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ORIGINAL PAPER

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# Evaluation of *Kluyveromyces marxianus* FII 510700 grown on a lactose-based medium as a source of a natural bioemulsifier

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**Abstract** Mannoprotein with emulsification properties was extracted from the cell walls of *Kluyveromyces marxianus* grown on a lactose-based medium by autoclaving cells in a citrate buffer at pH 7.The purified product was evaluated for chemical and physical stability to establish its potential use as a natural emulsifier in processed foods. The yield of purified bioemulsifier from this strain of *K. marxianus* was 4–7% of the original dry cell weight. The purified product, at a concentration of 12 g $l^{-1}$, formed emulsions that were stable for 3 months when subjected to a range of pH (3–11) and NaCl concentrations (2–50 g $l^{-1}$). The composition of this mannoprotein was 90% carbohydrate (mannan) and 4–6% protein. These values are similar to mannoprotein extracted from cells of *Saccharomyces cerevisiae*, which is the traditional source. Consequently *K. marxianus* cultivated on a low-cost lactose-based medium such as whey, a lactose-rich clean waste of the dairy industry, could be developed as a source of bioemulsifier for use in the food industry.

**Keywords** Bioemulsifier · Extraction · *K. marxianus* · Mannoprotein · Yeast

## Introduction

In recent years there has been increasing interest in the isolation and identification of new microbial emulsifiers and surfactants [3, 8, 12, 38, 39, 45, 48] that might have application in the oil [7, 10] pharmaceutical [34, 41, 52, 53], mining [14], food [11, 15, 50] and other industries [31, 47]. The majority of microbial emulsifiers have been reported in bacteria [25, 27, 29, 30,35, 36, 38, 42]. However, a large number of emulsifiers from bacterial sources are not suitable for use in foods due to the pathogenic nature of the producer organisms [50].

Recently a filamentous fungus was reported to produce an agent containing a polysaccharide-protein complex capable of stabilising oil in water emulsions [45]. Yeasts on the other hand, have been used extensively for the production of emulsifiers. Bioemulsifier-producing strains include *Candida petrophilum* [32], *C. tropicalis* [37], *Torulopsis petrophilum* [23], *C. lipolytica* [18,19] and *C. bombicola* [13]. However, emulsifying agents from these sources are produced only in the presence of water-immiscible substrates, such as alkanes and oils, which appear to facilitate the necessary metabolism [17] and these water-immiscible substrates pose a challenge in the isolation of the produced bioemulsifier, requiring enzyme digestion, foam fractionation and repeated extraction with solutions of chloroform-methanol. Furthermore, the emulsifier yields obtained at the end of these elaborate protocols are low, hence commercial development of bioemulsifiers from these yeast strains is unattractive [17].

Since, due to consumer demand, natural emulsifiers are becoming increasingly important in the food industry over synthetic emulsifying agents, which are suffering diminishing popularity due to high production costs and potential human health hazards, the quest to find reliable sources of these natural emulsifiers has intensified [51]. Consequently, strains of *Saccharomyces* produced by low-cost biotechnology methods using water-soluble substrates, as well as spent brewer's yeast from the wine and brewing industries, have become important sources from which bioemulsifiers are extracted [9, 51]. These sources offer the advantages of low cost and high volume of yeast biomass, which translates into high bioemulsifier yields in comparison with synthetic sources [9, 51].

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Bioemulsifiers derived from these yeast sources are mostly mannoprotein [9, 17, 54]. Two classes of mannoproteins have been extracted from yeast. First, structural mannoproteins, which contain approximately 90% mannose and 4–6% protein, the two components considered necessary for their action as emulsifiers [5, 6] and which are interspersed within a network of glucan to form the outer layer in the yeast cell wall [49]. The second class of mannoproteins are the mannan enzymes, which are approximately 30–50% protein and the remainder carbohydrate, and these are located mainly in the periplasmic space between the plasma membrane and the cell wall [2, 20, 33].

This paper proposes use of the yeast *Kluyveromyces marxianus* grown on a lactose-based medium as a possible source of these natural bioemulsifiers. Such bioemulsifiers could provide higher value products and a means of utilising the abundant lactose present in whey, a clean waste of the dairy industry, 50% of which goes to waste worldwide.

## Materials and methods

### Chemicals

All chemicals used were of analytical grade. Tri-potassium citrate, potassium metabisulphate, ethanol 950% (v/v), acetic acid and Cetavlon were obtained from Merck (Sydney, Australia). Corn oil was purchased from a Nature First, Victoria, Australia.

### Yeast strain, medium and growth conditions

A strain of *K. marxianus* FII 510700 (FRR 1586) previously cultivated on casein whey [40] was obtained from the Culture Collection of the University of New South Wales, UNSW 248 (World Data Centre for Microorganisms). The yeast was cultured and maintained on slants of malt extract and stored at 4°C. A fresh culture was prepared on malt extract nutrient again on Petri dishes every 2 months from the primary culture. Purity of the culture was confirmed before use by further streaking on plates of malt extract nutrient agar and by microscopic observation.

*K. marxianus* strain FII 510700 was grown in a deproteinated lactose medium (DPP), (Murray Goulburn Co-operative, Melbourne) containing (g $l^{-1}$): lactose, 40; $(NH_4)_2SO_4$, 5; $MgSO_4 \cdot 7H_2O$, 2; $KH_2PO_4$, 4; yeast extract, 2. The culture was grown by transferring 3 l sterilised medium (121°C, 15 min) into a pre-sterilised Biostat B (Braun, Melsungen, Germany) vessel and inoculating it with seed culture. The temperature, pH and dissolved oxygen level, were controlled at 30°C, 5.0 and greater than 20% air saturation, respectively.

### Extraction of emulsifier

The bioemulsifier was extracted from cells of *K. marxianus* by the method of Torabizadeh et al. [51]. Yeast (20 g dry weight) was suspended in 100 ml 0.1 M potassium citrate and 0.02 M potassium metabisulfite buffer, pH 7, and autoclaved (121°C) for various periods (30–120 min). After thorough mixing, the resulting suspensions were centrifuged at 500 *g* for 10 min at room temperature. The supernatant was retained and mixed with three volumes of 95% (v/v) ethanol containing 1% (v/v) acetic acid and incubated overnight at 3–4°C to allow complete precipitation. The precipitates were recovered by centrifugation at 5,000 *g* for 10 min and freeze-dried for 48 h (Model D125; Dynavac, Sydney, Australia).

### Purification of bioemulsifier

Bioemulsifier was purified by the method of Nakajima and Ballou [44]. Crude bioemulsifier (4 g) obtained by hot citrate extraction as described above was dissolved in 100 ml water to which 4 g Cetavlon (hexadecytrimethylammonium bromide) in 50ml water was added. The mixture was allowed to form a precipitate by standing at room temperature for 4 h. The precipitate was removed by centrifugation (5,000 *g*) and the supernatant retained. The precipitate was washed twice with a total of 50 ml of water by centrifuging as described above. The washings were combined and added to 100 ml 1% boric acid in a beaker and stirred. The pH was then adjusted to 8.8 with 2 M NaOH. After precipitation was complete (2 h), the solid residue was collected and washed with 0.5% sodium acetate, pH 8.8. This precipitate was then dissolved in 50 ml acetic acid to which 1 g sodium acetate and three volumes ethanol were added. The precipitate was collected by centrifugation and washed with 2% acetic acid in ethanol. The ethanol precipitate was then dissolved in water and the pH adjusted to 7 with 2% (w/v) NaOH, dialysed against distilled water (48 h) and lyophilised.

### Analysis of bioemulsifier

#### *Molecular weight*

Preliminary investigations were made to determine the molecular weight of the mannoprotein bioemulsifier by sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) with a 100 g $l^{-1}$ resolving gel [46]. A solution (18 µl) containing 0.5 mg $ml^{-1}$ mannoprotein was mixed with 6 µl loading buffer and boiled in a water bath for 10 min. After cooling, the solution was loaded onto a gel, and placed in an electrophoresis tank (Novex, San Diego, Calif.) operating at 125 V (constant) for 90 min.

#### *Total carbohydrate*

Total carbohydrate was determined colorimetrically by the method of Dubois et al. [24].

#### *Monosaccharide composition*

Monosaccharide composition was assayed by the method of Harrison et al. [28]. A stock solution containing bioemulsifier was made by dissolving 1 mg in 10 ml water. From this stock solution, 20 µl was pipetted into three screw-capped Eppendorf tubes. To each aliquot, 0.1 ml 4 M trifluoroacetic acid (TFA) was added and the solutions mixed thoroughly. The saccharides were then hydrolysed by placing the solution in a heating block at 80°C for 4 h after which time the TFA was evaporated under vacuum (Savant Speed-vac, Halbrook, N.Y.). The dried residue was resuspended in 50 µl water to which 1 mg $ml^{-1}$ deoxy-D-glucose was added as an internal standard. Finally, the mixture was centrifuged at 5,000 *g* for 2 min and the supernatant was maintained at 4°C until it was analysed. Aliquots (10 µl) were analysed by HPLC using a Dionex Carbopac PA10 column and an ED40-Dionex electrochemical detector. The eluent was 18 mM NaOH pumped at a flow rate of 1.0 ml $min^{-1}$ using a GP 40 Dionex gradient pump. The pressure range was 9,653–11,032 kPa. Standard solutions of glucose and mannose (concentrations in the range 1–5 mg $ml^{-1}$) were similarly hydrolysed and analysed by HPLC.

*Protein*

Protein was estimated by the Folin-Ciocalteau reagent as outlined by Lowry et al. [43]. Optical density was read at 500 nm. A standard curve (0–200 mg $l^{-1}$) was constructed with bovine serum albumin (BSA) as the standard.

*Assay of emulsification activity*

Emulsification activity was evaluated as described by Akit et al. [1] and Cameron et al. [15]. Dry bioemulsifier (0.12 g) was dissolved in 4 ml distilled water in a test tube to which 6 ml corn oil was added. The tube was vortexed to homogeneity and left to stand at room temperature. After 1 h, when an emulsion formed, the height (mm) of the volume occupied by the emulsion phase was divided by the height (mm) of the total volume occupied by the water and the corn oil added and the percentage of emulsion calculated. A blank containing 4 ml oil and 6 ml water, vigorously vortexed and left to stand at room temperature for 1 h was used as the control in all experiments

Physical and chemical stability of emulsion

*Effect of pH*

The stability of emulsions was evaluated for 90 days over a range of chemical and physical conditions. The effect of pH on the stability of the emulsion was tested in the range 2–11. Dry bioemulsifier (0.12 g) was dissolved in test tubes containing 4 ml water in which the pH had been adjusted with 0.1 M HCl or 0.1 M NaOH in the pH range 2–11. Corn oil (6 ml) was added to each test-tube and the solutions were vortexed to homogeneity. The emulsion phase was compared with the total volume of water and corn oil added. The experimental control was prepared as described above.

*Effect of NaCl*

The effect of various concentrations of NaCl on the stability of the emulsion was determined. Dry bioemulsifier (0.12 g) was dissolved in 4 ml water containing varying amounts of NaCl (5–50 g $l^{-1}$) in test tubes. Corn oil (6 ml) was added to each test tube and the solutions were vortexed to homogeneity. The emulsion phase was then compared with the total volume of water added. The tubes were kept for 90 days and the stability of the emulsion was compared with a blank, which was prepared as described above.

*Effect of bioemulsifier concentration*

The effect of various concentrations of bioemulsifier on the stability of the emulsion was determined using the methods of Torabizadeh et al. [51] and Cameron et al. [17]. Various amounts of bioemulsifier were dissolved in 4 ml water in test tubes for a range of concentrations (1–50 g $l^{-1}$). Again, 6 ml corn oil was added to each test tube and solutions vigorously vortexed to homogeneity. The emulsion phase was then compared with the total volume of water added. The tubes were kept for 90 days and the stability of the emulsion recorded. The experimental control was prepared as described above.

## Results

Effect of extraction time on emulsion

The effect of extraction time on purified bioemulsifier yield and stability after 90 days at 4°C are summarised in Table 1. The yield increased with increasing time in the autoclave. However, bioemulsifier extracted for 150 min at 121°C produced the least amount of purified emulsifier. Extraction times of 30–120 min produced emulsions that remained stable after 90 days at 4°C. However, extraction for longer than 120 min yielded a product that had no emulsification properties.

**Table 1** Effect of extraction time on bioemulsifier yield and stability. The bioemulsifier was extracted from dried yeast cells of *Kluyveromyces marxianus* grown in a lactose-based medium (DPP). The yield and stability of corn oil-water emulsions containing 12 g $l^{-1}$ purified bioemulsifier extracted at various holding times were evaluated after 90 days. Values given are the mean of duplicate analyses. *DCW* Dry cell weight

| Holding time in autoclave (min) | Purified emulsifier yield (% DCW) | Emulsion phase (%) after 90 days at 4°C |
|---|---|---|
| 0 | 0 | 0 |
| 30 | 4.2 | 75 |
| 60 | 4.8 | 75 |
| 90 | 6.0 | 77 |
| 120 | 7.0 | 77 |
| 150 | 0.7 | 0 |

Carbohydrate and protein content

The carbohydrate content determined in the purified bioemulsifier at various extraction times was 90% in all cases while the protein content was estimated to be 4.2, 4.8, 6 and 7% at the extraction times of 30, 60, 90 and 120 min, respectively.

Molecular weight determination

Protein bands were not observed in the lower molecular weight region due to smearing by contaminating glucans and/or other polysaccharides of the cell envelope. However, even with contamination, bands were observed in the higher molecular weight region (66,000–97,00 Da) in proteins extracted for 30, 60, 90 and 120 min. No bands were observed for emulsifier extracted for 150 min, most likely due to protein denaturation or interference by polysaccharides.

Effect of bioemulsifier concentration

The effect of purified bioemulsifier concentration (1–50 g $l^{-1}$) extracted for 90 min at 121°C on the stability of the emulsion is presented in Table 2. The largest emulsion phase, which was stable for 90 days, was obtained with a concentration of 12.0 g $l^{-1}$. Increasing the amount of bioemulsifier did not produce any added benefits. No emulsion was formed at bioemulsifier concentrations of less than 4 g $l^{-1}$; however, emulsions formed at concentrations above this level were stable for 90 days.

**Table 2** Effect of bioemulsifier concentration extracted for 120 min at 121°C on the stability of corn oil-water emulsions. The percentage emulsion phase and stability were determined after 90 days at 4°C

| Bioemulsifier concentration of (g $l^{-1}$) | Emulsion phase (%) | Stability of emulsion after 90 days at 4°C |
|---|---|---|
| 1 | No emulsion | No emulsion |
| 2 | No emulsion | No emulsion |
| 4 | 60 | Stable |
| 6 | 65 | Stable |
| 8 | 70 | Stable |
| 10 | 73 | Stable |
| 12 | 76 | Stable |
| 16 | 74 | Stable |
| 20 | 74 | Stable |
| 30 | 74 | Stable |

### Effect of pH

The effect of pH (2–11) on the stability of emulsion containing 12 g $l^{-1}$ purified emulsifier indicated that the emulsion was stable only in the range 4–11. No emulsion was formed below pH 4 (Table 3).

### Effect of sodium chloride

Emulsions containing 2–50 g $l^{-1}$ NaCl were all stable. However, the volume of the emulsion phase decreased from 75–70% when the concentration of sodium chloride was increased from 2 g $l^{-1}$ to 50 g $l^{-1}$.

**Table 3** Effect of pH and NaCl on the stability of corn oil-water emulsions containing purified emulsifier (12 g $l^{-1}$) over a 90 day period. The percentage emulsion phase and stability after 90 days at 4°C were determined. The bioemulsifier was extracted for 120 min at 121°C from *K. marxianus* grown under batch culture in DPP medium at 30°C, pH 5.0, and dissolved oxygen concentration greater than 20%. Values are the mean of duplicate analyses

| Parameter | Emulsion phase (%)[a] | Stability of emulsion after 90 days at 4°C |
|---|---|---|
| pH | | |
| 2 | No emulsion | No emulsion |
| 4 | 72 | Stable |
| 6 | 72 | Stable |
| 7 | 76 | Stable |
| 9 | 78 | Stable |
| 10 | 76 | Stable |
| 11 | 76 | Stable |
| NaCl (g $l^{-1}$) | | |
| 2 | 75 | Stable |
| 5 | 74 | Stable |
| 10 | 72 | Stable |
| 20 | 72 | Stable |
| 30 | 71 | Stable |
| 40 | 70 | Stable |
| 50 | 70 | Stable |

## Discussion

Liquid heat extraction by autoclaving allowed the isolation of crude emulsifier from cells of *K. marxianus*. The yield of both crude and purified emulsifier varied with extraction time from 7–14% and 4.2–7% of the cell dry weight, respectively. From these yields, it is likely that the emulsifier consisted mainly of structural mannoproteins, as the mannan enzymes, which appear to be periplasmic, do not cosediment with cell wall fragments after mechanical breakage of the cells [17]. The yields of emulsifier were similar to those obtained from *S. cerevisiae* reported by Cameron et al. [17].

The best conditions for extraction of the mannoprotein of *K. marxianus* were autoclaving of the cell suspension for 120 min at 121°C. Extracting emulsifier for longer than 120 min caused denaturation of mannoprotein molecules and elimination of its emulsification properties. This observation is in agreement with that of Torabizadeh et al. [51], who showed that the optimum time for *S. cerevisiae* bioemulsifier extraction was 120 min at 121°C.

The purified bioemulsifier was tested for its stability over a range of chemical and physical conditions that might be encountered in various applications. The relationship between the fraction of the corn oil phase emulsified and the concentration of purified emulsifier (Table 2) indicated that a maximum of 76% of the corn oil phase could be emulsified with 12 g $l^{-1}$ purified emulsifier. The use of more emulsifier beyond this concentration did not result in increased emulsification of the corn oil phase. The concentration of bioemulsifier used could be significant for industrial applications due to the high cost that may be associated with the use of large quantities, and when making a choice between a bioemulsifier and a synthetic emulsifier.

When subjected to varying conditions of pH and sodium chloride concentrations, the results obtained (Table 3) suggest that the bioemulsifier extracted from *K. marxianus* is stable in oil-in-water emulsions over a wide range of physical and chemical conditions. It may therefore have commercial applications in the production of foods such as mayonnaise, biscuits, crackers, cake, ice cream and meat products such as sausages, and other food products [51].

After purification, preliminary investigations indicated the emulsifying agent had several protein bands with molecular weights in the range 66,000–97,000 Da. This is in agreement with the findings of others [4, 6, 9, 16, 26, 54] who found large ranges of molecular weights from 25,000 to at least 1,000,000 Da. The mannose and protein in the emulsifier are necessary for its action as an emulsifier [17]. Furthermore, the presence of hydrophilic mannose polymers covalently attached to the protein backbone [21], provides the mannoprotein with the amphiphilic structure common to surface-active agents. Effective emulsifiers [22] have compositions in the range 90% carbohydrates and 4–6% protein.

The emulsification properties of the bioemulsifier from *K. marxianus* are similar to those of *S. cerevisiae* reported by Cameron et al. [17] and Torabizadeh et al. [51]. As an emulsifying agent, mannoprotein from *K. marxianus* grown on a lactose-based medium may present certain advantages over other yeast species. First, the yeast can be grown on whey, a clean waste of the dairy industry, which is rich in lactose. Second, the difficulty of removing residual hydrocarbons from bioemulsifiers from alkane-grown yeasts would preclude their use in certain applications such as food products. Since *K. marxianus* is classified as having a 'generally regarded as safe' (GRAS) status, it is expected that its mannoprotein bioemulsifier would be non-toxic. Third, it is stable over a wide range of pH from 3 to 11. Finally, it can be used in formulations containing a wide range of NaCl concentrations from 2 to 50 g $l^{-1}$

A novel bioemulsifier from *K. marxianus* has been successfully isolated and evaluated for its emulsification properties and potential use in the food industry, where emulsification plays an important role in forming the consistency and texture as well as in phase dispersion [8]. Furthermore, surface-active compounds are widely used in bakery and meat products, where they influence the rheological characteristics of flour and the emulsification of partially broken fat tissue [55]. Lecithin and its derivatives are currently in use as emulsifiers in the food industry worldwide while *Candida utilis* bioemulsifier is used in salad dressing [50].

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