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BIOPROCESSING WHEY PERMEATE FOR YEAST BIOMASS PRODUCTION

[36]

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ABSTRACT

Media and procedures were tested for propagation of the lactose-utilizing yeast, *Kluyveromyces marxianus* ATCC 36907. Detailed techniques for yeast propagation in shake-flasks and in a 14-liter bioreactor were described. Abundant growth was obtained in whey permeate supplemented with mineral salts [$MgSO_4$ $7H_2O$+$(NH_4)_2$ SO_4+ K_2HPO_4 + KH_2PO_4] and trace elements ($ZnSO_4$ + $FeCl_3$ + $NaMoO_4$ + $MnSO_4$ + $CuSO_4$). Of these components, ammonium sulfate and the trace elements were the essential factors for growth enhancement, as the highest biomass crop was achieved either in the shake-flasks or in the bioreactor.

Key Words: *Kluyveromyces marxianus*, Biomass, Whey permeate, Fermentation, Bioreactor

INTRODUCTION

Because of certain desirable properties, there may be reasons to consider processes based on *Kluyveromyces marxianus* (formerly designated *Saccharomyces fragilis).* Among these desirable features are the ability of at least some strains to grow at high temperatures (35-40°C), ability to use lactose, and ability to ferment sugars to produce ethyl alcohol. These cultures have long been used as a " food yeast" or nutritional yeast", and are regarded as safe for use in foods or as food supplements (**Harrison, 1993**).

One of the reasons for interest in *K. marxianus* is the possible use of this culture for processes including the use of dairy wastes, such as whey and permeates. The latter waste is formed during recovery of milk proteins by ultrafiltration. These materials have a high BOD due to their lactose content, which presents a problem for simple disposal, but also suggests the potential for developing bioprocesses based on use of the lactose as a microbial growth substrate (**Yang**

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et al **1992; Ali and Roushdy, 1998).** Over the years a number of studies have demonstrated the technical feasibility of processes using lactose-utilizing yeasts, such as the sporeforming species of *Kluyveromyces* (K. *marxianus*, *K. lactis* etc.) and their imperfect counterparts, *Candida pseudotropicalis*, etc. Processes have been designed to produce yeast biomass, ethyl alcohol and bioemulsifiers from whey or permeate **(Capoor & Singh, 1985 and 1986; Magdoub *et al* 1992a&b; Fayed *et al* 1992; Harrison, 1993 and Roushdy, 1997).**

The present study was undertaken to improve growth of the lactose utilizing yeast, *Kluyveromyces marxianus*, on whey permeate in shake flasks and in a 14-liter bioreactor. Such yeast cell crop can be used as a substrate in many different applications.

MATERIAL AND METHODS

Strains and Maintenance

The lactose utilizing yeast *Kluyveromyces marxianus* ATCC 36907 (formerly *K. fragilis*) was used in this study. Culture was propagated in Yeast-Peptone-Agar (YPD) at 32°C and maintained at 4°C on slants and plates of YPD agar.

Whey permeate

A sample of whey permeate powder was kindly provided as a gift by Foremost Farm USA, Baraboo, Wisconsin. This commercial product, designed as Permalac, Code 396, typically has a protein content of about 3.5% and lactose content of 82%.

Fermentation medium

A) Whey permeate

For shake-flasks, a solution was prepared by dissolving 60g of the whey permeate powder (Permalac; Foremost Farms, Code 396) in 800ml of deionized water, adjusting the pH to 4.5 with 6N HCl, and bringing the final volume to 900ml. Each 250ml-flask received 45ml of the whey permeate solution and was autoclaved.

For the fermenter experiments, a solution containing 600g of the why permeate powder was prepared into the 14-liter fermentor vessel, using approximately 8.5 liters of deionized water. The pH was adjusted to 4.5 with 6N HCl; the total volume was adjusted to 9.5 liters with deionized water and 1 ml of antifoam DF-204 (Sigma A-6207) was added, then the vessel was autoclaved for 60 min.

B) Supplement solution

Solution "A" containing $(NH_4)_2 SO_4$, 4g; $MgSO_4$ $7H_2O$, 0.1g plus 50ml of deionized water, was autoclaved. After cooling, 1.0 ml of a filter-sterilized trace elements solution was aseptically added. The trace elements solution consisted of: $ZnSO_4$ $7H_2O$, 800 mg; $FeCl_3$ $6H_2O$, 800 mg; $NaMoO_4$ H_2O, 800 mg; $MnSO_4$ H_2O, 400 mg; $CuSO_4$ $5H_2O$, 80mg; being dissolved in 50 ml 0.1N HCl, then brought to 1000ml with deionized water. Solution "B" containing K_2HPO_4, 0.7g; KH_2PO_4, 0.3g plus 50 ml deionized water was autoclaved.

Experimental procedures

For propagation in shake-flasks, all flasks have aseptically received one or more of three sterile supplement solutions (2.2 ml of solution "A"; 2.5 ml of solution "B" or 1.0 ml of 10% yeast extract solution). From active 24-hr yeast culture, inoculum of 2.5% (v/v) was transferred into 250-ml Erlenmeyer flasks containing 50 ml of medium and incubated at 32°C with shaking at 180 rpm.

To the whey permeate in the fermenter vessel, 250ml of sterile double strength solution "A" and 250ml of active yeast culture were aseptically added.

Determinations

Lactose content was determined by the phenol-sulfuric acid method as described by **Gerhart (1981)**. Growth intensity was assessed by measuring the Optical Density (OD) at 600nm, using a double beam spectrophotometer (Spectronic 20+/20D, Spectronic Unicam, New York, USA). Values of pH were followed by using a pH meter (Orion, model A400, Orion Research Laboratory Research group, Boston, USA).

RESULTS AND DISCUSSION

Shake flasks

Yeast propagation in shake flasks containing whey permeate medium at different pH values as determined by OD measurements is shown in Table (1). It was concluded that pH adjustment was not a critical factor for growths in the whey permeate medium, but at the low pH values a clearer medium was observed. Also, it was found that after autoclaving the whey permeate and addition of the sterile supplements (mineral salts solutions A and B, and yeast extract), the pH of the final medium was about 0.5-pH unit higher than that of the whey solutions alone. The clearer medium makes optical density measurements simpler, so for routine purposes it was decided to use an initial pH adjustment of the whey solution to pH4.5.

It could, however, also be appeared from the result in Table (1) that adjusting the pH to 3.5 before sterilization may result in growth at the early incubation period (23 hours). The effect of very low pH on promoting the early growth of the culture would allow a great increase in the efficiency of the process. Some possible interpretations of such an effect might be that the low pH (or sterilization at the low pH) would solubilize some constituent (such as iron, phosphate, or other minerals), or that the low pH (or sterilization at low pH) allows hydrolysis of some component to form a beneficial nutrient. These results coincide with those obtained by **Capoor and Singh (1986) and Magdoub *et al* (1992b)** who observed that the decrease in initial pH of either whey or whey permeate down to 4.5 did favor lactose utilization and thus biomass and ethanol production by *Kluyveromyces* strains.

The effects of the three "supplements" that had been added to the whey permeate (mineral salts solutions "A" and "B", and yeast extract) are shown in Table (2). At the 24-hour sampling

Table 1. Effect of initial pH adjustment on growth of *Kluyveromyces marxianus* ATCC 36907 in supplemented whey permeate medium [a]

Treatment	Growth [b]					
	Zero time		23 Hrs		44 Hrs	
	pH	OD_{600nm}	pH	OD_{600nm}	pH	OD_{600nm}
No pH adjustment	6.0	0.310	6.35	1.115	4.75	8.12
pH 5.0	5.5	0.117	6.10	0.98	4.55	7.48
pH 4.5	5.0	0.084	5.90	1.19	4.15	8.62
pH 4.0	4.4	0.044	5.65	2.715	3.85	9.87
pH 3.5	4.2	0.045	4.05	6.57	4.00	7.60

[a] Each flasks contained 45 ml whey permeate medium aseptically received three sterile supplement solutions (2.2 ml of solution "A"; 2.5 ml of solution "B" and 1.0 ml of 10% yeast extract solution.

[b] Values are the average for duplicate flasks.

Table 2. Effect of supplements [a] on growth of *Kluyveromyces marxianus* ATCC 36907 in whey permeate medium inoculated with 2.5% culture and incubated at 32°C with shaking at 180 rpm.

Supplement	Growth [b]			
	24 Hr		48 Hrs	
	pH	OD_{600nm}	pH	OD_{600nm}
A + B + YE	6.15	1.187	4.9	6.22
A + B	6.0	1.087	3.85	9.33
A	5.9	1.152	3.5	9.14
A + YE	6.0	1.695	3.9	9.07
B	6.0	0.910	5.85	1.42
B + YE	6.05	1.990	4.95	8.3
YE	6.0	1.872	4.5	8.0

[a] Flasks contained 45 ml whey permeate medium aseptically received one or more of the following sterile suppement solutions: 2.2 ml of solution "A"; 2.5 ml of solution "B" or 1.0 ml of YE (10% yeast extract solution).

[b] Values are the average for duplicate flasks.

period there seemed to be little difference in the growth being achieved with various combinations of the three supplements. After 48 hours incubation, there was an abundant growth in all of the examined treatments, except the addition of solution "B" alone. The combination of all three supplements (solutions "A", "B" and yeast extract) resulted in somewhat less growth than the other treatments. The major conclusion from Table (2) seemed to be that whey permeate plus solution "A" gave an abundant growth. Also, because the culture was shown to require vitamins, it is obvious that the whey permeate contains a sufficient amount of the needed vitamins to allow significant growth. The additions of yeast extract and/or solution "B" would involve additional expense, and as they did not yield any greater growth than solution "A" alone, so they seemed of little value and consequently, no further studies on them were done.

Next, an examination was undertaken on the growth enhancement obtained with the components of solution "A". The three components of solution "A" are ammonium sulfate, magnesium sulfate and a mixture of trace metals. Because of the known iron-binding properties of lactoferrin (which would be largely removed during ultrafiltration of whey to produce the whey permeate), it was thought that possibly iron might be a limiting factor in the whey permeate solution. This indicates that the iron may be the major factor to the growth enhancement by Solution A. However, as can be seen in Figure (1), a combination of all of the three components of solution "A" was important to promote the growth in whey permeate medium. Addition of either ammonium sulfate, magnesium sulfate or trace metals alone did not results in a greater growth than occurred in the unsupplemented whey permeate. Also, this experiment showed that there was no growth enhancement by addition of ferrous sulfate alone (in this case the final concentration of iron in the medium was about 30 times greater than provided by the trace elements solution contained in solution "A").

Further testing of the solution "A" component revealed that the essential factors were ammonium sulfate and the trace elements (Figure 2). It could be noted that there was minimal growth in whey permeate alone, but abundant when solution "A" was added. Omission of magnesium sulfate from solution "A" gave little if any effect, but omission of ammonium sulfate reduced the growth enhancment effect by about 50%. Omitting the trace elements solution abolished completely the growth enhancing effect of solution "A". Increasing the concentration of magnesium sulfate upto five folds did not increase the growth enhancement produced by solution "A". These results are in agreement with **Capoor and Singh (1986) and Fayed *et al* (1992)**, who stated that lactose fermenting yeasts such as *Kluyveromyces* sp. were successfully propagated in milk or whey permeates with producing remarkable yields of biomass and ethanol, specially in the presence of certain limiting nutrients such as ammonium sulfate and yeast extract.

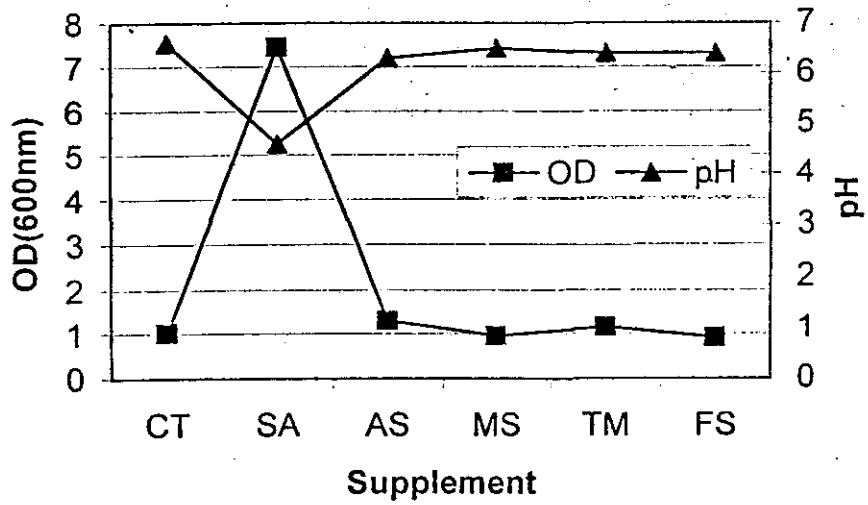

Fig. 1. Requirement for components of solution "A"[a] for growth of *Kluyveromyces marxianus* ATCC 36907 in whey permeate medium inoculated with 2.5% culture and incubated for 45 hrs at 32°C with shaking at 180 rpm
CT: Control; SA: Solution A; AS; Ammonium sulfate only; MS: Magnesium sulfate only; TM: Trace metals only; FS: Ferrous sulfate only (0.012 mg/ml final concentration)

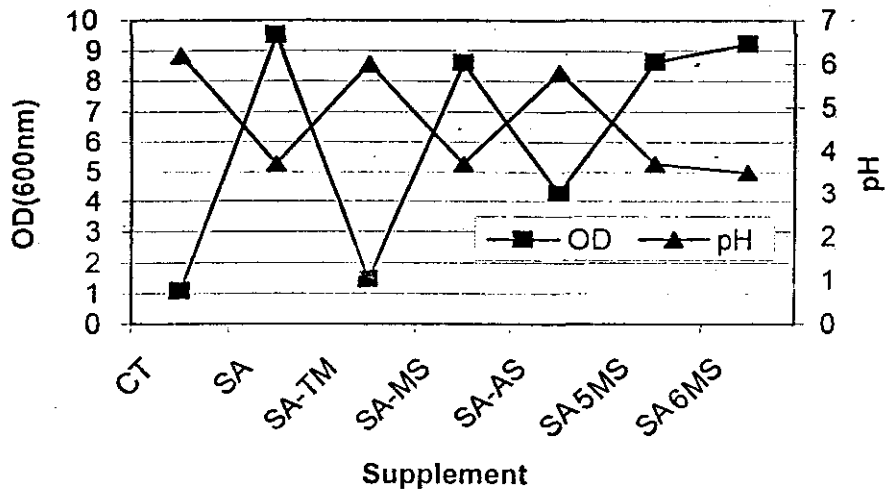

Fig. 2. Requirement for components of solution "A"[a] for growth of *Kluyveromyces marxianus* ATCC 36907 in whey permeate medium inoculated with 2.5% culture and incubated for 45 hrs at 32°C with shaking at 180 rpm
CT: Control; SA: Solution A; SA-TM; SA minus trace metals; SA-MS: SA minus MgSO4; SA-AS: SA minus NH4SO4; SA5MS: SA but 5X MgSO4; SA6MS: SA but 6X MgSO4 and 2X trace metals

Fermenter

In shake flasks, there is a lack of *in situ* probes to monitor pH and dissolved oxygen. Other problems include possible insufficient aeration; inadequate sample size and frequency...etc. Therefore, study of growth kinetics can preferably be achieved using bioreactors.

It was considered that the use of 6% whey permeate solution supplemented with solution "A" would be sufficient for our need to obtain some data on growth of the culture in a bioreactor. Experiments were carried out using the 14 liter fermenter (Bioflo 3000, New Brunswick Scientific Co.) charged with 10 liters of whey permeate solution (6%w/v, adjusted to pH 4.5 before sterilization) supplemented with solution "A". To control excess foaming, 1 ml of synthetic antifoam No.204 (Sigma A6207) was included. The fermenter was inoculated with a shake flask culture in the same medium at the rate of 2.5% (i.e., 250 ml of culture for 10 liters of medium), and it was maintained at 32°C without pH control. Sterile air was supplied at a flow rate of 10 liters/minute (i.e., 1 vol air/vol medium /min) and the initial agitation rate was 400 rpm.

Data obtained from the fermenter experiments are presented in Table (3). It could be noted that there was a transient drop in dissolved oxygen during the first several hours of incubation, followed by a temporary rise, and finally by a decline to very low levels during the exponential growth phase. In contrast, the pH increased steadily during the first 9 hours of incubation (from pH 4.85 to 5.80), and then steadily declined to a final pH of 2.73 after 17 hours. By 11 hours of incubation the culture reached a significant cell density (OD of 4.30) and the dissolved oxygen had dropped to negligible levels. Besides, when the agitation rate was manually increased to 500 rpm at 12 hours, the dissolved oxygen (DO) level obviously rose. This emphasizes the difficulty of supplying adequate dissolved oxygen for aerobic respiration, even in stirred and aerated bioreactors, and the value of using an automatic DO control procedure (which will increase agitation rate as needed to keep the DO level above the set point). In this whey permeate fermentation process the dissolved oxygen concentration probably is a limiting factor, and to maintain an adequate DO it will be necessary to use very high agitation rates during the period of rapid growth when the cell density begins to exceed OD values of 2.0 to 3.0. The values shown in Table (3) indicate that during this period of rapid (and apparently exponential) growth, between 10 and 12 hours of incubation for example, the doubling time was approximately 2 hours. The experiment was terminated after 17 hours of incubation, at which time the culture had reached an OD of 12.6. Growth was no longer exponential, but had not completely stopped.

The lactose content was initially higher than would have been predicted by the supplier's analysis, and the lactose was only partially used during growth of the culture (approximately 42.5% consumed). If lactose utilization is incomplete, as these data suggest, then it might be possible to attain a yield of cell crops higher than that seen in Table 3 through use of a "fed batch" procedure. If lactose

Table 3. Batch-culture growth of *K. marxianus* ATCC 36907 in a 14-liter stirred bioreactor (Bioflo 3000, New Brunswick Sci. Co.) containing 10 liters of supplemented whey permeates solution and 2.5% inoculum. The fermenter was maintained at 32°C, with an aeration rate of 10 liters/min (i.e., 1.0 vol/vol/min).

Incubation (Hours)	OD_{600nm}	pH	DO[a] (%)	Agitation (rpm)	Lactose[b] (mg/ml)
0	0.268	4.85	96	400	61.2
1	0.292	4.82	97	400	ND[d]
3	0.409	4.90	89.7	400	ND
4	0.564	5.12	83.3	400	ND
5	0.715	5.56	87.8	400	58.8
6	1.040	5.71	94.9	400	ND
7	1.332	5.73	88.8	400	56.4
8	1.580	5.80	76.5	400	ND
9	2.016	5.80	58	400	ND
10	2.690	5.72	28.6	400	ND
11	4.300	5.53	0.8	400	ND
12	5.600	5.17	2.3	400	ND
13	8.080	4.46	20.0	500	49.6
14	9.80	3.41	23.7	500	ND
15	10.90	3.04	25.7	500	37.6
16	11.76	2.90	29.4	500	ND
17	12.60	2.73	33.3	500	35.2

[a] DO : dissolved oxygen
[b] Lactose content was measured by the phenol-sulfuric acid method.
[c] ND, not determined.

is not the limiting factor for cell growth, then perhaps addition of an appropriate nutritional supplement after the cell density has reached a suitable level (e.g., an OD value of 6.0 to 8.0) would give greater cell crops.

CONCLUSSION

Abundant growth of *K. marxianus* was obtained in whey permeate supplemented with mineral salts [$MgSO_4$ $7H_2O$ + $(NH_4)_2$ SO_4+ K_2HPO_4 + KH_2PO_4] and trace elements ($ZnSO_4$ + $FeCl_3$ + $NaMoO_4$ + $MnSO_4$ + $CuSO_4$). Of these components, ammonium sulfate and the trace elements were the essential factors for growth enhancement, as the highest biomass crop was achieved either in the shake-flasks or in the 14-liter bioreactor.

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مجلة حوليات العلوم الزراعية ، كلية الزراعة ، جامعة عين شمس ، القاهرة ، ٤٧(٢) ،٥٦٥ - ٥٧٤، ٢٠٠٢

معاملة راشح الشرش حيويا لإنتاج خلايا الخميرة

[٣٦]

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استهدف هذا البحث دراسة ظروف وبيئة النمو لأحد انواع الخمائر المخمرة للاكتـــوز Kluyveromyces marxianus ATTC 36097 وذلك بإستخدام تقنيه التخمر المهتز وكذلـــك بإستخدام جهاز تخمير سعة ١٤ لتر ، حيـث تم دراسة تأثير كل من الـ pH والأمـــلاح المعدنية (كبريتات الماغنسيوم و كبريتـــات الامونيوم وفوسفات البوتاســـيوم الاحاديـــة وفوســـفات البوتاســـيوم الثنائيـــة) وبعـــض العناصر النادرة على كثافة نمو الخميرة فـــى بيئة راشح الشرش .

وقد أوضحت هذه الدراسة أن تدعيم بيئة راشح الشرش بالأملاح المعدنيـــة خاصـــة كبريتات الأمونيوم مـــع بعـــض العنـــاصر النادرة أدى الى الحصول على أعلى كثافـــه لنمو هذه السلاله.

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