used as a coating and packaging material, as a sizing agent for paper, as a starch substitute in low-calorie food formulations, in cosmetic emulsions, and in other industrial and medicinal applications [3].

The production of pullulan from a chemically defined medium by different strains of *A. pullulans* has been described [6,16,19] However, utilization of sucrose or glucose as carbon source is not economical, and a less expensive carbohydrate source would be beneficial. LeDuy *et al* [10] studied the production of pullulan from lactose by coculture of *A. pullulans* and *Ceratocystis ulmi*, while Israilides *et al* [7] used beet molasses to produce pullulan by *A. pullulans* NRRLY-6220. LeDuy and Boa [9] and Leathers and Gupta [8] employed peat hydrolysate and fuel ethanol byproducts, respectively, as fermentation substrates for pullulan production. Recently, a considerable interest has been shown in using agricultural crops such as Jerusalem artichoke tubers and carob pods for polysaccharide production [13,20]. Pullulan production from deproteinized whey by *A. pullulans* has not been investigated.

The aim of this investigation was to examine whey as a potential source for pullulan production by *A. pullulans*. *A. pullulans* does not possess constitutive enzymes for the fermentation of lactose [10]. In order to overcome this problem three different techniques were used: the culture adaptation technique, mixed cultures of *A. pullulans* with other microorganisms which produce β-galactosidase for the hydrolysis of lactose, and acidic or enzymic hydrolysis of lactose with concentrated HCl or β-galactosidase.

Materials and methods

Microorganisms and culture conditions

Aureobasidium pullulans P56, a strain deficient in melanin production, was kindly supplied by Professor A Mersmann of the Technical University of Munich, Germany. The strains *Lactobacillus brevis* X20 and *Debaryomyces hansenii* 194 were provided from our institute culture collection. *A. pullulans* was maintained on potato dextrose agar plates at 4°C and subcultured every 3 weeks. Cells for inoculation of the culture medium were obtained from cultures grown on potato dextrose agar plates at 28°C for 48 h. From the petri dish, two loops of *A. pullulans* cells were transferred to 500-ml conical flasks containing 100 ml of culture medium (pH 5.5) of the following composition (g L^{-1}): sucrose 30, $(NH_4)_2SO_4$ 0.6, yeast extract 0.4, K_2HPO_4 5, $MgSO_4{\cdot}7H_2O$ 0.2, and NaCl 1. The flasks were incubated at 28°C for 48 h in a rotary shaker incubator (Lab Line Orbit-Environ Shaker, Lab-Line Instr, Melrose Park, IL, USA) at 200 rpm. These cultures were used to inoculate the production medium at a level of 5% (v/v).

The culture adaptation technique was carried out in 500-ml conical flasks containing 100 ml culture medium (pH 5.5) which had the composition described above, but instead of 3% sucrose it contained 5% lactose. The flasks were inoculated with 5 ml of the inoculum and incubated

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under the conditions described above. A 7-day-old culture was used to inoculate the fresh medium at a ratio of 5% (v/v). This procedure was repeated 10 times. Five milliliters of the final culture were used to inoculate 100 ml of deproteinized whey for the production of polysaccharide. *Lactobacillus brevis* X20 and *Debaryomyces hansenii* 194 were propagated in MRS broth (Merk) and MYGP medium (malt extract 0.3%, yeast extract 0.3%, glucose 1%, and peptone 0.5%), respectively at 30°C for 48 h. *Aspergillus niger* ATCC 9142 was grown on potato dextrose agar slants at 30°C for 5 days. Spores were suspended in 5 ml sterile distilled water and shaken vigorously for 1 min. The above cultures were used to inoculate the medium at a ratio of 2% (v/v).

Treatment of whey

Cheese whey was obtained from a local feta cheese plant. It contained 5% (w/v) lactose and had a pH of 5.5. Protein precipitation was induced by heating the whey at 90°C for 20 min. Precipitated proteins were removed by centrifugation at $5000 \times g$ for 15 min. One liter of the supernatant (pH 6.5) was hydrolysed with 2.0 ml of β-galactosidase (C Hansen's Ha-Lactase, Horsholm, Denmark) at 40°C for 5 h in a rotary shaker/incubator at 120 rpm. After hydrolysis, the solution was sterilized at 121°C for 20 min. In the case of acidic hydrolysis, the pH of the supernatant was adjusted to 1.5 with concentrated HCl and the medium was heated at 121°C for 30 min. After cooling the medium, its pH was adjusted to 6.5 with 10 N NaOH.

Study of fermentation parameters

Single- and mixed-cultures: One-hundred milliliters of deproteinized whey (pH 6.5) were inoculated with 5 ml of the single culture or the adapted *A. pullulans* or the mixed cultures of *A. pullulans* and/or *L. brevis*, *D. hansenii* and *A. niger* at a ratio of 5% (v/v) *A. pullulans* and 2% (v/v) the other cultures. The fermentation was carried out at 30°C in a rotary shaker/incubator at 200 rpm for 10 days.

A series of conical flasks containing 100 ml acidic or enzymic hydrolysed whey (50 or 25 g L^{-1} initial lactose, pH 6.5) were inoculated with 5 ml *A. pullulans* and incubated as described above.

Nutrients: A set of experiments was performed in conical flasks at different concentrations of nutrients, to investigate the effect of supplements on pullulan production. The flasks containing 100 ml hydrolysed whey (enzymic hydrolysed, 25 g L^{-1} lactose, pH 6.5) were supplied with K_2HPO_4, l-glutamic acid, olive oil and Tween 80 at various concentrations. The flasks were inoculated with 5 ml of the inoculum and incubated at 30°C in a rotary shaker/incubator at 200 rpm for 10 days.

Initial pH: A series of conical flasks containing 100 ml hydrolysed whey (enzymic hydrolysed, 25 g L^{-1} lactose) were supplemented with 0.5 g K_2HPO_4, 1 g l-glutamic acid, 2.5 ml olive oil, and 0.5 ml Tween 80. The pH of the medium was adjusted to 4.5, 5.5, 6.5 or 7.5 with HCl or NaOH and the substrate was inoculated with 5 ml of the inoculum. The flasks were incubated under the conditions described above.

Analytical techniques

At specific time intervals, the flasks were removed and the fermentation broth was analyzed. Total biomass (mycelial and yeast cells) dry weight was determined by centrifugation of the fermentation broth at $10\,000 \times g$ for 20 min, washing the sediment with distilled water, and drying it at 105°C overnight. The first supernatant was combined with the washing, and the polysaccharide was precipitated with 2 volumes of ethanol at 4°C for 1 h. The precipitate was filtered through a pre-weighed whatman GF/A filter and dried at 105°C overnight. Residual sugars as glucose were determined in the filtrate according to Dubois *et al* [4]. Polysaccharide yield was expressed as polysaccharide per 100 g of sugar consumed, whereas sugar utilization was taken as a ratio of sugar consumed over the total amount of added sugar multiplied by 100.

Each experiment was repeated three times and the results were reported as averages ±SD of three repetitions.

In the case of the addition of nutrients, the pure pullulan content of the crude polysaccharide was determined. When the maximum concentration of polysaccharide was observed, 100 ml of the fermentation broth was centrifuged at $10\,000 \times g$ for 20 min. Polysaccharide was precipitated from the supernatant with two volumes of ethanol. After drying the precipitate, it was resuspended in 0.05 M sodium acetate (pH 5.0) at a concentration of 1 mg ml^{-1}. To a 1-ml sample, 0.1 U ml^{-1} Pullulanase (Sigma, EC 3.2.1.41) was added and the mixture was incubated for 21 h at 25°C [21]. Enzyme was also added to a pure sample of pullulan (Sigma, P-4516) of the same concentration as described above. The glucose equivalents were quantitated using the reducing sugar assay of Dygert *et al* [5] and the pure pullulan content of the crude polysaccharide was determined.

Results and discussion

Polysaccharide production from deproteinized whey

The production of polysaccharide from deproteinized whey by monoculture, adaptation, and mixed culture techniques is shown in Table 1. In all culture systems the polysaccharide concentration and the sugar utilization were very low (up to 2.8 g L^{-1} and 20%, respectively). This may be explained by the fact that the activity of β-galactosidase produced by *A. pullulans*, by the adaptation technique, and by the mixed culture system was unsatisfactory for the hydrolysis of lactose. Thus, the polysaccharide concentration was low due to insufficient accumulation of utilizable sugar. LeDuy *et al* [10] who studied the production of polysaccharide from lactose by adaptation of different strains of *A. pullulans* found that strains 142 and 140B did not increase the production of polysaccharide, while strain 2552 increased the concentration of polysaccharide from 0.05 to 10.5 g L^{-1}. The same authors found that coculture of *A. pullulans* and *Ceratocystis ulmi* produced 15.5 g L^{-1} polysaccharide in lactose medium. With a mixed culture of *A. pullulans* and *Kluyveromyces fragilis*, Shin *et al* [20] obtained a polysaccharide concentration of 17.5 g L^{-1} and 15.5 g L^{-1} using inulin and jerusalem artichoke extracts as

Table 1 Polysaccharide production from deproteinized whey by monoculture[a], adaptation[b] and mixed culture[c]. Each value is the mean ± SD of three repetitions (fermentation time 10 days)

Culture	Polysaccharide ($g L^{-1}$)	Biomass dry wt ($g L^{-1}$)	Polysaccharide yield (%)	Sugar utilization (%)
A. pullulans	2.0 ± 0.2	7.0 ± 0.5	28.5 ± 1.7	14.0 ± 0.7
Adaptation of *A. pullulans*	2.8 ± 0.1	7.5 ± 0.5	28.0 ± 1.8	20.0 ± 1.2
A. pullulans and *L. brevis* X20	2.3 ± 0.2	6.5 ± 0.4	35.4 ± 2.0	13.0 ± 0.8
A. pullulans and *D. hansenii* 194	2.0 ± 0.1	4.8 ± 0.2	66.7 ± 3.0	6.0 ± 0.3
A. pullulans and *A. niger*	2.0 ± 0.1	5.7 ± 0.3	50.0 ± 2.5	8.0 ± 0.4

[a]*A. pullulans*.
[b]Adaptation of *A. pullulans* after 10 repeated inoculations.
[c]*A. pullulans* and *L. brevis* X20, *A. pullulans* and *D. hansenii* 194 or *A. pullulans* and *A. niger*.

carbon sources. These results indicate that the production of polysaccharide depends on the strain used and the chemical composition of the substrate.

Polysaccharide production from hydrolysed whey

To enhance the production of polysaccharide from whey, acidic or enzymic hydrolysis of lactose was required. As shown in Table 2, the enzymic treatment resulted in a slightly better polysaccharide concentration compared to acidic hydrolysis (6.0 *vs* 5.0 $g L^{-1}$). On the other hand, the acidic treatment gave higher biomass concentration and sugar utilization. This was due to differences in substrate availability and salt concentration. Acid hydrolysis might cause degradation of various substances of the whey and formation of nutrient ingredients. Microbial growth could also be stimulated by the presence of a low salt (NaCl) concentration [15] formed during the pH adjustment. The polysaccharide concentration remained constant as initial lactose concentration increased, while the biomass concentration increased as the lactose concentration increased (Table 2). On the other hand, the polysaccharide yield and the sugar utilization were higher in the culture grown at an initial lactose concentration of 25 $g L^{-1}$ than those grown at 50 $g L^{-1}$ of lactose. These observations may imply the presence of inhibitory factors in the whey (possibly residual proteins, proteose or peptides, which are not precipitated during heating of the whey) that exert a detrimental effect on biosynthesis of extracellular glucan when present at high concentration. Roukas and Biliaderis [13], Seviour and Kristiansen [17], Bulmer et al [2], and Auer and Seviour [1] found that the addition of different nitrogen sources in the medium (at a concentration higher than 0.6 $g L^{-1}$ led to a reduction in polysaccharide concentration and an increase in biomass. There are several possible explanations for the action of the ammonium ion. According to Seviour and Kristiansen [17], the cells may become irreversibly committed, in response to high initial ammonium levels, to a continued carbon flow into biomass rather than polysaccharide synthesis. Auer and Seviour [1] further suggested that both type and concentration of the nitrogen source may be important in regulating key enzyme systems involved in nitrogen assimilation, thereby affecting the pool of intracellular metabolites. The latter, acting as allosteric effectors of carbon flow in the cells, could divert cell metabolism toward growth or polysaccharide synthesis. Roukas and Biliaderis [13] reported a 6 $g L^{-1}$ polysaccharide concentration from carob pod extract in shake flask culture, whereas in similar experiments by LeDuy and Boa [9], maximum polysaccharide levels of 12–14 $g L^{-1}$ were found for strains of *A. pullulans* grown in peat hydrolysates. Leathers and Gupta [8] reported that a maximum concentration of 4.5 $g L^{-1}$ polysaccharide was obtained when *Aureobasidium* sp strain NRRL Y-12,974 was grown in fuel ethanol byproducts in batch culture. Several factors could account for the variation in yield reported in these studies; these include the strain of microorganism, the chemical composition and any impurities present in the medium, the fermentation system, and the conditions employed during fermentation. Generally, the results of these studies showed that maximal polysaccharide concentration (6 $g L^{-1}$) was obtained from enzyme-hydrolysed whey containing 25 $g L^{-1}$ initial lactose.

Effect of added nutrients

As shown in Table 3, all the fermentation parameters increased with the addition of nutrients. The polysaccharide concentration increased significantly from 6.0 to 11.0 $g L^{-1}$ when the medium was supplemented with K_2HPO_4, l-glutamic acid, olive oil and Tween 80. The increase of polysaccharide concentration may be explained by the fact that the addition of glutamic acid, olive oil and Tween 80 suppressed the morphogenetic shift from yeast-like cells to

Table 2 Kinetic parameters describing fermentation of acid- or enzyme-hydrolysed deproteinized whey by *A. pullulans*. Each value is the mean ± SD of three repetitions (fermentation time 10 days)

Substrate	Polysaccharide ($g L^{-1}$)	Biomass dry wt ($g L^{-1}$)	Polysaccharide yield (%)	Sugar utilization (%)
Acid-hydrolysed whey (5% lactose)	5.0 ± 0.3	15.2 ± 1.0	11.5 ± 0.5	87.0 ± 4.2
Enzyme-hydrolysed whey (5% lactose)	6.0 ± 0.2	9.7 ± 0.5	20.0 ± 1.5	60.0 ± 2.8
Enzyme-hydrolysed whey (2.5% lactose)	6.0 ± 0.1	6.5 ± 0.2	31.5 ± 1.7	76.0 ± 2.7

Table 3 Effect of nutrients on kinetic parameters during fermentation of enzyme-hydrolysed deproteinized whey by *A. pullulans*. Each value is the mean ± SD of three repetitions (fermentation time 10 days)

Nutrient supply	Polysaccharide (g L^{-1})	Biomass dry wt (g L^{-1})	Polysaccharide yield (%)	Sugar utilization (%)
None	6.0 ± 0.1	6.5 ± 0.2	31.5 ± 1.7	76.0 ± 2.7
K_2HPO_4 (0.5% w/v)	7.5 ± 0.4	8.5 ± 0.5	36.5 ± 1.5	82.0 ± 3.5
l-Glutamic acid (1% w/v)	6.5 ± 0.2	7.0 ± 0.3	32.2 ± 1.0	86.0 ± 4.0
Olive oil (2.5% v/v) + Tween 80 (0.5% v/v)	8.5 ± 0.5	9.5 ± 0.5	38.3 ± 1.7	88.8 ± 3.8
K_2HPO_4 (0.5% w/v) + l-glutamic acid (1% w/v) + Olive oil (2.5% v/v) + Tween 80 (0.5% v/v)	11.0 ± 0.5	10.5 ± 0.4	47.2 ± 1.8	93.2 ± 2.8

filamentous growth [19]. On the other hand, Simon *et al* [18] reported that *A. pullulans* is a polymorphic microfungus that produces polysaccharide with a life-cycle involving hyphae, blastospores, chlamydospores and intermediate forms. The chlamydospores are responsible for synthesis of extracellular polysaccharide. The swollen cells may in fact be responsible for synthesis of other polysaccharides with a possible further transformation to polysaccharide when the cell develops into a chlamydospore. The hyphae do not play a role in biosynthesis of extracellular polysaccharide [18]. Our results showed that the external addition of supplements improved the production of crude polysaccharide.

The pure pullulan concentration was determined in medium supplemented with K_2HPO_4, l-glutamic acid, olive oil and Tween 80. The results showed that the pullulan proportion of the crude polysaccharide was 40%. Thus *A. pullulans* grown on deproteinized hydrolysed whey produced other polysaccharides. In unpublished work from the author's laboratory, *A. pullulans* P56 produced only pullulan from synthetic medium. In a previous study [13] on the production of pullulan from carob pod extract by *A. pullulans* SU-M18, pure pullulan made up 70% of the total polysaccharide. West and Reed-Hamer [21] reported that the pullulan content of the crude polysaccharide was 54 and 100% when *A. pullulans* ATCC 42023 was grown on sucrose or corn syrup as a carbon source, respectively. Thus, the pullulan content of the crude polysaccharide depends on the strain of microorganism and the chemical composition of the substrate.

Effect of initial pH

The pH of the substrate is important in polysaccharide elaboration by *A. pullulans* and affects the morphology of the organism which influences polysaccharide production. The effect of the initial pH (4.5–7.5) on the kinetic aspects during fermentation of hydrolysed whey by *A. pullulans* is shown in Figure 1. The polysaccharide concentration and yield increased with increasing initial pH up to 6.5 and then decreased. Sugar utilization did not significantly change with respect to pH. Maximum polysaccharide concentration (11.0 ± 0.5 g L^{-1}), polysaccharide yield (47.2 ± 1.8%), and sugar utilization (93.2 ± 2.8%) were obtained at pH 6.5. In contrast, maximum biomass (13.5 ± 0.6 g L^{-1}) was obtained in culture grown at an initial pH of 5.5. In agreement with these findings, Roukas and Biliaderis [13] found maximum

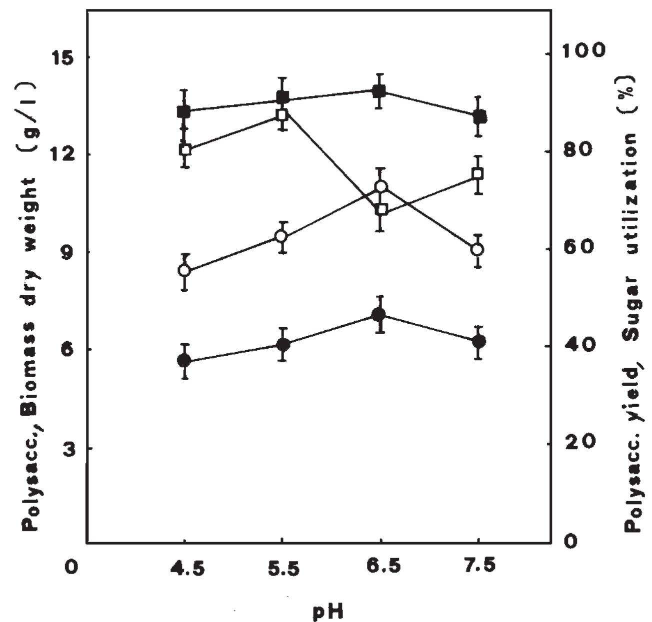

Figure 1 Kinetic parameters during fermentation of enzyme deproteinized hydrolysed whey by *A. pullulans* at different pH values. ○, Polysaccharide concentration; ●, polysaccharide yield; □ biomass dry weight; ■, sugar utilization. Each point is the mean ±SD of three repetitions (fermentation time 10 days).

polysaccharide at pH 6.5 when *A. pullulans* was grown in carob pod extract in shake flask culture. Auer and Seviour [1] observed maximum polysaccharide at an initial pH of 7.5. Papon *et al* [12] reported that a pH of 7.0 is optimal for polysaccharide production in batch culture, whereas Ono *et al* [11], using synthetic medium, found optimum polysaccharide production at a pH of 6.0. Overall, the observed variation in optimum pH is due to the strain of microorganism as well as the substrate composition (eg amount and type of nitrogen source) and the various fermentation systems used in these studies. The results shown in Figure 1 indicate the importance of initial pH of the medium on growth and polysaccharide production.

In conclusion, our results showed some important aspects of pullulan production from deproteinized whey by *A. pullulans* in shake flask culture. An adaptation technique and mixed culture system did not improve the production of polysaccharide. Enzyme hydrolysed whey gave higher polysaccharide concentration and yield than acidic hydro-

lysed whey. Maximum polysaccharide was obtained at pH 6.5. Addition of nutrients to the medium significantly increased the production of polysaccharide. *A. pullulans* grown on deproteinized whey produced a mixture of pullulan and other polysaccharides.

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