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Utilization of molasses for the production of fat by an oleaginous yeast, *Rhodotorula glutinis* IIP-30

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SUMMARY

Rhodotorula glutinis is known to produce fat when cultivated under nitrogen-limiting conditions. Economically, molasses is an ideal substrate, however, due to the presence of nitrogen in molasses, the lipid yield obtained is much lower than that obtained from glucose or sucrose. Higher yields were obtained using molasses in a fed batch fermentation supplemented with glucose or sucrose during the lipid accumulation phase. The fatty acids profile of the lipids thus produced, using a very simple and economical medium, was similar to that obtained from glucose and sucrose.

INTRODUCTION

Molasses is a complex mixture of sugars and nitrogenous material arising from sugar processing and is obtained as a waste product of the sugar industry. In India there is a large area under sugar cane cultivation, producing around 150–180 million tonnes of molasses annually. Because of its easy availability and low cost (10% of pure sugar cost) it is considered a promising carbon substrate for fermentation in India.

Recently, there has been renewed interest in lipid accumulation by microorganisms as a means of utilizing waste materials as fermentation substrates [5,13]. Use of molasses for lipid production has long been suggested [13,16] and serious efforts made in this direction in India, Cuba and Poland have recently been described [1,7,10–12]. Molasses has 50% (w/w) fermentable sugar and 0.5–1% (w/w) nitrogen; therefore the carbon to nitrogen ratio is unfavourable for fat production. Nayak et al. [12], in their efforts to remove nitrogen from molasses, found that the nitrogen was not precipitable by trichloroacetic acid. Because of this inherent disadvantage, the use of molasses as a raw material for lipid production has not been encouraged.

As little information and process data are available on oil production with molasses [14], investigations were carried out to explore the possibility of using molasses as a fermentable substrate for lipid production by an oleaginous yeast, *Rhodotorula glutinis* IIP-30.

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MATERIALS AND METHODS

Microorganism and growth medium

Rhodotorula glutinis IIP-30, reported previously by us [9] was used in the present study. Two types of media, namely OP24 and M5, were used employing an initial sugar concentration of 30 g L^{-1} (Table 1). Most of the studies were conducted with OP24 medium.

TABLE 1

Composition of fermentation media

Content		Medium OP24	Medium M5
Ammonium sulphate	g L^{-1}	2.00	2.00
Disodium hydrogen phosphate	g L^{-1}	0.75	3.00
Potassium dihydrogen phosphate	g L^{-1}	1.26	—
Yeast extract	g L^{-1}	1.00	—
Magnesium sulphate	g L^{-1}	0.70	—
Zinc sulphate	mg L^{-1}	4.40	4.40
Copper sulphate	mg L^{-1}	0.30	0.30
Calcium chloride	mg L^{-1}	25.00	—
Ferrous sulphate	mg L^{-1}	16.00	—
Manganese sulphate	mg L^{-1}	0.05	0.05
Cobalt nitrate	mg L^{-1}	0.05	0.05
Boric acid	mg L^{-1}	0.05	0.05
Ammonium molybdate	mg L^{-1}	0.05	0.05

Fermentation conditions

Fermentation studies were conducted in a fermentor (Gallenkemp, Loughborough, Leics, UK) with a working volume of 1 litre at 30 °C and pH 4.0. It was inoculated with a 12 h-old shake flask-grown culture, in exponential phase, so as to have an initial cell concentration of 0.1 g L^{-1}. Air was sparged at a flow rate of 1 v.v.m.; stirrer speed was kept at 600 r.p.m. and pH was maintained at 4.

The fermentation was carried out in two phases. The first phase was the growth phase (a batch fermentation) of 24 h; the second was the lipid accumulation phase (fed batch fermentation) of 96 h conducted under nitrogen-limiting conditions by the addition of sugar at regular intervals, maintaining its concentration between 5–30 g L^{-1} throughout the fermentation cycle and controlling the pH at 4.0 by adding 1 N NaOH.

Carbon substrates, glucose, sucrose, molasses alone or in combination were used in various modes as detailed below:

(A) Batch fermentation using a single substrate.
(B) Batch fermentation followed by fed batch fermentation using a single substrate in both phases.
(C) Batch fermentation with molasses followed by fed batch fermentation with glucose or sucrose.
(D) Same as mode C, except using medium M5 in place of OP24.

Analytical methods

Biomass was determined in triplicate by centrifugation of 10-ml samples of culture liquid at 10 000 *g* for 15 min at room temperature. After washing with distilled water, the cell pellets were dried for 24 h at 105 °C. Lipids were extracted and estimated by the method of Folch et al. [3]. Methyl esters of fatty acids were prepared by using BF3 methanol and analysed by gas chromatography on a Shimadzu GC-15A instrument (Kyoto, Japan) using a stainless steel column (1.52 m × 3.17 mm) having 10% diethylene glycol succinate (DEGS) on 80–100 mesh chromosorb W at 180 °C, using N_2 as a carrier gas (30 ml min^{-1}) as described earlier [8]. Sugar was estimated by the anthrone method [4] and nitrogen by the Kjeldahl method [15].

Pretreatment of molasses

Molasses was diluted to 40% (w/w); the pH of the solution was brought to 2.0 with the addition of H_2SO_4 and was heated to boiling for 30 min. The solution was cooled and used after removing the sludge by centrifugation.

RESULTS AND DISCUSSION

Studies with a single substrate

Rhodotorula glutinis IIP-30, an oleaginous yeast strain [8] has shown a maximum specific growth rate of 0.40 h^{-1} with molasses, 0.34 h^{-1} for glucose and 0.31 h^{-1} for sucrose. Recently, Granger et al. [6] reported a maximum specific growth rate of 0.26 h^{-1} with *R. glutinis*. Jacob and Krishnamurthy [7] studied different *Rhodotorula* strains and found that

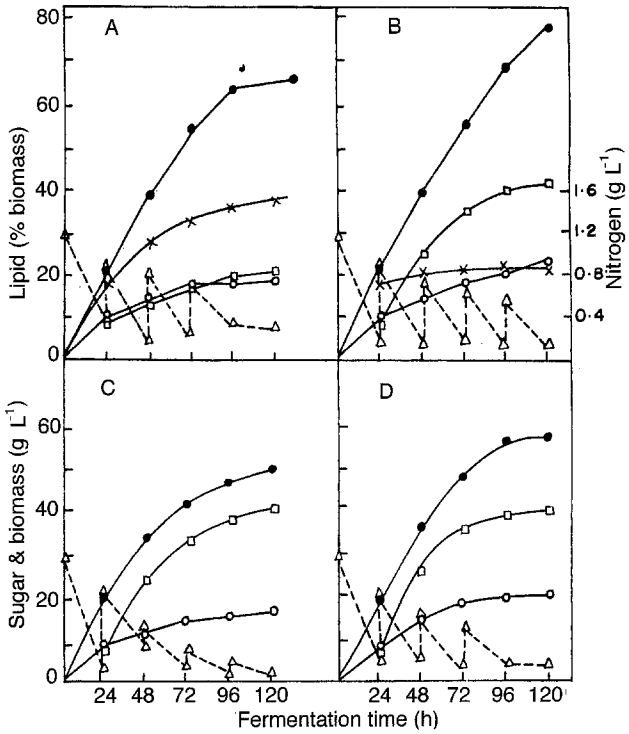

Fig. 1. Biomass (○), Sugar (Δ), Nitrogen (X), Sugar consumed (●) and lipid contents of biomass (□) during fed batch fermentation. (A) Substrate molasses, mode A up to 24 h, mode B 24–120 h. (B) Substrate molasses followed by glucose, mode C. (C) Substrate molasses followed by sucrose, mode C. (D) Substrate molasses followed by sucrose, medium M5, mode D.

R. gracilis CFR.1 exhibited the highest specific growth rate on molasses of 0.16 h^{-1}.

The lipid content of the biomass produced by batch fermentation of molasses as the sole carbon substrate (mode A) was only 8% (Fig. 1(A)), which gave a fat coefficient (g lipid produced per 100 g sugar consumed) of only 3.7. These figures improved considerably with the introduction of fed batch fermentation, (mode B), using molasses in both the phases (Fig. 1(A) and Table 2). Lipid produced during fed batch fermen-

TABLE 2

Biomass concentration and lipid contents of *Rhodotorula glutinis* IIP-30, produced by batch (mode A) and fed batch fermentation (mode B) using single substrate

Substrate	Biomass conc. (g L^{-1})	Sugar consumed (g L^{-1})	Lipid yield (g L^{-1})	(% biomass)	Fat coefficient
Mode A					
Molasses	9.80	20.95	0.78	8.00	3.72
Glucose	5.10	11.00	0.38	7.50	3.48
Sucrose	6.00	17.00	0.39	6.48	2.29
Mode B					
Molasses	18.37	65.30	4.00	21.76	6.13
Glucose	22.30	81.00	14.78	66.30	18.24
Sucrose	6.90	34.40	3.45	50.00	10.02

tation with molasses was very low, nearly half in comparison to the lipid produced from other carbon sources such as glucose (66%) and sucrose (50%) under nitrogen-limiting conditions during fed batch fermentation (mode B). Similar observations of a lower yield of lipids on molasses than on pure sucrose have been reported by Atamanyuk and Vekar [2]. This may be due to the fact that when glucose and sucrose were used for lipid production, the nitrogen in the medium was exhausted within 17–22 h during the growth phase and favourable nitrogen-limiting conditions were readily established in the lipid accumulation phase. Upon continuous feedding of these substrates, they were converted into lipids. However, in the case of molasses, nitrogen does not become exhausted during the continuous feeding as molasses itself contains nitrogen, so nitrogen-limiting conditions cannot be established. Due to this, a lower production of lipid was obtained with molasses added in fed-batch fermentation as compared to other sugars (Table 2).

Studies with mixed substrate

As the nitrogen content in molasses interfered during molasses feeding in the fed batch fermentation, studies were conducted to use it along with other substrates, where the batch fermentation was started with molasses as the carbon substrate. After the growth phase, glucose or sucrose feeding was started at regular intervals during the fat accumulation phase (modes C and D). The course of fermentation by these modes is shown in Fig. 1(B) and (C) respectively. Lipid contents of 42% (fat coefficient 13.5) and 40% (fat co-efficient 13.7) were obtained with glucose and sucrose respectively (Table 3). These observations also confirm that nitrogen limitation is essential for lipid accumulation and the presence of nitrogen in molasses is not desirable for its use in the fat accumulation phase. However, molasses, a cheaper substrate, can be used for the growth phase in order to replace costly glucose or sucrose.

Sucrose utilization was higher (50 g L^{-1}) when used with molasses (Table 3), as compared to 34.4 g L^{-1}, when it was tried alone in both the phases (Table 2). It also resulted in a higher biomass yield (17.2 g L^{-1}), as compared to 6.9 g L^{-1} with sucrose alone. Interestingly, a fat coefficient of 13.7 was obtained with mixed substrate, as compared to 10.0 with sucrose alone, which seems more favourable for fat production. Thus, molasses in the growth phase and sucrose in the lipid accumulation phase were found to be better than the use of only sucrose in both phases.

Studies with revised and low value medium

As molasses itself contains growth factors, vitamins and nutrient salts, the medium OP24 was simplified as M5. The lipid contents of the biomass, produced using the simplified medium with combined substrate molasses and sucrose was found to be 39% (Fig. 1(D)) which is very close to that obtained with OP24 medium. This indicates that medium OP24 can be replaced by the simpler and more economical medium M5.

Fatty acid profile

All of the lipid samples were analysed for their fatty acid composition (Table 4). There does not seem to be significant variation in fatty acid profiles among the lipids produced under different modes of fermentation.

In conclusion, the results reported here show that easily available and inexpensive molasses, which is a poor substrate for lipid production, can be utilized in the growth phase along with glucose or sucrose in a fed batch fermentation. Thus, higher priced glucose and sucrose can be replaced partially by a waste material of the sugar industry. This seems to be an interesting strategy for process development and economic production.

TABLE 4

Fatty acid profile of *Rhodotorula glutinis* IIP-30 produced by fermentation using various modes of substrate feeding

Fermentation condition	C16:0	C18:0	C18:1	C18:2	C18:3
Molasses mode A	23.4	3.5	34.7	25.1	11.4
Molasses mode B	23.5	4.7	36.4	25.3	8.3
Glucose mode C	25.4	5.7	42.9	18.9	4.3
Sucrose mode C	24.5	5.2	40.0	22.4	6.5
Sucrose mode D	24.8	4.8	39.2	22.2	7.8

TABLE 3

Biomass concentration and lipid contents of *Rhodotorula glutinis* IIP-30, produced by mixed substrates

Fermentation condition	Biomass (g L^{-1})	Sugar consumed (g L^{-1})	Lipid yield (g L^{-1})	(% biomass)	Fat coefficient
Mode C					
Glucose in lipid phase	25.0	77.50	10.51	42.07	13.56
Sucrose in lipid phase	17.20	50.00	6.88	40.00	13.76
Mode D					
Sucrose in lipid phase; medium M5	19.30	56.80	7.52	39.01	13.25

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