Synthesis of Mannose-Rich Exopolysaccharide by *Rhodotorula glutinis 16P* Co-Cultured with Yeast or Bacteria

Emilina D. Simova*, Ginka I. Frengova and Dora M. Beshkova

Laboratory of Applied Microbiology, Institute of Microbiology, Bulgarian Academy of Sciences, 4002 Plovdiv, 26 Marizta Blvd., Plovdiv, Bulgaria.
E-mail: lbambas@hiffi-plovdiv.acad.bg

* Author for correspondence and reprint requests

Z. Naturforsch. **55c,** 540–545 (2000); received December 12, 1999/February 14, 2000

Rhodotorula glutinis, *Kluyveromyces lactis*, *Lactobacillus helveticus*, Exopolysaccharides

Exopolysaccharides from the lactose-negative yeast *Rhodotorula glutinis 16P* were synthesized by co-cultivation with the yeast *Kluyveromyces lactis MP11* or with the homofermentative lactic acid bacteria *Lactobacillus helveticus 9A* in a cheese whey ultrafiltrate. Exopolysaccharides were produced by lactose hydrolysis, performed by two pathways: with β-galactosidase from *Kluyveromyces lactis MP11* which assimilates glucose and galactose; with β-galactosidase and *Lactobacillus helveticus 9A* which uses lactic acid. By growing the two mixed cultures maximum yield was obtained as follows: 11.4 g/l and 15.8 g/l, respectively. Structural units of the carbohydrate composition of the two polymers are mannose (72.4–63.5%), glucose (2.0–15.9%), galactose (25.3–19.8%) and xylose (3.6–4.3%). Mannose dominated in the polysaccharide compositions.

Introduction

The interest in microbial exopolysaccharides is constantly expanding because of their specific rheological, chemical, biological and pharmaceutical properties, and their wide-range applications in fields of industry and medicine. Exopolysaccharides are synthesized by microorganisms of various taxonomy (bacteria, fungi, yeast) (Calvo *et al.*, 1995; Schuster *et al.*, 1993; Sarkar *et al.*, 1986; Igochi *et al.*, 1990; Adami and Cavazzoni, 1990). Yeast polysaccharides can be used to obtain glucan, mannan and glucomannan (Sarkar *et al.*, 1986; Elinov *et al.*, 1979; Chiura *et al.*, 1982). They have a protective function which boosts the specific immunobiological reactivity of the microorganism (Gurina *et al.*, 1988; Elinov *et al.*, 1988). Yeast mannans have been found to possess anti-virus activity while their high-molecular fractions also have fibrinolytic activity (Kovalenko *et al.*, 1991; Elinov *et al.*, 1988).

Cultivated on synthetic growth media containing glucose, yeast of the species *Moniella*, *Candida*, *Lypomyces*, *Hansenula*, *Sporobolomyces*, *Bullera*, *Trichosporon*, and *Rhodotorula*, synthesize exopolysaccharides containing mannose (Sarkar *et al.*, 1986; Adami and Cavazzoni, 1990; Elinov *et al.*, 1979). For synthesis of exopolysaccharides lactic-based substrate (native whey) (Igochi *et al.*, 1990) and whey hydrolyzed with the enzyme preparation "β-galactosidase" (Stauffer *et al.*, 1978) have been used.

The strain producer of exopolysaccharides, *Rhodotorula glutinis 16P*, which was used in this study, does not assimilate lactose, however, it actively assimilates glucose, galactose and lactic acid. The search for ways of biotransformation of lactose for the purpose of using it as a substrate for exopolysaccharides synthesis has led to the idea to form an association of microorganisms which, as monocultures, have limited ability to synthesize this metabolite. It is only possible to synthesize exopolysaccharides from the lactose-negative yeast *Rhodotorula glutinis 16P* by enzyme hydrolysis of lactose to assimilable carbon sources as follows: hydrolysis of lactose with *Kluyveromyces lactis MP11* – produced β-galactosidase to glucose and galactose during co-cultivation the exopolysaccharide strain producer *Rhodotorula glutinis 16P* in a cheese whey ultrafiltrate; hydrolysis of lactose with β-galactosidase and enzyme systems produced by the homofermentative lactic acid bacteria *Lactobacillus helveticus 9A* to lactic acid during co-cultivation with the exopolysaccharide

0939–5075/2000/0700–0540 $ 06.00

strain producer *Rhodotorula glutinis 16P* in a cheese whey ultrafiltrate.

There are no quotations of studies by other authors on the synthesis of exopolysaccharides by lactose-negative yeast grown in association with other microorganisms in lactose-containing substrates. In the present article we report the results from our study of exopolysaccharides production by the lactose-negative strain *Rhodotorula glutinis 16P*, cultivated in cheese whey ultrafiltrate (WU) in association with *Kluyveromyces lactis MP11* and with *Lactobacillus helveticus 9A*.

Materials and Methods

Microorganisms

The strain *Rhodotorula glutinis 16P* was selected by a multistage selection according to the ability to synthesize exopolysaccharides in a medium containing (g/l): glucose, 40.0; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 0.5; $(NH_4)_2SO_4$, 2.5; NaCl, 0.1; $CaCl \cdot 2H_2O$, 0.1; yeast extract, 3.0, pH = 5.3. The National Bank for Industrial Microorganisms and Cellular Cultures supplied 25 cultures of yeasts form the genera *Rhodotorula*, *Sporobolomyces* and *Cryptococcus*. The strain *Rhodotorula glutinis 16P* was identified using the characterization of Kreger van Rij (1984). The culture was maintained by monthly transfers onto 2.0% malt extract agar slants and stored at 4 °C.

Kluyveromyces lactis MP11 strain was selected as an active producer of intracellular β-galactosidase from 15 cultures of yeasts. They were supplied by the collection of the Laboratory of Applied Microbiology at the Institute of Microbiology, Bulgarian Academy of Sciences. The strain was identified according to Kreger van Rij (1984). It was maintained by monthly transfers in a medium containing (g/l): lactose, 40.0; $(NH_4)_2HPO_4$, 6.0; $MnSO_4$, 0.5; yeast extract, 1.0; agar, 20.0 and stored at 4 °C.

Lactobacillus helveticus 9A strain was selected on the basis of the comparative exopolysaccharide-synthesizing activity of the strain producer *Rhodotorula glutinis 16P* cultivated in association with various species of lactic acid bacteria (*Lactobacillus bulgaricus, Lactobacillus helveticus, Lactobacillus casei, Lactobacillus acidophilus*). They were supplied by the Milk Technology Department at the Higher Institute of Food and Flavour Industries. Strain *Lactobacillus helveticus 9A* was identified with the determiner of Bergey (1986). The culture was maintained by transferring it every week on skim milk and MRS broth, and stored at 4 °C. The following microbe associations were formed to synthesize exopolysaccharides: *Rh. glutinis 16P + K. lactis MP11*; *Rh. glutinis 16P + L. helveticus 9A*.

Medium Composition

The composition of the fermentation medium was as follows (g/l): WU containing lactose, 42.0; $(NH_4)_2SO_4$, 2.5; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 0.5; $MnSO_4$, 0.5; yeast extract, 3.0, pH = 5.3–5.5. The manufacture of ultrafiltrate was given earlier (Frengova *et al.*, 1994).

Inocula

The inoculum of *Rh. glutinis 16P* was grown in 1000 ml Erlenmeyer flasks containing 100 ml culture medium with 2.0% malt extract, at 29 °C, in the course of 48 h, on a rotary shaker with 220 rpm. The inoculum size for all fermentations was 6.0% (v/v) and its cell concentration was about 1.2 g dry cell/l. The *K. lactis MP11* inoculum was grown by cultivation on a rotary shaker with 220 rpm in 1000 ml Erlenmeyer flasks containing 100 ml culture medium with the following composition (g/l): WU – lactose, 42.0; $(NH_4)_2HPO_4$, 6.0; yeast extract, 1.0, pH = 5.0, at 29 °C for 24 hours. The inoculum size for all fermentations was 3.0% (v/v) and its cell concentration was about 1.5 g dry cell/l. The inoculum of *L. helveticus 9A* was grown in skim milk, in a thermostat, at 37 °C for 24 h. It was introduced into the fermentation medium in a quantity of 1.0% (v/v, $4–6 \cdot 10^7$ cells/ml).

The batch cultivation of the two microbial associations *Rh. glutinis 16P + K. lactis MP11* and *Rh. glutinis 16P + L. helveticus 9A* was performed in a 15L fermentor MBR AG (Switzerland, Zurich) at 26 °C, air-flow rate 0.7 l/l min, agitation 220 rpm for 144 hours. The pH of the fermentation system was not adjusted during the growth period.

Analytical methods

The viable counts of *Rh. glutinis 16P, K. lactis MP11* and *L. helveticus 9A* cells were given earlier (Frengova *et al.*, 1994).

Dry cell weight was determined at 105 °C to a constant weight. Lactose, glucose, galactose, L- and D-lactic acid were determined by enzymatic methods as described by Boehringer Mannheim. β-galactosidase was detrmined by a method described by Roger (1976).

The exopolysaccharide content in the supernatant medium was measured after precipitating the biopolymer with acetone according to the method of Adami and Cavazzoni (1990). The total amount of carbohydrates in the polysaccharides was determined using the phenol-sulphuric acid method (Dubois *et al.*, 1956). The carbohydrate composition was determined by gas-chromatography using Fractovap 2407 (Carlo Erba, Italy, Milano) after hydrolysis with 4n H_2SO_4 for 8 h at 105 °C. Chromatography conditions: flame ionization detector temperature 350 °C, 4×2000 mm steel column, 2.0% SE-54W80/100 mesh silanised chromosorb, carrier gas N_2 at 35 cm^3/min, programmed temperature 160 °C increased to 300 °C by 4 °C steps, injector temperature 350 °C, Autolab 6300-02 injector and chart speed 10 mm/min.

Results and Discussion

When *Rh. glutinis 16P* was grown in association with *K. lactis MP11* the synthesis of exopolysaccharides correlates with the synthesis of cell mass, and their maximum yields at the 96th h were 11.4 g/l and 19.0 g/l, respectively (Fig. 1). The production of exopolysaccharides followed the natural course of pH change of the fermentation medium. The pH-interval for active synthesis of β-galactosidase established agrees with the pH-interval for intensive synthesis of polysaccharides (pH = 4.8–6.0), which is a precondition for symbiotic growth between the two yeast species. *K. lactis MP11* – produced β-galactosidase hydrolyses lactose to assimilable glucose and galactose, which stimulates *Rh. glutinis 16P* growth and exopolysaccharide activity. After the 48th h, *Rh. glutinis 16P* cells were observed to predominate accompanied by an intensive synthesis of exopolysaccharides (Figs 1, 2). By the 24th h 50% of the lactose was assimilated by the yeast cultures and by the end of the process it was entirely used up. In our previous studies the maximum amount of β-galactosidase was produced by *K. lactis MP11* monoculture by the 22nd–24th h (1300 U/ml culture liquid) (Roshkova *et al.*, 1992). For the conditions of mixed cultivation of the two yeast species we established prolonged β-galactosidase synthesis up to the 96th h with a maximum amount at the 48th h. During the fermentation process there was no glucose in the culture medium and no galactose after the 48th h. The absence of glucose in the process of joint growth can be explained with its fast assimilation by both yeast species. The morphological differences of the two yeast species made their differentiation easier in the course of the process. The shape of *K. lactis MP11* cells is elliptical to oblong and their size is (2.0–6.6)×(3.0–8.0) μm, while *Rh. glutinis 16P* cells are oval, sized (4.0–8.0) μm. Until the 24th h we observed intensive growth of *K. lactis MP11* – young, budding cells and slow growth of *Rh. glutinis 16P* – single, non-budding but well-shaped cells. After the 48th h *Rh. glutinis 16P* dominated with intensively budding cells up to the 72nd h; enlarged to physiological maturity by the 96th h.

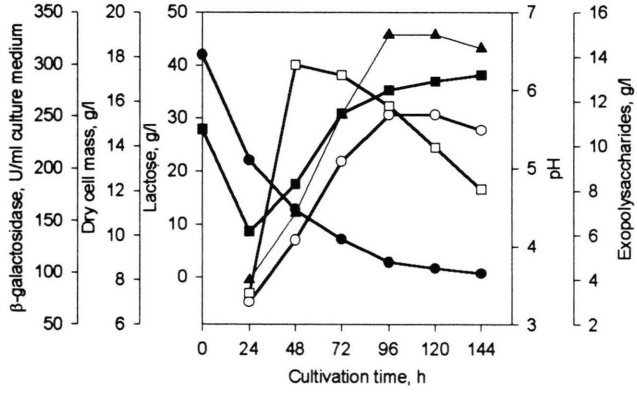

Fig. 1. Growth dynamics, production of exopolysaccharides and β-galactosidase activity of the mixed culture *Rh. glutinis 16P* and *K. lactis MP11* in cheese whey ultrafiltrate. —●— lactose, —○— exopolysaccharides, —■— pH, —□— β-galactosidase, —▲— dry cell mass.

Rh. glutinis 16P growth in association with the lactic acid strain *L. helveticus 9A* manifested comparatively higher activity of exopolysaccharides formation (15.8 g/l at the 96th h) than the yeast association (11.4 g/l at the 96th h) (Figs 1, 3). During the yeast association's growth, glucose takes part in the constructional exchange of both species, while in the yeast – lactic-acid bacteria association the lactic acid is a dominating energy source for *Rh. glutinis 16P.* In associated cultivation the microaerophil *L. helveticus 9A* quickly overcomes the high oxygen concentrations and develops intensively. The viable cells of the lactobacillus dominated over the yeast until the 36th h (Fig. 4), and by the 48th h about 50% of lactose

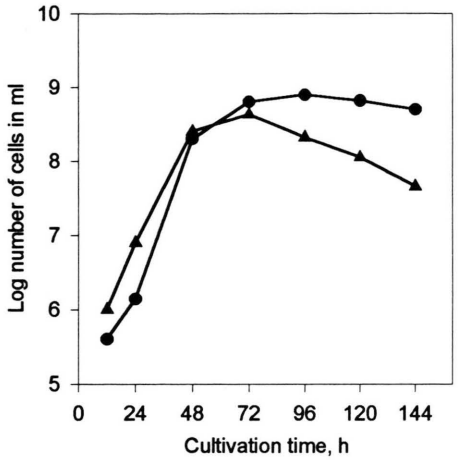

Fig. 4. Growth of mixed culture of *Rh. glutinis 16P* and *L. helveticus 9A* in cheese whey ultrafiltrate. —●— *Rh. glutinis 16P* —▲— *L. helveticus 9A.*

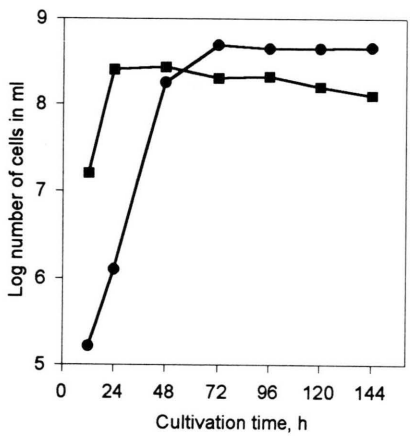

Fig. 2. Growth of mixed culture of *Rh. glutinis 16P* and *K. lactis MP11* in cheese whey ultrafiltrate. —●— *Rh. glutinis 16P,* —■— *K. lactis MP11.*

was assimilated (Fig. 3). The development of the microaerophil *L. helveticus 9A* in conditions of intensive aeration is probably related with the stimulating effect of the products of the yeast metabolism. After a 10-hour log-phase the yeast population was budding intensely, and after the 48th h it was ahead of the development of the lactic-acid bacteria (Fig. 4). There was an abundant growth of young oval budding cells of *Rh. glutinis 16P* sized (6.0–9.0) μm. The young bacterial cells sized (0.5–0.6)×(3.0–6.5) μm were positioned in singles, in pairs and in short chains. During the process we found in the culture medium insignificant concentrations of lactic acid or its traces, and

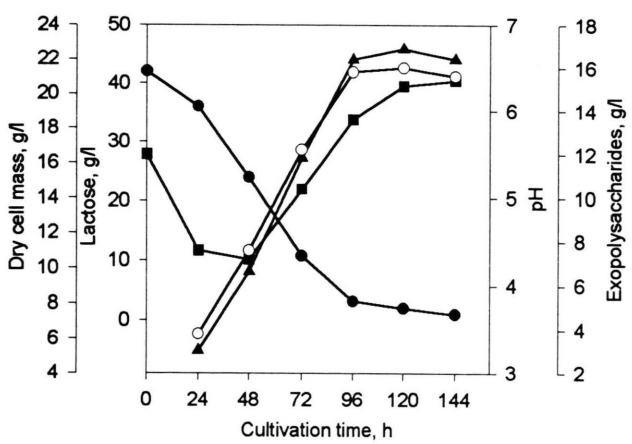

Fig. 3. Time course of growth and exopolysaccharides production by a mixed culture of *Rh. glutinis 16P* and *L. helveticus 9A* in cheese whey ultrafiltrate. —●— lactose, —■— pH, —▲— dry cell mass, —○— exopolysaccharides.

the biomass was increasing up to the 96th h (22.0 g/l) at nearly 90% assimilated lactose. This suggests the fast inclusion of the lactic acid produced by *L. helveticus 9A* D(−) and L(+) in the energy exchange of the yeast, which rapidly entered the phase of exponential growth in a medium in which the carbon substrate is impossible to assimilate directly. It was established that the stimulating effect of the lactic acid on *Rh. glutinis 16P* for the production of exopolysaccharides was higher than that of the glucose and galactose released at the β-galactosidase hydrolysis of lactose by *K. lactis MP11* (Figs 1, 3).

Native milk whey and whey hydrolysed with the industrial preparation "β-galactosidase" have been used for synthesis of exopolysaccharides by monocultural yeast of the *Hansenula*, *Cryptococcus* and *Candida* genera (Stauffer *et al.*, 1978; Igochi *et al.*, 1990). Published literature does not contain information about the production of exopolysaccharides by yeast cultivated in association with other microorganisms. The strain producer *Rh. glutinis 16P* grown in association with *L. helveticus 9A* in cheese whey ultrafiltrate manifested higher exopolysaccharide activity (15.8 g/l) compared to the previously used monocultural yeast *Hansenula*, *Cryptococcus* and *Candida* in milk whey (5.7–13.0 g/l) (Stauffer *et al.*, 1978; Igochi *et al.*, 1990).

The proof of the symbiotic relationships between the microorganism species in the two associations for exopolysaccharides synthesis is the assimilation of the carbon substrate and the active production of exopolysaccharides. These studies reveal possibilities for forming associations which can be used to assimilate lactose by lactose-negative yeast-producers of biologically active substances through mixed cultivation with other microorganism species in cheese whey ultrafiltrate. The comparative studies of the monosaccharide composition of the exopolysaccharides synthesized by *Rh. glutinis 16P*, cultivated in association with *K. lactis MP11* or with the homofermentative lactic-acid bacteria, showed that D-mannose, D-glucose, D-galactose and D-xylose are structural units of the synthesized polymers (Table I). Mannose dominated in the polysaccharide compositions and was higher in the polysaccharide produced by *Rh. glutinis 16P* cultivated with *K. lactis MP11*. There have been reports on species of the *Rhodotorula* genus which, when grown in synthetic glucose media, can produce exopolysaccharides in whose compositions the dominating element is mannose (Adami and Cavazzoni, 1990). According to Elinov *et al.* (1988) mannose, whose concentration is higher than 50% in the polysaccharide macromolecule, is determinative of the biological activity of the synthesized biopolymer.

Table I. Carbohydrate composition of exopolysaccharides synthesized by *Rh. glutinis 16P* grown in association with *K. lactis MP11* or with *L. helveticus 9A* in WU*.

Microbial associations	Carbohydrate (% dry weight)	D-Monosacharides (% carbohydrate) Mannose	Glucose	Galactose	Xylose	Fucose
Rh. glutinis 16P + *K. lactis MP11*	86.3	72.4	2.0	25.3	3.6	–
Rh. glutinis 16P + *L. helveticus 9A*	78.6	63.5	15.9	19.8	4.3	–

K. lactis MP11 and *L. helveticus 9A* cultivated as monocultures in WU do not produce exopolysaccharides.
* WU – cheese whey ultrafiltrate.

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