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ORIGINAL ARTICLE

# Comparison of ethanol production from cheese whey permeate by two yeast strains

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**Abstract** The objective of the present laboratory scale experiment was to compare ethanol production by Kluyveromyces marxianus strain ATCC8554 and Candida kefyr ATCC 14245 from unconcentrated and concentrated cheese whey permeate. The results indicated that ethanol production was greater when using concentrated whey permeate (9.8% lactose) compared to unconcentrated whey permeate (4.9% lactose) by both the yeasts, especially in presence of growth supplements. The rate and extent of ethanol formation increased noticeably and partly linearly for both the yeasts with sharp and partly linear decrease in both lactose and Chemical Oxygen Demand (COD), especially after the first 10 h of fermentation; total time of fermentation was 60 h. The optimum pH and temperature conditions for ethanol production were 4.8 and 30º C respectively. Klu. marxianus strain had greater ethanol producing ability from cheese permeate whey than Can. kefyr.

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## Introduction

Dairy industries generate significant liquid waste, of which, cheese whey is the most abundant. Whey is the liquid resulting from the coagulation of milk and is generated from cheese manufacture. Sweet whey, with a pH of at least 5.6, originates from rennet-coagulated cheese production such as Cheddar, Mozzarella, Swiss and other hard cheeses. Acid whey, with a pH no higher than 5.1, is obtained from the manufacture of acid-coagulated cheeses viz., cottage. About 9 liter of whey is generated for every kilogram of cheese manufactured (Jelen 2003, Onwulata and Huth 2008). The composition of whey varies with the composition of milk, the variety of cheese made, and the cheese-making process employed. Cheese whey contains about 7% solids comprising of about 10–12% proteins, the rest being lactose (74%), minerals (8%) and fat (3%) (Morr 1989). The major whey proteins are α-lactalbumin, β-lactoglobulin, bovine serum albumin, and the heavy and light chain immunoglobulins. Other minor but important proteins include lactoferrin and lactoperoxidase (Onwulata and Huth 2008). Whey protein is used in many food applications because of its functionality and nutritive value. Whey may also include the proteose-peptone components, glycomacropeptides and low molecular weight products formed by the enzymatic degradation of the caseins during the cheese-making process (De Wit 1989). The world production of cheese whey is estimated at over $10^8$ tonnes per year yielding an important environmental pollution source (Grba et al. 2002, Ozmihci and Kargi 2007). Large volumes of cheese whey and permeates are produced annually in Iran from traditional and cheese making via

**Table 1** Physico-chemical characteristics of UF whey permeate supernatant[b]

| Day | pH | Dry matter, % | Specific gravity, g/cm$^3$[a] | Fat, % | Protein, % | Lactose, % |
|---|---|---|---|---|---|---|
| First | 6.4 | 6.9 | 1.028 | 0.05 | 0.90 | 4.91 |
| Second | 6.3 | 6.9 | 1.029 | 0.05 | 0.95 | 4.89 |
| Third | 6.0 | 6.7 | 1.028 | 0.06 | 0.90 | 4.90 |
| Fourth | 5.9 | 6.8 | 1.027 | 0.07 | 0.85 | 4.92 |
| Fifth | 6.1 | 6.8 | 1.026 | 0.06 | 0.90 | 4.91 |
| Sixth | 6.1 | 6.7 | 1.028 | 0.06 | 0.92 | 4.90 |
| Average | 6.0 | 6.8 | 1.028 | 0.05 | 0.90 | 4.90 |

[a]Specific gravity determined at 20 °C ($n$=2)

[b]each value is a mean of 3 replicates

ultrafiltration. Their combined annual production is $1.15\times10^6$ tonnes (Koushki 2009). The disposal of whey remains a significant worldwide problem for the dairy industry. Whey is a potent pollutant with a biological oxygen demand (BOD) of 35–45 kg/l (Onwulata and Tomasula 2004) and chemical oxygen demand (COD) of about 60–80 kg $m^{-3}$ (Marwaha and Kennedy 1988). Today, whey is evolving into a sought-after product because of the nutrients it contains as well as the functional properties it imparts to food (Onwulata and Tomasula 2004). Several value-added products obtained from whey include single cell protein, ethanol, organic acids, biopolymers and biodegradable plastics. Cheese whey has been used as an inexpensive and nutritionally rich raw material for ethanol production (Marwaha and Kennedy 1988, Ozmihci and Kargi 2008). Ethanol production from cheese whey has been studied by many investigators due to high carbohydrate content and availability of cheese whey (Moulin et al. 1980, Mahmoud and Kosikowski 1982, Domingues et al. 2001, Grba et al. 2002, Kourkoutas et al. 2002, Silveira et al. 2005, Zafar and Owais 2006, Kargi and Ozmichi 2006, Ozmihci and Kargi 2007). The distillation cost for ethanol separation from dilute fermentation broths (2–3% ethyl alcohol) is expensive in fermenting whey by yeast (Ozmihci and Kargi 2008). It is clear that many yeast strains are not capable of fermenting lactose to ethanol. Most of the *Saccharomyces* species cannot ferment lactose to ethanol due to the lack of galactose fermenting enzymes (Kargi and Ozmichi 2006). *Kluyveromyces* species and partly *Candida kefyr* (formerly *Candida pseudotropicalis)* have been widely used to ferment lactose in whey to ethanol. This ethanol is used in pharmaceuticals, perfumes, inks, vinegar, alcoholic beverages, etc. In Comparison to the production of ethanol from glucose by traditional *Sacch. cerevisiae* strains, organisms fermenting lactose exhibit inferior production rates and yields (Terrel et al. 1984, Coté et al. 2004). Typical ethanol yield from lactose is reported as 80–85% of theoretical (Mawson 1994) and higher yield of up to 93% is favorable (Ingledew 1995). In this experiment, unconcentrated and concentrated cheese whey was used because direct fermentation of cheese whey with low lactose content (3–5% *w*/*v*) led to low ethanol concentrations (2–3% *v*/*v*) at the end of fermentation. These two yeasts have been studied individually (Zafar and Owais 2006, Ghaly and El-Taweel 1997), but in none of the literature studies their fermenting ability has been compared. Thus, the present study was undertaken to investigate which out of *Klu. marxianus* or *Can. kefyr* is best suited for conversion of whey into ethanol.

**Table 2** Ethanol production efficiency in normal and concentrated whey permeate supernatant (WPS) added (a) with and (b) without growth supplements[a]

| Yeast and medium | Lactose, % | | Lactose utilized by yeast, % | Ethanol production, % | | Alcohol production efficiency, % |
|---|---|---|---|---|---|---|
| | | | | w/v | v/v | |
| *Klu. marixanus* in WPS | a | 4.9 | 96.2 | 2.2 | 2.8 | 88.6 |
| | b | 4.9 | 88.2 | 2.0 | 2.5 | 86.2 |
| *Klu. marixanus* in concentrated WPS | a | 9.8 | 96.5 | 4.6 | 5.8 | 91.3 |
| | b | 9.8 | 88.5 | 4.1 | 5.1 | 87.5 |
| *Can. kefyr* in WPS | a | 4.9 | 94.2 | 2.0 | 2.5 | 80.6 |
| | b | 4.9 | 88.1 | 1.8 | 2.2 | 75.9 |
| *Candida kefyr* in concentrated WPS | a | 9.8 | 94.0 | 4.0 | 5.0 | 80.8 |
| | b | 9.8 | 87.5 | 3.8 | 4.4 | 76.4 |

[a]each value is a mean of 3 replicates and different letters ( a & b) in the respective row are significantly different at $\alpha$=0.05

**Table 3** The effect of pH on ethanol production by yeasts in normal and concentrated whey permeate supernatant with growth supplements[b]

| *pH* | Primary lactose, %[a] | Utilized lactose by *Klu. marixanus*, % | Ethanol production by *Klu. marixanus*, % | | Alcohol production efficiency, % | Utilized lactose by *Can. kefyr*, % | Ethanol production by *Can. kefyr*,% | | Alcohol production efficiency, % |
|---|---|---|---|---|---|---|---|---|---|
| | | | w/v | v/v | | | w/v | v/v | |
| 4.1(N) | 4.9 | 91.6 | 2.1 | 2.6 | 86.4 | 89.5 | 1.8 | 2.3 | 78.2 |
| 4.1(C) | 9.8 | 91.1 | 4.1 | 5.1 | 85.1 | 89 | 3.6 | 4.5 | 76.8 |
| 4.8(N) | 4.9 | 99.2 | 2.5 | 3.1 | 94.9 | 96.1 | 2.2 | 2.7 | 85.4 |
| 4.8(C) | 9.8 | 99.5 | 5.0 | 6.2 | 94.6 | 96.7 | 4.4 | 5.5 | 86.5 |
| 5.4(N) | 4.9 | 92.5 | 2.2 | 2.7 | 88.8 | 89.9 | 1.9 | 2.4 | 81.1 |
| 5.4(C) | 9.8 | 93.1 | 4.3 | 5.4 | 88.1 | 91.1 | 3.9 | 4.9 | 81.8 |
| 5.8(N) | 4.9 | 90.5 | 2.2 | 2.7 | 96.6 | 88.2 | 1.8 | 2.2 | 75.9 |
| 5.8(C) | 9.8 | 91.2 | 4.3 | 5.4 | 90.0 | 89 | 3.6 | 4.5 | 76.8 |

*N* Normal whey permeate supernatant, *C* Concentrated whey permeate supernatant

[a]Primary lactose content showed in the table was same for both the yeasts in the fermented whey

[b]each value is a mean of 3 replicates and different letters ( N & C) in the respective row are significantly different at $\alpha$=0.05

## Materials and methods

*Microorganisms Klu. marxianus* ATCC 8554 and *Can. kefyr ATCC 14245* obtained in lyophilized forms were used in all experiments. They were grown aerobically at 30±2 °C for 48 h. *Klu. marxianus* ATCC 8554 was maintained at 4 °C in nutrient broth containing 1 g/l of $KH_2PO_4$ as well as $(NH_4)_2SO_4$, 4 g/l of yeast extract, 5 g/l of $MgSO_4$ and 50 g/l of glucose (Fluka, Buchs, Switzerland). *Can. kefyr ATCC 14245* was also maintained at 25 °C in yeast mold agar (Merck, Germany). The synthetic media were sterilized at 121 °C for 15 min prior to use. The wet cells were harvested using a small centrifuge (Z 200A, Hermle Labortechnik, Germany) at 5000 rpm for 10 min and then were used.

*Substrate* Whey permeate from ultrafiltration (UF) based cheese making was obtained from Shiraz Dairy Company, Shiraz, Iran. UF whey permeate supernatant for ethanol production contained 6–7% solids, with a lactose content of between 45 and 55 g/l. Typically, volumes of 4 l were obtained and stored at 4 °C until used. The whey permeate then was heated in 250 ml Erlenmeyer flask for 15 min in 95 °C in order to coagulate its proteins. Next, it was filtered and the supernatant was collected. Half of the supernatant was concentrated at 60ºc and 93 mbar using a Rotary Vacuum Evaporator (Hei-Vap Precision, Heidolph, Germany) till the lactose content doubled. Higher supernatant concentrations reduced both the specific growth rate of the yeasts and the substrate utilization rate because of the

**Table 4** The effect of temperature on ethanol production by yeasts in normal and concentrated whey permeate supernatant with growth supplements[b]

| T, °C | Primary lactose,% [a] | Utilized lactose by *Klu. marixanus*, % | Ethanol production by *Klu. marixanus* ,% | | Alcohol production efficiency, % | Utilized lactose by *Can. kefyr*, % | Ethanol production by *Can. kefyr* ,% | | Alcohol production efficiency, % |
|---|---|---|---|---|---|---|---|---|---|
| | | | *w/v* | v/v | | | *w/v* | *v/v* | |
| *25(N)* | 4.9 | 93.5 | 2.2 | 2.7 | 87.9 | 92.1 | 1.9 | 2.4 | 79.2 |
| *25(C)* | 9.8 | 94.0 | 4.4 | 5.5 | 88.8 | 93.0 | 3.9 | 4.9 | 80.0 |
| *28(N)* | 4.9 | 98.5 | 2.3 | 2.9 | 89.5 | 93.8 | 2.0 | 2.5 | 81.2 |
| *28(C)* | 9.8 | 98.7 | 4.7 | 5.9 | 90.9 | 92.6 | 3.8 | 4.8 | 78.8 |
| *30(N)* | 4.9 | 99.2 | 2.5 | 3.1 | 94.9 | 96.1 | 2.2 | 2.7 | 85.4 |
| *30(C)* | 9.8 | 99.5 | 5.0 | 6.2 | 94.6 | 96.7 | 4.4 | 5.5 | 86.5 |
| *37(N)* | 4.9 | 87.8 | 2.0 | 2.5 | 96.5 | 89.3 | 1.8 | 2.3 | 78.2 |
| *37(C)* | 9.8 | 86.5 | 3.8 | 4.8 | 84.3 | 90.5 | 3.8 | 4.8 | 80.7 |

*T* Temperature, *N* Normal whey permeate supernatant, *C* Concentrated whey permeate supernatant

[a]Primary lactose content showed in the table was same for both the yeasts in the fermented whey

[b]each value is a mean of 3 replicates and different letters (N & C) in the respective row are significantly different at $\alpha$=0.05

substrate inhibition phenomenon (Ghaly and El-Taweel 1994). The concentrated and unconcentrated samples were heated at 121 °C for 5 min, followed by cooling to 30 °C. For alcohol production by yeasts, two substrates were used namely (a) unconcentrated and concentrated supernatants as such, and (b) both supernatants supplemented with growth substances viz., ammonium hydroxide ($NH_4OH$, 0.07%) and monoammonium phosphate ($NH_4H_2PO_4$, 0.25%) (Merck, Germany) (Fox 1982). A control flask free of yeast cells was used to determine any ethanol formation or sugar utilization in the absence of yeast cells.

In order to determine the effects of pH and temperature on ethanol production, both the yeasts were exposed to varying pH (4.1–5.8) and temperature (25–37ºc) in unconcentrated and concentrated whey permeate supernatant. The pH of UF permeate supernatant was adjusted to 4.5±0.1 using concentrated citric acid (Merck, Germany) since growth substances as basic salts did not make any difference in pH adjustment (Ghaly and El-Taweel 1997, Fox 1982). The fermented broth was transferred to the vessel up to 3/4 of its capacity for distillation. The distilled product, having an alcoholic concentration of ~50%, was poured into glass bottles. Theoretically, 1 pound of lactose would yield 0.538 pound of ethanol (Ling 2008). In other words, the lactose in whey (~ 5%) would yield ~2.5% ethanol assuming 100% efficiency. Alcohol production efficiency is produced alcohol per theoretically maximum produced alcohol multiplying by 100.

*Chemical analysis of whey* All the chemical parameters were measured at intervals of 6 successive days. The pH value of UF permeate supernatant was determined at 20 °C by a pH meter, (Model-pH 209 Hanna Instruments, Portugal). Its Specific gravity was determined using Pycnometer (Glacier Glass, India) at 20 °C. Fat content of whey was determined by Gerber method (ISO 2008b) using skimmed milk butyrometer

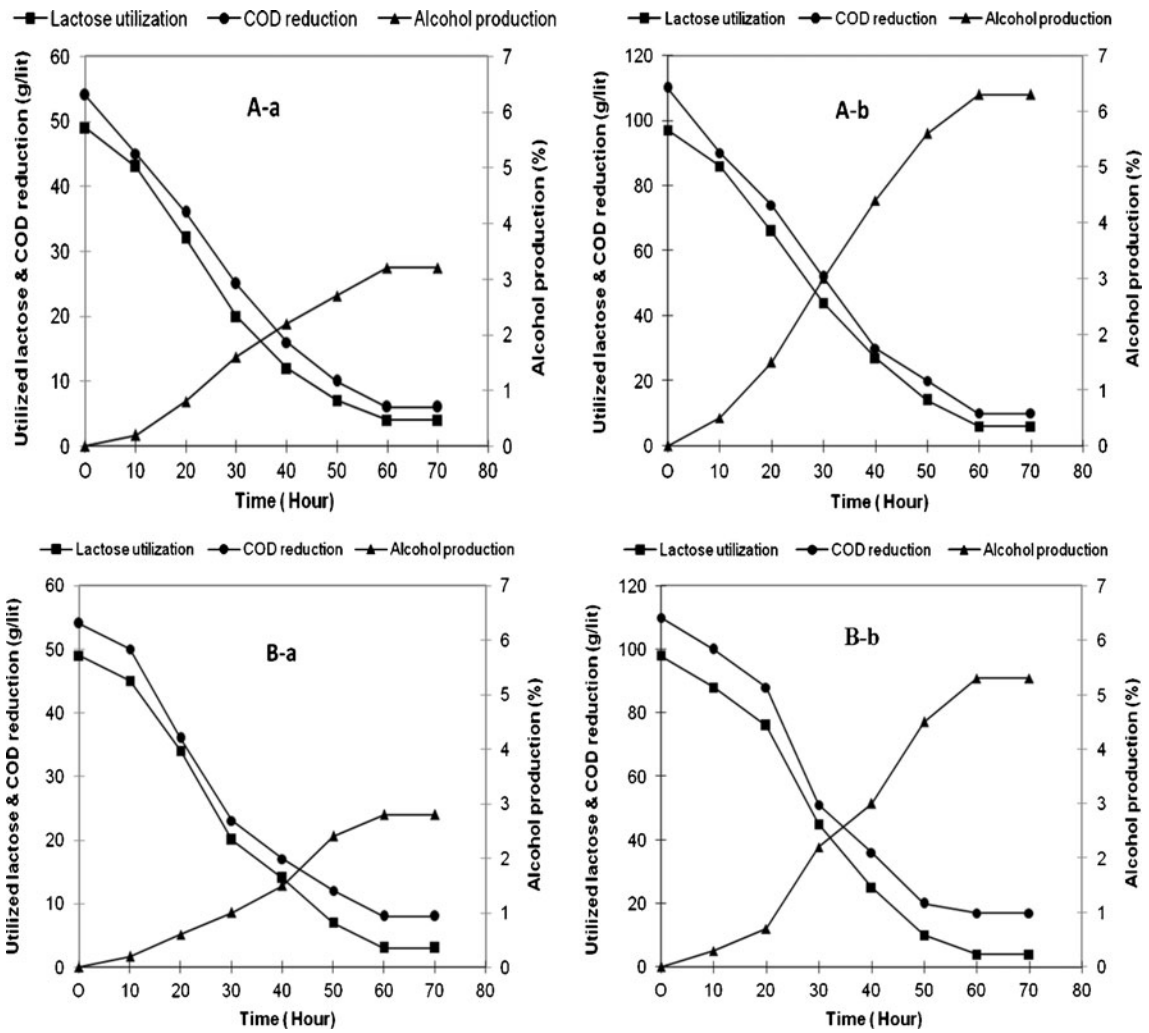


**Fig 1** Alcohol production, lactose utilization and COD reduction factor for **a** *Klu .marixanus* and **b** *Can. kefyr* in (*a*) normal and (*b*) concentrated whey permeate supernatant

based on the recommendation of ISO on Gerber butyrometers (ISO 2008a). Lactose and protein content of UF permeate supernatant was measured using Lane-Eynon (ISO 1981) and Kjeldahl method (ISO 2001), respectively. Dry matter of whey was determined by reference method (ISO 2004). Its COD was measured using ISO (1989) method.

*Statistical analyses* All measurements were repeated 3 times on each treatment. The analysis of variance (ANOVA) was used to analyse the data (Steel and Torrie 1980). The means obtained from each set were compared using the Duncan's multiple's range test at 0.05 confidence level (Duncan 1955).

## Results and discussion

The result of the chemical parameters determined in UF supernatant (WPS) is shown in Table 1. The composition was practically unaltered up to 6 days. The ethanol production by the two yeasts strains in unconcentrated (4.9% lactose) and concentrated (9.8% lactose) WPS added with or without growth supplements were studied. We expected increased ethanol production from whey concentrated up to reasonably higher total solids and then decreased for larger cheese whey concentrations due to substrate inhibition at high lactose concentrations (Ozmihci and Kargi 2008). No ethanol formation and sugar utilization was observed in the control flask. Comparing the results of Table 2, it is evident that presence of growth supplements resulted in an increased ethanol production by both the yeasts. The recent study well established that direct fermentation of CW to ethanol yields low ethanol concentrations because of low lactose content and therefore, is not economical (Ozmihci and Kargi 2008).

The rate of lactose utilization and ethanol formation increased linearly with increasing solids in whey (Ozmihci and Kargi 2007). Although the amount of lactose utilized by *Klu. Marxianus* and *Can. kefyr* in normal and concentrated WPS was almost the same, both the yeasts produced substantially more ethanol in concentrated WPS than in normal one (Tables 2 and 3). This result is in agreement with other workers (Ghaly and El-Taweel 1997, Zafar and Owais 2006). However, Klu. *marxianus* exhibited higher alcohol production efficiency over *Can. kefyr* in normal and concentrated WPS, irrespective of presence of growth supplements. The alcohol produced by *Klu. Marxianus* and *Can. kefyr* in normal WPS (with growth supplements) was 2.2 and 2% (*w*/*v*) respectively, whereas the corresponding figures for concentrated WPS was 4.6 and 4.0% (*w*/*v*) respectively. The optimum pH and temperature for the yeasts were 4.8 and 30ºc respectively with regard to alcohol production efficiency (Tables 3 and 4). Alcohol production, lactose utilization and COD reduction with regard to use of *Klu. marxianus* and *Can. kefyr* in normal and concentrated WPS are depicted in Fig. 1. There was a slight reduction in the amount of lactose utilized by *Klu. Marxianus* as well as the *COD* factor within the first 10 h, limiting alcohol production. However, there was a sharp drop with time in utilized lactose and COD factor, whereas ethanol production by *Klu. Marxianus* showed a significant increase. This finding is in agreement with the results of Zafar and Owais (2006). Total fermentation time required for *Klu. Marxianus* as well as *Can. kefyr* was 60 h in normal and concentrated WPS. After 60 h, all the parameters studied remained constant. During the first 10 h there was a slight decline in lactose utilization and COD reduction curves, and slight increase in alcohol production curve in normal whey (Fig. 1b-a). Afterwards, there was a sudden and noticeable decrease in lactose consumption and COD reduction factor, and sudden and a corresponding sharp increase in alcohol production. There was a sharp drop and a significant rise in lactose utilization and COD reduction, and rise in alcohol production in concentrated WPS (Fig. 1b-b). Zafar and Owais (2006) reported an inhibitory effect of ethanol produced on the growth rate of yeast at specific rise in concentration of alcohol, slowing down the alcohol production.

## Conclusion

The use of microorganisms as catalyst is an alternative technology for biological treatment of cheese whey. Cheese whey constitutes an inexpensive and nutritionally rich raw material for production of ethanol by fermentation especially in developing countries like Iran. The conclusion to be drawn from the findings is that *Kluyveromyces marxianus* is more promising for ethanol production from whey permeate than *Candida kefyr.* The rate and extent of ethanol formation partly increased with increased solids concentration in UF permeate supernatant. The optimal conditions for alcohol production by the yeasts were pH value of 4.8 and temperature of 30 °C. In today's environmentally sensitive world, ethanol production can be a cost effective method of whey utilization and disposal.

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