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Enzymatic digestion of spent yeast cells for nutrient recycling in inulase production

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SUMMARY

An enzyme complex capable of lysing yeast cells was produced by *Arthrobacter* sp. in a medium containing live cells of *Kluyveromyces fragilis* as the sole source of nutrients. The enzyme complex caused a 90% reduction in the optical density of viable yeast cells in 6 h at 25°C. This yeast cell hydrolysate can be used as a source of nitrogen, vitamins and minerals for subsequent growth of yeast cells (8.3 mg/ml) and further production of inulase (167 U/ml) representing 88 and 87% yield respectively, compared to cells grown on a standard yeast extract (1%) and sucrose (2%) medium.

INTRODUCTION

Processing of D- fructans from the roots and tubers of, for example, Jerusalem artichoke, chicory or dahlia for the manufacture of high fructose syrups involves a hydrolysis step. In comparison to acid hydrolysis, with its side-reactions, enzymatic hydrolysis, because of the mild reaction conditions, yields a more acceptable product stream for further refining [2,17]. D-Fructans of the inulin type are composed of linear chains of varying lengths of D-fructopyranosyl residues with a terminal D-glucose residue. Many microbial enzymes (inulases) are known to split the (2→1)-β–D-fructofuranosidic bonds of inulin, but they differ widely in their mode of action [17]. Of numerous microorganisms known to produce inulase (EC 3.2.1.26), the food yeast, *Kluyveromyces fragilis*, seems to be one of the best sources [3,9,12,18].

Strains of *Kluyveromyces fragilis* have been selected and fermentation conditions optimized for the production of inulase [3–5,15,16]. The maximum enzyme yield achieved in continuous culture represents about 7.5% dry cell weight, of which half is cell-wall associated and the other half is in the medium [4,10]. Thus, after enzyme extraction of the cellular fraction [10], about 96% of the total yeast biomass is left as by-product. Under commercial conditions it would either have a low market value or, if not marketable, a negative value as a waste. Recycling of the biomass may provide a means to enhance its value. Inasmuch as inulase fermentation is growth associated [3,4], recycling of whole yeast cells would not improve the product yield per unit amount of net nutrient input. It would also not improve the volumetric productivity of the continuous fermentation because this is limited by the mass transfer capacity of the fermentor. Therefore, we are seeking biomass recycling in the form of an enzyme digest.

Various microorganisms are known to produce extracellular enzyme complexes with lytic activities toward yeast cells [1,6,8,13,14]. An *Arthrobacter* sp., isolated by Rowley and Bull [13], is able to lyse living cells of *K. fragilis*. The extracellular lytic enzyme complex produced by this bacterum contains β-1,3-glucanase, mannan mannohydrolase and protease activities. The work described in this paper outlines some characteristics of the enzyme complex from *Arthrobacter* sp., which lyses yeast cells. The use of this complex in the recycling of *K. fragilis* cells to provide a source of nitrogen, vitamins and minerals for the subsequent yeast fermentation and inulase production is presented.

MATERIALS AND METHODS

Microorganisms. The yeast strain of *K. fragilis* ATCC 52466 [10] was used as the substrate for the production and assay of lytic activity. *Arthrobacter* sp. [13] was kindly provided by Dr. A.T. Bull, University of Wales Institute of Science and Technology, Wales.

Medium and culture conditions. Shake flask cultures (25 ml) of *K. fragilis* ATCC 52466 in 250 ml Erlenmeyer flasks were grown on medium containing yeast extract

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10 g, and sucrose 20 g, in 1 l of distilled water with no pH adjustment. *Arthrobacter* sp. was maintained on agar slants prepared by adding living *K. fragilis* cells (1% w/v) to 2.5% (w/v) sterile agar (Difco) at 50°C and allowing them to solidify. Slant cultures were grown at 30°C for 4 days and stored at 4°C for less than 1 week. Inocula for liquid cultures were prepared by suspending the cells from one agar slant in 5 ml of water, and using 0.5 ml per 25 ml of medium. The medium for the production of lytic enzyme complex was composed of living yeast cells (9.5 mg dry weight/ml) in sterile water with no pH adjustment.

Lytic enzyme assay. All lytic enzyme assays were made with clarified culture supernatant fluid prepared by centrifugation at 17000 × *g* for 15 min. Yeast lytic activity was determined by measuring the reduction in absorbance of reaction mixture at 520 nm. The reaction mixture contained 3.0 ml of a suspension of *K. fragilis* cells (80 mg dry wt./ml) freshly harvested from shake flask culture (24 h-old), 20 ml of lytic enzyme solution (supernatant from *Arthrobacter* sp. culture, approximately 0.35 mg protein/ml), and 2.0 ml of water. The reaction mixture (25 ml) was shaken (50 rpm) in an incubator shaker at 25°C. Cell lysis was measured over a period of 3 h. One unit of lytic activity is defined as the amount of enzyme complex that produces a decrease at 520 nm of one absorbance unit per hour.

Biomass and protein determination. Dry biomass yield was measured by membrane filtration (0.45 μm, Millipore Corp.) of culture samples, freeze-drying the resultant yeast mat, and weighing. Inulase activity was measured by the method of Lam and GrootWassink [10]. Protein concentration was estimated according to Lowry et al. [11] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

A water suspension of *K. fragilis* cells, which had been extracted for cell-wall associated inulase using 0.3 M potassium phosphate and 8 mM L-cysteine at pH 7.0 and 25°C for 30 min [10], was tested as a growth substrate for the production of the lytic enzyme complex by *Arthrobacter* sp. The extraction did not reduce the viability of the yeast cells [10]. No lytic activity was observed during the first 20 h cultivation of *Arthrobacter* sp. in this medium (Fig. 1). Lytic activity initially appeared at 24 h and reached a maximum activity of about 2 U/ml of culture (5.9 U/mg protein) at 28–29 h. Microscopic examination revealed that the yeast cells were intact at 24 h but were completely digested 4–5 h later. The yeast lytic activity was stable at least up to a culture age of 44 h. No lytic activity was detected in the *Arthrobacter* sp. culture grown in a medium containing 1% yeast extract and 2% sucrose (Fig. 1). The pH profile (Fig. 1) and the maximum dry cell weight (6.8 mg/ml) of the *Arthrobacter* cultures in these two media were however very similar. This confirms the early finding that the yeast lytic enzyme activity from *Arthrobacter* sp. is induced by whole yeast cells [13].

The supernatant fluid of a 34 h-old *Arthrobacter* culture was used to check the time course of lytic enzyme action. The turbidity of the reaction mixture decreased rapidly and became approximately constant after an incubation period of 6 h at 25°C (Fig. 2). At this time, no intact cells could be found microscopically. From the decrease in the turbidity, it was estimated that over 90% of the yeast cells had been digested. Although no buffer was added the pH of the lytic medium remained constant at around 7 (Fig. 2). Fig. 3 shows the activity profile of lytic enzyme complex at different pH. The lytic activity

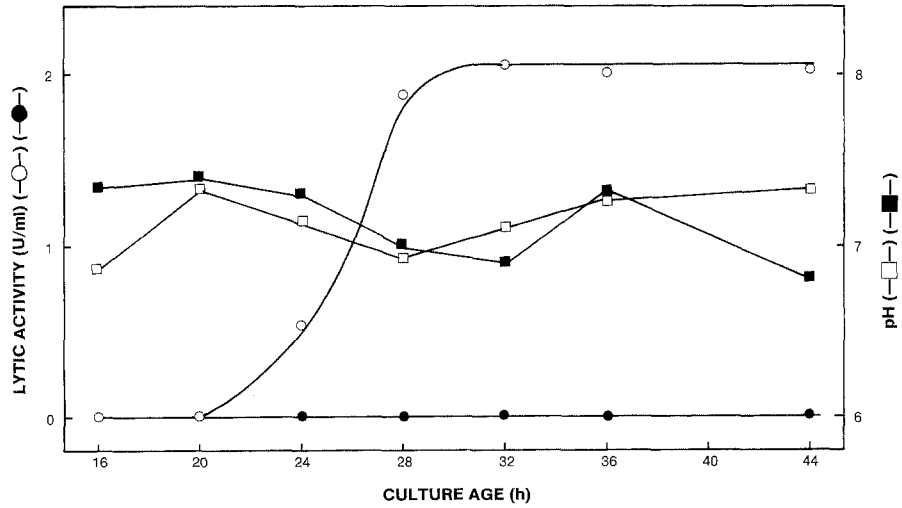

Fig. 1. Lytic enzyme activity during batch cultivation of *Arthrobacter* sp. on *K. fragilis* cells medium (open symbols) and yeast extract (1%)-sucrose (2%) medium (closed symbols).

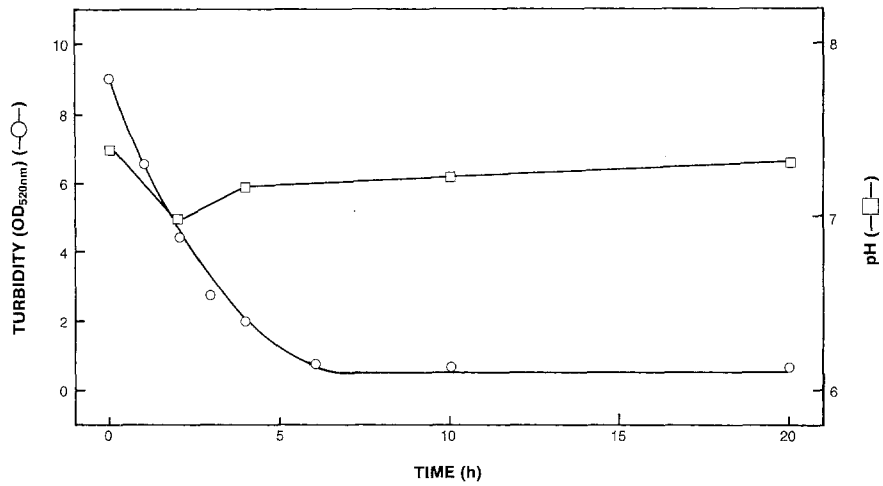

Fig. 2. Time course of lysis of viable yeast cells by lytic enzyme complex from *Arthrobacter* sp. at 25°C. The total volume of the reaction mixture was 25 ml. The yeast cell concentration and lytic enzyme used in this experiment were 9.8 mg/ml and 1.93 U/ml, respectively.

had a pH optium from pH 6–7. Therefore in future assays, no addition of buffer components was made.

K. fragilis can be grown and will produce inulase in a simple growth medium containing sucrose as a carbon source and yeast extract as the source of nitrogen, minerals and vitamins [3]. It thus seemed reasonable to assume that the cell components of *K. fragilis* after being extracted for inulase, could be recycled by adding sucrose to the soluble portion of *Arthrobacter* sp. lytic reaction mixture. To test this possibility, a suspension of *K. fragilis* cells (9.5 mg/ml) which had been extracted for cellular inulase [10], were incubated with lytic enzyme complex (the supernatant fluid of a 34 h culture of *Arthrobacter* sp.,

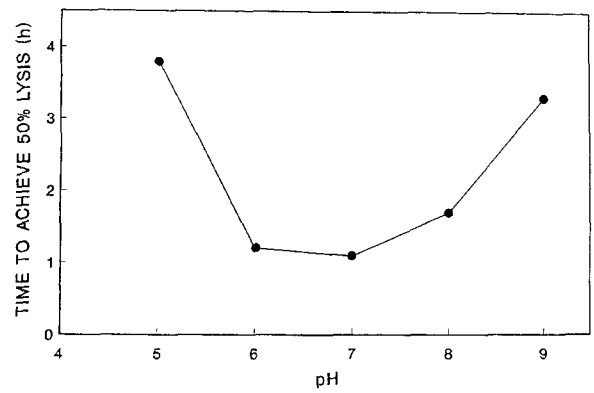

Fig. 3. Effect of pH on lysis of viable yeast cells by lytic enzyme complex from *Arthrobacter* sp. at 25°C. The buffers used in this experiment were 0.1 M sodium acetate, pH 5.0; 0.1 M sodium citrate, pH 6.0; 0.1 M potassium phosphate, pH 7.0; 0.1 M Tris-HCl, pH 8.0; and 0.1 M bicine, pH 9.0. The yeast cell concentration and lytic enzyme in this experiment were 9.3 mg/ml and 1.97 U/ml, respectively.

2.02 U/ml) for 12 h. After this time the reaction mixture was heated at 100°C for 5 min to stop the lytic reaction and then centrifuged at 17000 × *g* for 15 min. To the clear supernatant fluid was added sucrose (final concentration of 2%, w/v), the mixture (recycled cell-sucrose medium) was autoclaved and when cool, inoculated with *K. fragilis*. The autoclaved supernatant fluid without addition of sucrose (recycled cell medium) was inoculated with *K. fragilis* to serve as a control. The above media were compared with the standard yeast extract (1%)-sucrose (2%) medium for both the growth of and inulase production by *K. fragilis*. The results are summarized in Table 1 for the cultures removed 30 h after inoculation. The results show that the recycled cell-sucrose medium is capable of supporting both the growth (8.3 mg/ml) of and inulase production (167 U/ml) by *K. fragilis*. Although only 87–88% as efficient as the standard yeast extract-sucrose medium, the specific activity (20 U/mg dry wt.) is the same in both media. This indicates that the recycled cell-sucrose medium is limiting in some growth nutrient(s). The recycled cell medium supported very poor cell growth (1.3 mg/ml) and inulase production (31 U/ml) indicating the requirement for additional carbon source.

From the above studies, we demonstrate the utilization of the waste generated from the inulase fermentation for further production of inulase. After inulase extraction, about 96% of the total yeast cell mass is left as by-product. We were able to use this by-product for the production of yeast lytic enzyme complex from *Arthrobacter* sp. The lytic enzyme complex was then used to hydrolyze the waste yeast cells to generate nutrients for the subsequent growth of *K. fragilis* and further production of inulase. The above methods for generating yeast

TABLE 1

Comparison of the dry weight and inulase activity in shake flask culture[a] of *K. fragilis* grown in standard medium, recycled cell medium[b] and recycled cell-sucrose medium[c]

Media	Dry weight (mg/ml)	Inulase activity	
		U/ml	U/mg dry wt.
Yeast extract (1%)-sucrose (2%)	9.4 ± 0.5	191 ± 6	20.3 ± 1.3
Recycled cell	1.3 ± 0.2	31 ± 6	23.9 ± 2.0
Recycled cell-sucrose (2%)	8.3 ± 0.4	167 ± 11	20.1 ± 0.4

[a] The shake flask cultures of *K. fragilis* were incubated at 28°C and 250 rpm on a gyrotary shaker for 30 h before assayed for dry weight and inulase activity. This experiment was performed in triplicate and the average values of dry weight and inulase activity were reported.

[b] The recycled cell medium was composed of the autoclaved supernatant fluid of the yeast cell hydrolysate as the sole source of nutrients.

[c] The recycled cell-surcrose (2%) medium was the recycled cell medium supplemented with 2% sucrose.

lytic enzyme complex and hydrolysis of waste yeast cells are simple and easy for scale up. Using the same concentration of lytic enzyme complex (2 U/ml) as in the above studies, 45–50 mg/ml of yeast cells can be hydrolyzed within 12 h. Optimizing the concentration of lytic enzyme complex and temperature of the lytic reaction would further improve the above process.

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