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# Microbial production of single-cell protein from deproteinized whey concentrates

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**Abstract** Deproteinized sweet and sour cheese whey concentrates were investigated for their suitability as substrates for the production of single-cell protein with *Kluyveromyces marxianus* CBS 6556 up to a 100-l scale. An important factor for gaining high cell concentrations was the use of the Crabtree-negative strain *K. marxianus* CBS 6556. Supplements such as trace elements, ammonium and calcium were required for the complete conversion of sweet whey concentrates into biomass, whereas sour whey concentrates had to be supplemented with ammonium, trace elements and vitamins. After improvement, biomass dry concentrations of up to 50 g $l^{-1}$ could be reached with Yx/s values of 0.52 for sweet whey and of up to 65 g $l^{-1}$ with Yx/s values of 0.48 for sour whey concentrates. The chemical oxygen demand of the whey concentrates were reduced by 80%. The cells were used for the analysis of amino acid and ash composition, showing a distinct increase of eight out of ten essential amino acids compared to sweet and sour whey protein and exceeding the World Health Organisation guidelines for valine, leucine, isoleucine, threonine, phenylalanine and tyrosine.

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## Introduction

Cheese whey is a by-product of the dairy industry. Annually, 107 million tonnes of milk are produced in the European Union (Animal Health Online 2001; http://www.ticker-grosstiere.animal-health-online.de/20011004-00003/, http://www.animal-health-online.de/drms/rinder/ehs.htm) and 46 million tonnes of whey are produced in Europe (Sienkiewicz and Riedel 1990).

A frequently used option to exploit the worldwide enormous amount of whey is ultrafiltration to produce a protein concentrate. The filtrate (whey permeate) contains almost all of the total lactose and salts found in milk (Börgardts et al. 1998). Lactose can be isolated by crystallization, thereby representing approximately 70% of the whey solids. Another possibility is the deproteinization (by ultrafiltration) of whey, which results in lactalbumin and lactoferrin, proteins of pharmaceutical interest (de Wit 2001). For disposal of the remaining deproteinized whey, the chemical oxygen demand (COD) and therefore the costs for waste-water treatment are still high. A lactose content of up to 50 g $l^{-1}$ corresponds to a COD between 40,000 and 60,000 ppm, which may disrupt the biological process of sewage-disposal plants (Ghaly and Singh 1989).

These problems could be overcome by further use of deproteinized whey as substrate for micro-organisms and for the biotechnological production of compounds of worth such as "single-cell oil" (Daniel et al. 1999), biosurfactants, e.g. sophoroselipids (Otto et al. 1999), or "single-cell protein" (SCP), which is used as feed. As mentioned by Ghaly and Singh (1989), micro-organisms have to be able to metabolize lactose for this purpose, since it is the major constituent of whey.

A yeast of interest for single-cell protein production and used as feed organism in Germany [Futtermittelverordnung (FMVO), Anlage 1] is *Kluyveromyces marxianus* CBS 6556, which is considered as a "generally recognized as safe "(GRAS) organism (§4, BioStoffVO). It has been used for industrial purposes due to its physiological characteristics and bioproduct yields, in particular hydrolytic enzymes and food biomass (Belem and Lee 1998), ribonucleotides,

oligosaccharides and oligopeptides (Walker 1998). Furthermore, *K. marxianus* CBS 6556 was recently reported to be highly efficient in the expression of heterologous proteins (de Souza Galvão et al. 2001).

The aim of this study was the production of single-cell protein from deproteinized whey, comparing the use of pure deproteinized sweet or sour whey concentrates with lactose contents of up to 140 g $l^{-1}$ after sterilisation in a two-step filtration process as previously described in the literature (Daniel et al. 1999).

A problem that may occur at such high lactose concentrations is the production of ethanol at the expense of biomass formation, caused by the Crabtree effect (de Deken 1965). Ethanol formation by yeasts cultivated on whey at the expense of biomass formation was previously described in the literature (Willetts and Ugalde 1987). Van Urk et al. (1989) differentiate between Crabtree-positive and Crabtree-negative yeasts according to their glucose-uptake systems. For our experiments, we chose *K. marxianus* CBS 6556, a strain described as a Crabtree-negative yeast in the literature (van Urk et al. 1989).

Here we present results on the question of supplementation of sweet and sour whey concentrates for total conversion of the lactose to biomass and the composition of the single-cell protein produced in regard to its content of essential amino acids.

## Materials and methods

### Micro-organisms

*K. marxianus* CBS 6556 [taxonomic synonym, *Kluyveromyces fragilis* (A. Jörgensen) Van der Walt] was obtained from the Centraalbureau voor Schimmelcultures (CBS, Delft, the Netherlands).

### Media

*K. marxianus* CBS 6556 was cultivated on Petri dishes, using yeast–malt (YM) medium with the following composition: 5 g $l^{-1}$ peptone, 3 g $l^{-1}$ malt extract, 3 g $l^{-1}$ yeast extract, 10 g $l^{-1}$ glucose, 20 g $l^{-1}$ agar. Moreover, agar plates with sweet and sour whey were used for cultivation; 300 ml sweet whey contained 5 g $l^{-1}$ agar and 300 ml sour whey contained 10 g $l^{-1}$ agar.

Deproteinized sweet whey concentrates (DWC 20, 20% dry weight after water evaporation) were obtained from Milei GmbH (Leutkirch-Adrazhofen, Germany). DWC 20 contained approximately 120 g $l^{-1}$ lactose, 2.5 g $l^{-1}$ L-lactate, 400 mg $l^{-1}$ $NH_4^+(NH_4)_2SO_3$, 1.2 g $l^{-1}$ calcium (pH ~5.6). Mother liquid, deproteinized sour whey concentrate (140 g $l^{-1}$ lactose, 28 g $l^{-1}$ L-lactate, 400 mg $l^{-1}$ $NH_4^+$ $(NH_4)_2$ $SO_3$, 4.8 g $l^{-1}$ calcium, pH ~4.5) and sour whey concentrate (198 g $l^{-1}$ lactose, 13 g $l^{-1}$ L-lactate, 1.2 g $l^{-1}$ $NH_4^+$ $(NH_4)_2SO_3$, 3 g $l^{-1}$ calcium, pH ~5.5) were obtained from Nordmilch eG (Edewecht, Germany). The sterilisation of the whey was performed as described by Daniel et al. (1999). Trace elements and vitamin solutions were prepared according to Verduyn et al. (1992).

### Preparation for cultivation in a bioreactor

*K. marxianus* CBS 6556 was grown on agar plates with YM medium [3 g of yeast extract, 3 g of malt extract, 5 g of peptone, 10 g of glucose, 15 g of agar in 1,000 ml distilled water (Medium 186 DSMZ)] at 30°C and stored at 4°C.

Preculture 1 was made in a 250-ml shaking flask with four buffles and filled with 100 ml whey to which was added one inoculation loop of *K. marxianus* CBS 6556 for starting growth. After inoculation, the 250-ml shaking flask was incubated for 24 h with agitation at 75 rpm (New Brunswick Scientific, USA).

Preculture 2 was made in a 1,000-ml shaking flask with four buffles and filled with 400 ml whey. For the inoculation, 5 ml of preculture 1 was used followed by incubation of the 1,000-ml shaking flask for 16 h with agitation at 75 rpm.

For the inoculation of deproteinized sour whey concentrate and sour whey concentrate, 40 ml of preculture 1 was added to preculture 2. The 1,000-ml shaking flask was incubated for 24 h with agitation at 75 rpm.

*K. marxianus* CBS 6556 was cultivated at 30°C in a 30- or 100-l stirred bioreactor equipped with a pH, aeration, weight, temperature and agitation control (Bioengineering, Wald, Switzerland). The pH adjustment was made with 10% (w/w) NaOH (or 10% $H_3PO_4$ in the case of sour whey) and 25% (v/v) ammonia solution ($NH_3$) and kept at 5.8 in the case of sweet whey or 4.8 in the case of sour whey. The $pO_2$ value was adjusted maximally to 50% and minimally to 10%. The mixing speed was 200–1000 rpm and the airflow was 11–15 N l/min. The medium consisted of 13.5- or 55-l filter-sterilised DWC 20. Furthermore, 1.5 or 5.5 l of *K. marxianus* CBS 6556 preculture 2 was used to inoculate the reactor. The total volume added into the reactor was 15 or 60 l, respectively. Deproteinized sour whey concentrate was similarly used for the cultivation in a 30-l scale.

### Analytical assays

To examine the production of ethanol and the consumption of lactose in samples of the yeast culture during fermentation, commercially available enzymatic test kits were used (Boerhinger Mannheim, Germany).

Colorimetric test kits to control the decrease in calcium (Aquamerk-testkits, Merck, Darmstadt, Germany) and nitrogen (LCK 303 cuvette test, Dr. Lange, Hegnau, Switzerland) were also obtained commercially.

A flame ionisation detector (FID, Ratfisch, Germany) that was compatible with the bioreactor was used to analyse the ethanol content in the bioreactor outgoing gas stream.

Chemical oxygen demand was determined in a water research plant (Stuttgart-Büsnau, Germany) according to the literature (Deutsches Einheitsverzeichnis, DIN 38409,

Teil 1, H 41, Bestimmung des chemischen Sauerstoffbedarfs in Bereichen über 15 mg/l).

Cell growth kinetics was determined by measuring the increase of the cell dry mass during growth. Five-milliliter samples were added to preweighed tubes and immediately centrifuged for 10 min at 4,000 rpm (Megafuge 1.0, Fa. Heraeus Sepatech, Hanau, Germany). After two washing cycles (with 0.9% NaCl solution) the resulting pellet was dried in an oven at 105°C for 24 h. In addition, the optical density of the culture was measured at 620 nm in a spectrophotometer [Biosience, Ultrospec 2100 pro (UV/Vis) photometer].

### Amino acid and ash analysis of single-cell protein

The amino acid composition of the SCP from sweet and sour whey was determined commercially as described in the literature (Spackmann et al. 1958) by the workgroup of Prof. Dr. Henle at the Institute of Food Chemistry, Technical University of Dresden, Germany. The ash analysis (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten [VDLUFA] 1985) was performed by Milei for DWC 20 products and by Nordmilch for deproteinized sour whey concentrates.

## Results

For the successful production of SCP from sweet and sour whey with high Yx/s yields and high cell dry weights, the use of the Crabtree-negative strain *K. marxianus* CBS 6556 was particularly suitable. By using this strain we avoided the production of ethanol (Crabtree effect) provoked by the high amount of lactose in DWC 20 (deproteinized sweet whey concentrate) at the expense of biomass cell production. The Crabtree-positive strain *K. marxianus* DSM 5420 showed ethanol production of up to 10 g $l^{-1}$ on DWC 20 in an aerated bioreactor at $pO_2$ values of 50%. Under these conditions, growth was ended at batch and fed-batch cultivations at a dry biomass concentration of 25 g $l^{-1}$ and a Yx/s value of 0.18 (data not shown).

After improvement of the whey for *K. marxianus* CBS 6556, dry biomass concentrations of up to 50 g $l^{-1}$ could be reached with Yx/s values of 0.52 for sweet whey and of up to 65 g $l^{-1}$ with Yx/s values of 0.48 for sour whey concentrates. The optimal supplementation of sweet whey was 500 mg $l^{-1}$ $(NH_4)_2SO_4$, 1 g $l^{-1}$ $CaCl_2$ and 2.5 ml $l^{-1}$ trace element solution. The optimal supplementation of sour whey was 1 ml $l^{-1}$ trace element solution, 2.5 ml vitamins $l^{-1}$ and 1.5 g $l^{-1}$ $(NH_4)_2SO_4$. For comparison, the cultivation on DWC 20 without any supplementation ended growth at a dry biomass concentration of 25 g $l^{-1}$ Yx/s (efficiency yield) with 80 g $l^{-1}$ lactose remaining in the medium (data not shown).

The cultivation of *K. marxianus* CBS 6556 on deproteinized sweet whey concentrate enriched with unspecified and non-optimal supplements and the corresponding decrease of the lactose concentration is shown in Fig. 1. Between 20 and 26 h, the growth of *K. marxianus* CBS 6556 was very slow and the optical density remained at a value between 40 and 45. During this constant period, pulse experiments with $(NH_4)_2SO_4$ solution (250 mg $l^{-1}$), vitamins (2.5 ml $l^{-1}$), magnesium (0.2 g $l^{-1}$) and trace elements (2.5 ml $l^{-1}$) were done but they produced no positive effect on cell growth. The pulse with $(NH_4)_2SO_4$ solution was given sterile and at first to the bioreactor. The ammonia concentration in the culture broth became limiting at 20 h because it decreased from around 250 mg $ml^{-1}$ ammonia nitrogen to 4 mg $l^{-1}$ in the medium. The sharp increase in growth at 32 h cultivation time was due to a pulse of calcium (0.2 g $l^{-1}$) and to a second pulse with $(NH_4)_2SO_4$ (2 g $l^{-1}$) at the cultivation time of 31 h. That growth was accompanied and proved by a significant decrease in the oxygen partial pressure in the bioreactor (data not shown) and took almost 50 h until lactose was completely consumed (Fig. 1).

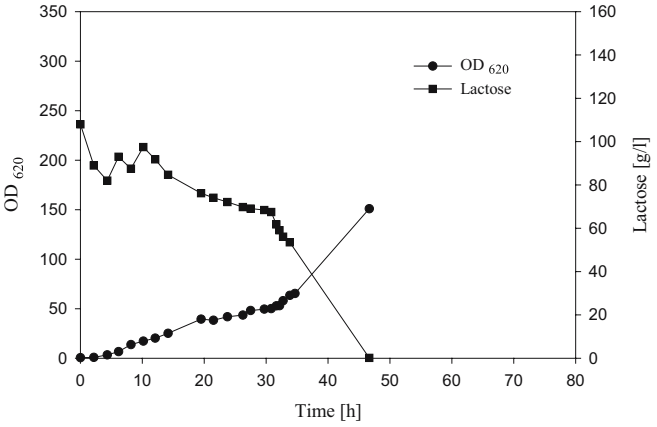


**Fig. 1** Lactose consumption during the growth of *K. marxianus* CBS 6556 on sweet whey enriched with unspecified and non-optimal supplements (cultivation 1)

In comparison to the growth of *K. marxianus* CBS 6556 shown in Fig. 1, the growth on optimally and minimally supplemented sweet whey is shown in Fig. 2. The growth under optimal conditions was exponential until lactose was consumed, which took around 30 h. A temporary lack of ammonia explains the break in growth observed at the cultivation time between 20 and 23 h.

Figures 3 and 4 show the influence of supplements on the growth curves of *K. marxianus* CBS 6556 on deproteinized sour whey concentrate and on deproteinized and supplemented sour whey concentrate, respectively. Supplementation experiments were done as described above for the case of sweet whey. The cultivation in the bioreactor shown in Fig. 3 was done only with the supplementation of vitamins (2.5 ml $l^{-1}$) at the 24th hour and no other supplements like $(NH_4)_2SO_4$ were added. However, until the end of the fermentation, vitamins had no visible positive effect on growth. The cultivation lasted for more than 70 h until the lactose was completely consumed.

With the supplementation of trace elements (1 ml $l^{-1}$), vitamins (2.5 ml $l^{-1}$) and $(NH_4)_2SO_4$ (1.5 g $l^{-1}$) from the start of the cultivation, the growth of *K. marxianus* CBS

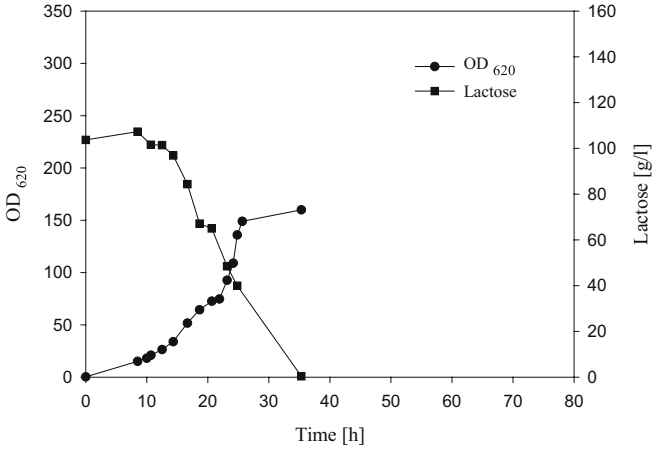


**Fig. 2** Lactose consumption during the growth of *K. marxianus* CBS 6556 on sweet whey with optimal supplements (cultivation 2)

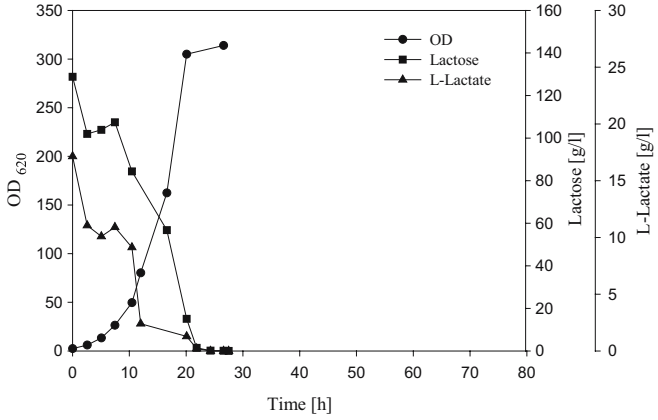


**Fig. 4** Lactose consumption during the growth of *K. marxianus* CBS 6556 on sour whey with supplements (cultivation 4)

6556 was optimal and was finished in less than 24 h (Fig. 4). Results from the experiments with deproteinized sour whey (Figs. 3 and 4) showed that without enough supplements the *K. marxianus* CBS 6556 initially seemed to consume L-lactate and then lactose, whereas with enough supplements the *K. marxianus* CBS 6556 consumed L-lactate and lactose at the same time. No further experiments were performed on this observation.

*K. marxianus* CBS 6556 can be grown in non-deproteinized sour whey concentrate supplemented with trace elements, and in this case vitamins were not needed (data not shown). For cultivation of *K. marxianus* CBS 6556 in deproteinized sour whey concentrate and in sour whey concentrate, the pH value should be kept below 4.9 to avoid the formation of crystals from calcium salts.

## Reduction of COD values in sweet and sour whey

Pure sweet whey has a COD of about 150,000 mg $l^{-1}$. After cultivation of *K. marxianus* CBS 6556, the COD value in the cell-free filtrate of sweet whey was reduced to about 15,000 mg $l^{-1}$, corresponding to a 90% total reduction of COD. Pure sour whey has a COD of about 193,000 mg $l^{-1}$. After cultivation of *K. marxianus* CBS 6556, the COD value in the cell-free filtrate of sour whey concentrate was reduced to about 32,533 mg $l^{-1}$, corresponding to a reduction of COD of around 83%. The probable reason for the still high remaining COD values is that *K. marxianus* CBS 6556 apparently does not consume the proteins of the whey concentrates.

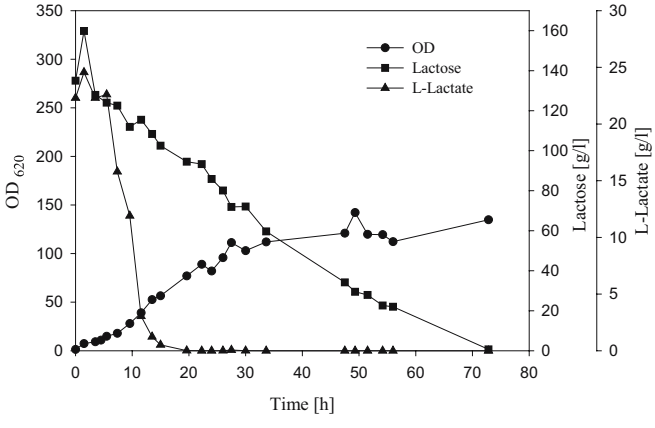


**Fig. 3** Lactose consumption during the growth of *K. marxianus* CBS 6556 on sour whey without supplements (cultivation 3)

## Ash composition in sweet and sour whey

Table 1 compares the ash composition of single-cell protein produced on deproteinized sweet whey concentrate (DWC 20) and on deproteinized sour whey concentrate to the ash composition of the cell-free supernatant of DWC 20 and of DWC 20 and deproteinized sour whey. Values in Table 1 are given in milligrams per 100 g, which is the amount of the elements of the ash (e.g. calcium) in 100 g of an examined sample of pure whey, supernatant or SCP. Deviations in the balance of the total ash composition of sweet and sour whey, cell-free supernatant and SCP in Table 1 are due to the pulse experiments of trace elements and other supplements like $(NH_4)_2SO_4$ in the bioreactor.

## Amino acid composition of sweet and sour whey

Table 2 compares the amino acid composition of DWC 20 and deproteinized sour whey with the produced single-cell protein and based on the standards set by the World Health Organisation (WHO) for single-cell protein. The composition of amino acids of the single-cell protein is shown in grams per 100 g of the weighed sample. A clear improvement of DWC 20 with regard to the content of essential amino acids was observed. Analysis of the amino acid composition of single-cell protein from DWC 20 shows an increase of eight essential amino acids compared to pure DWC 20. Furthermore, the guidelines of the WHO are

**Table 1** Ash composition (mg/100 g) of DWC 20 and deproteinized sour whey in comparison to the ash composition of single-cell protein produced from DWC 20 and deproteinized sour whey

| | DWC 20 | Supernatant (DWC 20) | SCP (DWC 20) | Deproteinized sour whey | SCP (deproteinized sour whey) |
|---|---|---|---|---|---|
| Calcium | 43 | 6 | 32 | 120 | 320 |
| Potassium | 1,007 | 948 | 759 | 150 | 30 |
| Sodium | 230 | 386 | 131 | 30 | 520 |
| Magnesium | 37 | 29 | 37 | <20 | 120 |
| Phosphorus | 212 | 203 | 370 | 70 | 350 |
| Chloride | 658 | 931 | 244 | ND | ND |

*ND* Not determined

positively exceeded by single-cell protein from DWC 20 in the case of valine, leucine, isoleucine, threonine and the aromatic amino acids phenylalanine and tyrosine. Methionine and histidine fall below WHO standards; however, they increased compared to the deproteinized sweet whey concentrate. When DWC 20 is used without supplements, single-cell protein is gained as well with almost the same percentage shares (data not shown). The production of single-cell protein on deproteinized sour whey concentrate resulted in the improvement of the five essential amino acids. Moreover, WHO standards were exceeded in the case of the five amino acids, valine, leucine, isoleucine, threonine and the aromatic amino acids phenylalanine and tyrosine.

## Discussion

A simple and especially efficient process by means of complete lactose consumption of up to 140 g $l^{-1}$ and Y x/s yields of 0.5 in around 30 h makes single-cell production from deproteinized sweet and sour whey concentrates attractive for industry. The application of the Crabtree-negative yeast (van Urk et al. 1989) *K. marxianus* CBS 6556 avoids the loss of lactose by conversion into ethanol and therefore is the main point in the production of SCP using concentrated whey. The simplicity of the described process with the *K. marxianus* CBS 6556 becomes clear when compared, for example, to processes where cultivation of yeast is started only on cheese whey saturated with oxygen (Ghaly and Kamal 2004).

For the complete consumption of lactose from deproteinized sweet and sour whey concentrates it is necessary to add supplements as shown in the results (Figs. 2 and 4). To avoid supplementation of whey and to save time and money it is possible to partly convert the lactose into SCP and to simplify drying thereby (Milei, personal communication), although the cultivation product still contained around 80 g $l^{-1}$ lactose. Nevertheless, the same promising composition of amino acids as with supplemented whey is achieved (Table 2). A lack of trace elements and stop in growth can be avoided by adding technical yeast extract as shown in a continuous process for the biological production of lactate from whey (Börgardts et al. 1998).

A fed-batch cultivation process to avoid the critical lactose concentration and the Crabtree effect resulted in the concentration of salts in the bioreactor and provoked the Crabtree-positive yeast *K. marxianus* DSM 5420 to produce even more ethanol (data not shown).

Analysis of the amino acid composition showed a distinct increase of eight out of ten essential amino acids compared to sweet and sour whey protein and exceeded the WHO guidelines (Moon et al. 1978) for valine, leucine, isoleucine, threonine, phenylalanine and tyrosine. Analysis of the amino acid composition of SCP generally show differences dependent on the yeast strain used for the cultivation (Sikka 1997) (in g 100 $g^{-1}$ protein): valine 5.1–6.6; leucine 5.9–9.9; isoleucine 3.5–7.3; methionine 1.2–3.5; threonine 4.6–7.0; histidine 1.5–4; tryptophan 0.5–1.7 (Spark 2004; Kihlberg 1972). The amino acid composition of the SCP produced by *K. marxianus* CBS 6556 and shown in Table 2 confirms these results. Generally, SCP from yeast is suitable for combination with feed from plants because of its high threonine content (Schiller et al 1972). The low content of sulphur-containing amino acids like methionine and cysteine might be a limiting factor when SCP from yeast is used in feed preparation (Mosenthin 1999; Spark 2004).

**Table 2** Amino acid composition (g/100 g protein) of sweet and sour whey in comparison to the amino acid composition of single-cell protein (SCP) produced from sweet and sour whey and to the guidelines of WHO

| Amino acid | Advice of WHO | DWC 20 | SCP (DWC 20) | Deproteinized sour whey | SCP (deproteinized sour whey) |
|---|---|---|---|---|---|
| Valine | 5 | 4.5 | 6.89 | 6.82 | 7.5 |
| Leucine | 7 | 5.07 | 7.62 | 5.82 | 7.74 |
| Isoleucine | 4 | 3.19 | 5.07 | 6.71 | 5.48 |
| Methionine | 3 | 0.73 | 0.95 | 1.11 | 0.77 |
| Phenylalanine | 6[a] | 1.95 | 3.67 | 2.42 | 3.58 |
| Tyrosine | 6[a] | 0.85 | 2.45 | 1.52 | 2.5 |
| Threonine | 4 | 5.47 | 7.45 | 9.2 | 6.94 |
| Histidine | 5.5 | 1.8 | 2.08 | 1.87 | 1.9 |
| Tryptophan | 1 | ND | ND | ND | ND |

[a]The recommendation is for the concentration of phenylalanine and tyrosine together
*ND* Not determined

The ash composition of yeasts varies and is dependent on the use of mineral buffers and trace elements during the cultivation process (Schulz 1975; Schulz and Oslage 1976; Spark 2004). Yeasts in general are rich in phosphorus and have a pure content of calcium (Gutierrez et al. 1999).

In 2004, Nordmilch estimated the SCP produced from sour whey concentrates as economically profitable with a possible price of 0.47 euros $kg^{-1}$ in the market, whereas whey powder (35%) had a price of 1.2 to 1.5, bakers yeast 066, whey powder (12%) 0.45 and shred protein (43–44%) 0.23 euros $kg^{-1}$ (H.-P. Hansen, personal communication by Nordmilch). The profitability of SCP becomes clear as it belongs to one of the cheapest protein products in the market.

With the objective of developing a more economical and profitable way for the production of single-cell protein in industry, some proposals will be discussed: (1) Instead of calcium chloride, calcium citrate could be used to provide additional calcium and is and easily available C source for the growth of *K. marxianus* CBS 6556. (2) Ascorbate could be a healthy additive when single-cell protein is used as animal feed. (3) A further option could be to replace the batch process by a repeated-batch process to minimise expenses during the preculture and the cleaning of the bioreactor after each cultivation. (4) A further aspect could be the direct use of whey after the first sterilisation step, by transferring it to the bioreactor and using it for the cultivation.

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