Arab Univ. J. Agric. Sci., Ain Shams Univ., Cairo, 11(1), 221 - 229, 2003

PROTEIN ISOLATES FROM *KLUYVEROMYCES MARXIANUS* GROWN IN WHEY PERMEATE

[17]

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ABSTRACT

Lactose-utilizing yeast, *Kluyveromyces marxianus* ATCC 36907 was grown in whey permeate supplemented with mineral salts and trace elements in a 14-liter bioreactor to produce protein isolates that could potentially be used as feed or food additives. After 20 h of batch fermentation, biomass productivity, yield coefficient, residual sugar and chemical oxygen demand (COD) reduction were 2.38 g/l/h, 180%, 34.58 g/l and 92%, respectively. *K. marxianus* showed relatively high protein content (43.18%) and ribonucleic acids (RNA) content (7.84%). To obtain a low nucleic acids-containing protein, different lytic enzyme concentrations and alkaline extraction at pH 7.5, 8.5 and 9.5 were studied. High level of protein extraction (51.31%) and protein precipitate (70.59%) with low nucleic acids (1.91%) were achieved by using Laticase enzyme (20 U/g cells) and alkaline extraction at pH 9.5. The amino acid pattern of *K. marxianus* protein isolate was similar to that of FAO/WHO index.

Key words: *Kluyveromyces marxianus*, Whey permeate, Fermentation, Bioreactor, Protein isolates, Nucleic acids reduction

INTRODUCTION

The great potential of yeasts as a source of proteins for feed or food purposes are well known (**Giec and Skupin, 1988; Paraju *et al* 1995**). For their use as food additives, it is necessary to disrupt the cell walls, to separate the intracellular proteins in the form of an isolate, to reduce the accompanying nucleic acids and to obtain the required functional properties (**Farber *et al* 1995**). Under mild conditions of alkaline extraction about 30-40% of the protein can be obtained from the native cells with a high proportion of peptides and amino acids (**Popova *et al* 1989**).

Biomass production from the whey by yeast fermentation has gained importance because of problems of pollution and an

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(Received December 28, 2002)
(Accepted January 4, 2003)

increasing demand for proteinaceous animal feed. Manufacture of cheese gives a fluid by-product called whey. In many countries, utilization and/or disposing whey, is still a serious problem. At the same time, cheese production is increasing worldwide, which complicates a full whey utilization program (**El-Samragy & Zall, 1988**). Although adequate technology is available to recover fat and protein component in whey, the lactose portion remains mostly unused and is often subjected to different waste treatments, lactose can be converted into ethanol (**Magdoub *et al* 1992 a&b**), single cell protein (**Shay & Wegner, 1986**), microbial polysaccharides (**Charles & Radji, 1977**), bioemulsifiers (**Roushdy, 1997**), proteases (**Ali and Roushdy, 1998**) and Yeast biomass production (**Abdel-Hafez *et al* 2002**). Such options are ultimately challenged by important and variable economic constraints.

In a previous study in our laboratory (**Abdel-Hafez *et al* 2002**), it was shown that ammonium sulfate and the trace elements ($ZnSO_4$ $7H_2O$, $FeCl_3$ $6H_2O$, $NaMoO_4$ H_2O, $MnSO_4$ H_2O and $CuSO_4$ $5H_2O$) were the essential factors for growth of *Kluyveromyces marxianus* ATCC 36907 in whey permeate medium and produce the highest biomass crop, either in the shake-flasks or in the bioreactor. Further experiments have been conducted with *Kluyveromyces marxianus* to demonstrate growth characteristics and extraction of yeast protein concentrates and isolates of this culture. The aim of the present investigation was therefore to achieve a protein isolate that could potentially be used as feed or food additives.

MATERIAL AND METHODS

Strains and growth condition

The experiments were performed using the lactose utilizing yeast *Kluyveromyces marxianus* ATCC 36907. Culture was propagated in Yeast-Peptone-Dextrose agar (YPD) at 32°C and maintained at 4°C on slant and plates of YPD agar.

The strain was cultivated in a 14-liter fermenter (New Brunswick Scientific Co.) charged with 9.5 liters of the whey permeate medium plus 1 ml of antifoam DF-204 (Sigma A6207). To obtain the highest crop of biomass in the bioreactor, 250ml of sterile double strength of the mineral solution containing [$MgSO_4$ $7H_2O$ + $(NH_4)_2$ SO_4 + $ZnSO_4$ $7H_2O$ + $FeCl_3$ $6H_2O$ + $NaMoO_4$ H_2O + $MnSO_4$ H_2O and $CuSO_4$ $5H_2O$] and 250ml of active yeast culture were aseptically added to the whey permeate in the fermenter vessel, as described in our previous study (**Abdel-Hafez *et al* 2002**).

The bioreactor was maintained at 32°C without pH control. Flow rate (1vol air/vol medium/min) of sterile air was supplied with agitation rate of 400rpm.

Reduction of nucleic acid content

Yeast suspension with reduced nucleic acid was obtained after treatment of cells (5 % suspension) at pH 4.5 in three steps (thermal shock at 60°C for 12s, hydrolysis at 45°C for 60min and hydrolysis at 60°C for 60min) as described by **Grigorova *et al* (1997)**. Intact cells of *K. marxianus* ATCC 36907 were treated with a Laticase enzyme system (Sigma L-4025) for cell lysis.

Yeasts suspensions with reduced nucleic acids were treated with 10, 20 and 50 U/g wet cells at 45°C for 60 min. The enzyme-treated yeasts were submitted to alkaline extraction at pH 7.5, 8.5, 9.5 for 30 min. The alkaline extract was separated by centrifugation and the protein was precipitated with 2N HCl at the isoelectric point at pH 4.2. The values of protein extraction were calculated as a ratio of protein estimated in the extract after enzyme treatment followed by an alkaline extraction, to the total proteins of the whole cells.

Analytical methods

The cell concentration was determined by spectophotometric measurement at 660 nm wavelengths, using a double beam spectrophotometer (Spectronic 20+/20D, Spectronic Unicam, New York, USA). The culture samples were diluted to within the absorbance range of 0-0.5 where a linear correlation between absorbance and cell density exists. To determine the maximum cell crop (g wet cells/liter) a 500 ml sample of the culture at the end of fermentation process was collected and centrifuged then the recovery cells were washed twice before weighing.

The cell-free fermentation broth was examined for residual lactose content by the phenol-sulfuric acid method (**Gerhart, 1981**) and chemical oxygen demand, COD (**APHA, 1980**). Yeast biomass was examined for crude protein content by Kjeldahl method (**APHA, 1980**). Protein isolate was examined for protein content by the dye-binding assay (**Bradford, 1976**), ribonucleic acids content by the orcinol method of **Schneider (1969)** and amino acid composition as described by **Sato and Hayakawa (1979)**.

RESULTS AND DISCUSSION

The growth characteristic of *K. marxianus* ATCC 36907 in whey permeate medium supplemented with mineral salts and trace elements has been investigated (Figure 1). It was noted that the optical density (OD) increased about 193% during the period from hour 10 to hour 14.5 (4.5 hours), and this would correspond to a doubling time of just over 2 hours during this stage of rapid growth. At the 11.5-hour incubation time, which was thought to be within exponential growth, a 1.0-liter sample of the culture was removed, the cells recovered by centrifugation, washed and weighed. Approximately 17.96 g wet weight of cells was obtained from 1 liter of the culture (OD of 4.73). Based on this value it is calculated that (in an exponentially growing culture) an OD reading of 1.0 corresponds to 3.8g wet cells per liter. This is a greater final cell crop than had been observed in the shake flask studies, and emphasizes the relatively poor predictability of the data obtained from growth studies in shake flasks. The highest biomass yield and productivity as well as the lowest residual lactose were recorded at 17h fermentation period (Figure 1).

After 20 h of batch fermentation, biomass productivity calculated on biomass produced per fermentation time was 2.38 g/l/h, yield coefficients calculated on biomass produced per lactose utilized was 180 %, while the residual sugar was 34.58 g/l (Table, 1). **Bayer (1983)** found that biomass productivity was increased enormously as

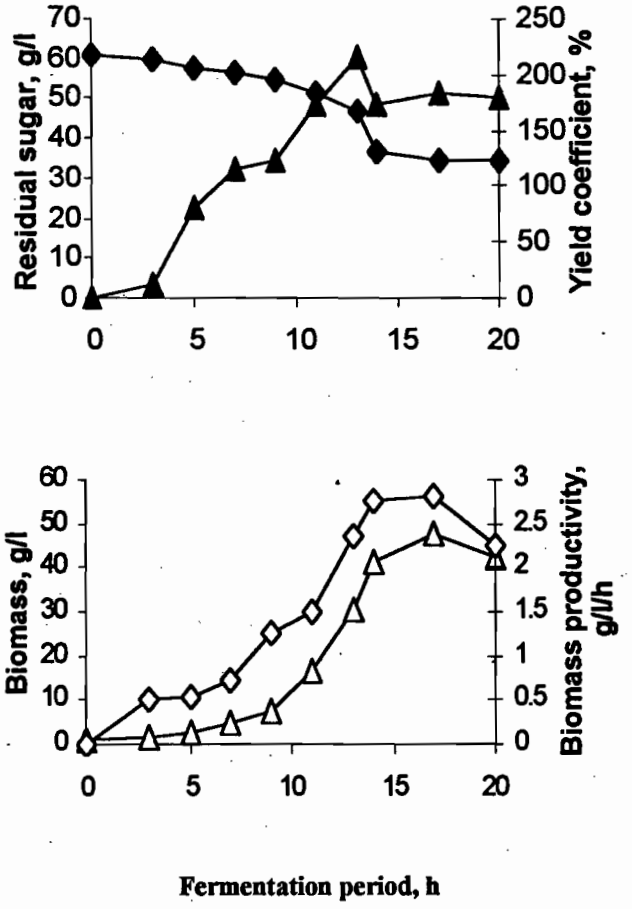

Figure 1. Parameters characterizing a batch culture growth of *K. marxianus* ATCC 36907 in whey permeate medium. Biomass (◇), biomass productivity (△), residual sugar (◆), Yield coefficient (▲)

Table 1.Characterization of whey permeate bioprocessing after 20 h batch fermentation by *K. marxianus* ATCC 36907

Characteristics	Value after 20 h whey permeate bioprocessing
*Yeast biomass, g/l	47.58
[a]Biomass productivity, g/l/h	2.38
*Residual sugar, g/l	34.40 ± 0.59
[b]Yield coefficient, %	180.23
*[c]COD, mgO_2/l	3551± 154
Decrease of COD by fermentation, %	92.80
*Crude protein N x 6.25, %	43.18 ± 0.24
*Nucleic acid content, %	7.84 ± 0.38

[a]Biomass productivity gram cell produced per hour of fermentation time
[b]Yield coefficient gram cell produced per gram lactose utilized, multiplied by 100
[c]COD of fermentation medium was 49240± 4258 mgO_2/l.
*Values represent the mean ± standard deviation from assays of three samples

a result of the stimulating effect of some trace element, when added to cheese whey.

Within the experiments mentioned, reduction of the chemical oxygen demands (COD) loads at the end of the fermentation process was determined (Table, 1). The COD load was reduced by *K. marxianus* by 92%, which means the reactor showed excellent COD removal. These results are comparable to those obtained by **Bayer (1983)**, who found that growing *Candida intermedia* in cheese whey reduced the COD load by 90%. Taking into account that the reductions of the COD reached 92%, protein isolate production from whey permeate by yeast should be feasible on a bigger scale.

Table (1) also shows crude protein content (43.18 %) and ribonucleic acids (RNA) content (7.84%) of *K. marxianus* ATCC 36907 cells grown on whey permeate medium after 20 h batch fermentation process. The *K. marxianus* showed relatively high protein and ribonucleic acids contents.

Finally, before yeast protein can be used as a significant source of protein for human consumption, the content of the nucleic acids present as RNA must be reduced so that daily intake of nucleic acid from yeast would not exceed 2 g on a dry weight basis (**Ohta *et al* 1971**). To use such a protein, in reasonable quantities, as a source of food proteins, reduction of the nucleic acids present in the yeast cells or protein preparation from

yeast cells is necessary. Cells were, therefore, subjected to the enzyme lysis. It was noticed that the reduction of the RNA was performed with thermal treatment that enhances the enzyme lysis of the whole cells (**Roshkova and Pavlova, 1988**).

The efficiency of the enzyme action was estimated by the degree of protein extraction and subsequent protein precipitation (Table, 2). With increasing enzyme concentration to 50 U/g cells a higher extraction of the cell protein was achieved (37.25 – 62.24 %) at the three-pH values used for extraction. However, the amount of the precipitated protein was lower as compared to that obtained at lower enzyme concentration. Keeping in mind the dependence of extractability on pH, it is understandable that the common procedure used for extraction of yeast protein is a treatment with alkaline solvents. The alkaline extract of yeast mass is always a multi-component system containing proteins, nucleic acids, lipids, carbohydrates and other low molecular weight components. To produce a product containing a high amount of protein and as few as possible other contaminating components, it is necessary to refine the extracted raw protein (**Grigorova *et al* 1997**). With the optimal quantity of the enzyme (20 U/g cells) suitable for extraction as well as for precipitation of the protein, 51.31 % protein extraction was achieved using alkaline extraction at pH 9.5, and the precipitate was 70.59% with 1.91% RNA. **Grigorova *et al* (1997)** reported that with increasing lytic enzyme concentration to 10U/g of cells, a higher extraction of the cell protein was achieved (53-75%). Extraction with alkaline media at pH 9-12 is the most practical procedure if the main purpose is to achieve a high protein yield. Several authors reported that for maximizing protein yields and minimizing contaminant nucleic acids, alkali extraction at elevated temperature (80°C) is the most practical procedures (**Lindblom, 1974; Vananuvat and Kinsella, 1975**).

Table 2. Effect of lytic enzyme concentration on the protein extraction and nucleic acid reduction from K. marxianus ATCC 36907

Enzyme concentration (U/g wet yeast)	Alkaline extraction at pH	Extraction* %	Precipitation* %	RNA content* %	RNA reduction %
10	7.5	21.81±1.22	89.24±1.52	2.31±0.13	70.53
	8.5	24.8±1.29	79.5±1.68	2.52±0.18	67.85
	9.5	35.3±1.34	72.8±1.67	3.19±0.22	59.31
20	7.5	25.15±1.25	79.19±1.28	1.57±0.08	97.97
	8.5	28.98±1.35	78.29±2.01	1.74±0.08	77.80
	9.5	51.31±1.87	70.59±1.78	1.91±0.13	75.63
50	7.5	37.25±1.64	51.42±1.64	1.31±0.12	83.29
	8.5	42.11±1.14	44.28±1.58	1.45±0.11	81.50
	9.5	62.24±1.89	34.58±1.57	1.68±0.09	78.57

*Values represent the mean ± standard deviation from assays of three samples

The essential amino acid composition of *K. marxianus* protein isolate was compared with the FAO/WHO reference (Table, 3). The amino acid pattern of *K. marxianus* protein isolate was similar to that of FAO/WHO index. On the other hand, slightly higher amino acids content of the isolate was shown with excluding the total sulpher containing amino acids. This is consistent with the report of **Viikari and Linko (1977),** who found that the concentration of most amino acids in the Pekilo-proteins, a single cell protein for human consumption prepared by using *Paecilomyces varioti* grown on spent corn liquor, was comparable to FAO/WHO SCP reference.

Table 3. Essential amino acids of *K. marxianus* ATCC 36907 protein (g/100g protein) after alkaline treatment for reduction of the RNA content

Amino acids	FAO/WHO Reference (1973)*	K. marxianus protein
Lysine	5.5	7.4
Theronine	4.0	5.7
Valine	5.0	5.9
Total S-amino acids	3.5	1.9
Isoleucine	4.0	5.1
Lucine	7.0	8.3
Phenylalanine	-	4.5

CONCLUSSION

Protein isolate from *K. marxianus* with accepted range of protein and nucleic acids content was achieved by using 20 U/g of lytic enzyme with alkaline extraction at pH 9.5.

ACKNOWLEDGMENTS

This research was supported by grant (930101) from the Egyptian -American Universities Linkage Project funded by the USAID through the FRCU. Appreciation would also be extended to Prof. Dr. M.N.I. Magdoub, Professor of Dairy Microbiology and Cairo MIRCEN Director, for his strong support.

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مجلة اتحاد الجامعات العربية للدراسات والبحوث الزراعية ، جامعة عين شمس ، القاهرة ، ١١(١) ، ٢٢١ - ٢٢٩ ، ٢٠٠٣

إنتاج معزول بروتين من خميرة *Kluyveromyces marxianus* المنماه فى راشح اللبن

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أستهدف هذا البحث انتاج بروتين ميكروبى ذو محتوى منخفض من الأحماض النوويه بإستخلاصه من خلايا الخميره *Kluyveromyces marxianus* (ATCC 36907) المنماه فى راشح اللبن المدعم ببعض الأملاح المعدنيه والعناصر النادرة فى مخمر سعة ١٤ لتر ، وذلك للإستفاده منه فى تدعيم الأغذية والعلائق.

وقد أوضحت الدراسة أنه بإجراء عملية التخمر لمدة ٢٠ ساعة ، أمكن الحصول على محصول خلايا قدره ٢,٣٨ جم/لتر/ساعة ، ومعامل محصول مقداره ١٨٠% ، فى حين بلغت كمية المتبقى من سكر اللاكتوز ٣٤,٥٨ جم/لتر وأنخفضت قيمة الاحتياج الكيماوى للأكسجين (COD) بنسبة ٩٢% . وأظهرت الدراسة احتواء محصول الخلايا الناتج على نسبة عالية من البروتين بلغت ٣٤,١٨% ومن الحمض النووى الريبوزى (RNA) بلغت ٧,٨٤% .

وقد لخصت الدراسة إلى أن معاملة خلايا الخميرة بالكمية المناسبة (٢٠ وحده/جم خلايا) من الانزيم Laticase مع إجراء عملية الاستخلاص على pH ٩,٥ ، قد أدى إلى الحصول على نسبة استخلاص عالية للبروتين بلغت ٥١,٣١% مع ارتفاع نسبة المترسب (٧٠,٥٩%) وانخفاض المحتوى من الأحماض النووية لأقل من ٢% ، مع تماثل محتواه من الأحماض الأمينية مع القيم المرجعية لمنظمة الصحة العالمية (WHO) ومنظمة الأغذية والزراعة (FAO) .

تحكيم: أ.د محمد نبيل إبراهيم المجدوب أ.د طه عبد الحليم نصيب