

**ORIGINAL RESEARCH PAPER**

**BIOETHANOL PRODUCTION BY SOLID STATE FERMENTATION  
FROM CHEESE WHEY MIXED WITH BREWER'S SPENT GRAINS**

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Food by-products, whey mixed with spent grains are renewable resources which can be used as fermentation substrates for bioethanol production using selected *Kluyveromyces* spp. yeast strains. These food by-products have extensive results as wastes in food industry, are cheap and readily available sources and their use has also important benefit for the environmental protection. The ability of some *Kluyveromyces* spp. yeast strains (commercial starter culture and wild culture) to ferment the carbohydrates mixture from a complex fermentation substrate based on hydrolyzed brewer's spent grains and cheese whey was analyzed. Three brewer's spent grains (hydrolyzed) and cheese whey (heat treated) ratios (1:1, 1:2 and 2:1) were considered in the study. Studies have shown that using an optimum combination of fermentation substrate, respectively hydrolyzed brewer's spent grains and heat treated cheese whey in ratio of 1:2 have influence on yeast fermentation behavior and yield ethanol production.

**Keywords:** cheese whey, brewer's spent grains, renewable resources, bio-ethanol, fermentation yield

### **Introduction**

In 2006 the worldwide bio-ethanol production was estimated to be about 40 Mt (70% of which coming from Brazil and USA), with a steady increasing demand as requested, for instance, by the guidelines defined by Kyoto Protocol. Nowadays, the bio-ethanol is obtained by fermentation of vegetable biomass, essentially sugar cane and cereals; thus contributing to the observed increase of foodstuffs price. It is therefore, necessary to identify alternative renewable and non-vegetable sources (paper waste, agri-food waste i.e.) for bio-fuels production oriented especially on the use of organic wastes with low prices (Sansonetti *et al.*, 2009).

Cheese whey is a rich source of fermentable carbohydrates, i.e. lactose. Production of cheese whey in the world is estimated to be over 10<sup>8</sup> tons per year and is increasing. Cheese whey is an important source of environmental pollution because 10 L of this waste per 1 kg of cheese result from milk processing. Uncontrolled overflow in aquatic media causes increase of COD (chemical oxygen demand)

levels of  $80 \text{ g}\cdot\text{L}^{-1}$ , approximately. Composition of cheese whey is the following: 5-6% lactose ( $\text{wv}^{-1}$ ), 0.8-1% proteins, and 0.06% fats constituting an inexpensive and nutritionally rich raw substrate for ethanol production by fermentation using selected yeast strains. Cheese whey has been used by many researchers as the raw material for ethanol fermentations because of its high carbohydrate content and organic compounds availability (Tomás-Pejó *et al.*, 2009, Ozmihci and Kargi, 2008).

Malt spent grains is the higher amount of waste resulting in beer factories, obtained after wort filtration. This by-product is rich in carbohydrates, proteins, fibers and fats. *K. marxianus* yeast presents a considerable attention due to its desirable biotechnological properties, including a broad spectrum of sugars utilization and a good metabolic adaptability in simple fermentation media (Lane and Morrissey, 2010). *K. marxianus* species includes yeast strains, capable for growing and fermenting natural substrates (especially containing lactose), with good fermentation yields at temperatures above  $40^{\circ}\text{C}$ , exploited for a wide range of applications (Tomás-Pejó *et al.*, 2009, Kourkoutas *et al.*, 2002, Zafar and Owais, 2006) such as enzyme production, (Tomás-Pejó *et al.*, 2009, Guadix *et al.*, 2004, Ghaly and El-Taweel, 1995) cell protein synthesis (Tomás-Pejó *et al.*, 2009, Ghaly and El-Taweel, 1997) and ethanol production (Tomás-Pejó *et al.*, 2009, Banat *et al.*, 1992, Banat and Marchant, 1995, Banat *et al.*, 1996).

In this study, a complex fermentation substrate based on brewer's spent grains (BSG) (by-product from beer industry) and cheese sweet whey, after chemical and thermal treatments, mixed in different ratio, were used for ethanol production by solid state fermentation process (SSF) using two selected yeast strains, *Kluyveromyces* spp. KV3 (wild strain) and *K. marxianus* (commercial starter culture), in order to increase the yield of the ethanol production.

## Materials and methods

### *Microorganisms*

*K. marxianus* NCIM 3465 commercial yeast starter was purchased from Danisco Company (Singapore) as lyophilized culture. *Kluyveromyces* spp. KV3 is part of the Collection of microorganisms (acronym MIUG) of Bioalimnet Research Platform of *Dunarea de Jos* University of Galati, Romania. The stock reactivated cultures were preserved on 20% glycerol at  $-70^{\circ}\text{C}$ .

### *Fermentative substrates*

**Sweet cheese whey** was provided from S.C. Lacta S.A. Giurgiu and frozen at  $-18^{\circ}\text{C}$  temperature during transport to the processing. It is presented as an opalescent liquid, yellow-green with characteristic fluidity. Physico-chemical properties were determined in the Laboratory of Milk Analysis using the device MILK Milk-Lab (Milk-Lab UK Ltd.). The sweet cheese whey was used untreated and heat-treated at  $80^{\circ}\text{C}$  for 10 min.

**Brewer's spent grains** by-product was provided from S.C. Martens S.A., Galati, as waste resulting from malt wort production. It appears as a brown mass with granular aspect and high dry matter content. Brewer's spent grains were used after

acid hydrolysis using 0.4% concentrated sulfuric acid (brewer's spent grains: concentrated sulfuric acid ratio of 125:1) at 121°C, 1.2 atm (autoclave AE 75-DRY, RAYPA, Spain) for 60 minutes.

The fermentation medium were obtained by mixing, in ratio of 1:1, 75 g hydrolyzed brewer's spent grains and 75 g cheese whey (thermally treated or untreated). Ratios such as 2:1 (100 g hydrolyzed brewer's spent grains and 50 g cheese whey) and 1:2 ratio (50 g hydrolyzed brewer's spent grains and 100 g cheese whey) were also investigated. The medium was transferred in Erlenmeyer flasks and then sterilized at 121°C for 20 minutes and, after cooling, the pH was adjusted to 5.0 with solution of 1 N NaOH.

#### ***Yeast inoculum production***

The pure stock cultures were reactivated on cultivation on malt extract-agar medium (MEA, Sigma). The culture medium was sterilized in an autoclave at 121°C for 20 min, then it was poured on Petri dishes for solidification. Then the yeast biomass was spread on surface and incubated at 28°C for 72 h. In order to be used in the fermentation process, cells were suspended in sterile dilution solution (0.9% NaCl) in a concentration of  $10^5$ - $10^7$  cells/ml. A concentration of 5 % (v/w) was used for fermentative medium inoculation.

#### ***Solid state fermentation***

Alcoholic fermentation was carried out in 250 mL Erlenmeyer flasks, coupled with special fermentation valve, for 120 hours (5 days) at temperature of 28°C, weighed every 24 hours for the evaluation of the CO<sub>2</sub> released. The experiments were performed in duplicate.

#### ***Fermentation parameters analysis***

The ethanol content expressed in % (v/w) was calculated using the following equation (Bonciu *et al.*, 2010):

$$E, \% = (G_1 - G_2) \cdot 1.045 \quad (1)$$

G<sub>1</sub>, G<sub>2</sub> = weight of fermentation vessel, g

G<sub>1</sub> – G<sub>2</sub> = CO<sub>2</sub> released during fermentation, g/100 g

1.045 = coefficient for transformation in the ethanol

Reducing sugars were determined and expressed as glucose equivalent concentration by using dinitrosalicylic acid (DNS) method (Miller, 1959). A standard curve was used.

#### ***Statistical analysis***

Statistical analysis was carried out using Excel Microsoft Office 2007 software. For all analytical determinations, three separate samples were analyzed and mean and standard deviation values were calculated.

### **Results and discussion**

Physico-chemical properties of sweet whey raw material were analyzed. Various samples were subjected to heat treatment (pasteurization at 95 °C for 30 minutes) after which the precipitate was removed by filtration. Data presented in Table 1

show that the dry matter and lactose content do not change significantly during thermal treatment and filtration.

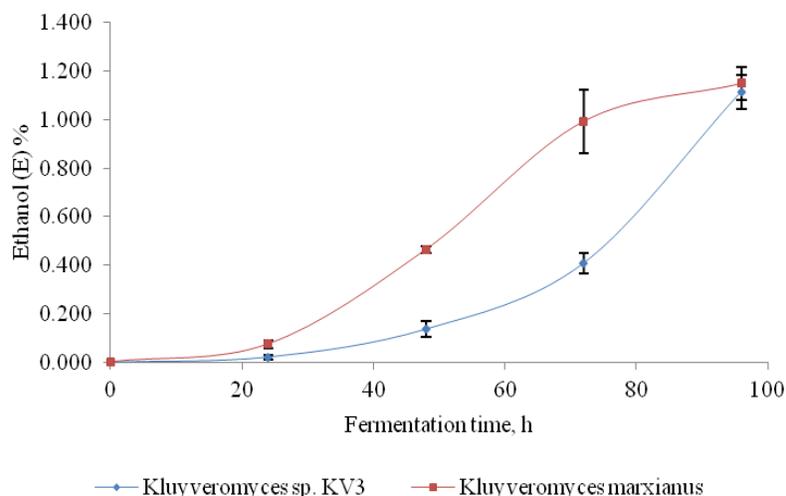
**Table 1.** Physical and chemical parameters of sweet whey

Physical and chemical parameters	Fat, %	Dry matter, %	Lactose, %	Proteins, %	Mineral substances, %
Raw whey	0.17	4.04	2.09	1.60	0.33
Whey after pasteurization, 95°C/30 min	0.00	3.36	1.66	1.33	0.26
Whey permeate	0.00	3.19	1.74	1.26	0.27

The sugar content, expressed as glucose, of hydrolyzed brewer's spent grains was 0.0021 g/g .

The fermentation ability of *Kluyveromyces* spp., commercial and wild strains, on solid state fermentation system, on medium based on sweet cheese whey (raw or heat-treated) and chemically hydrolyzed brewer's spent grains was analyzed.

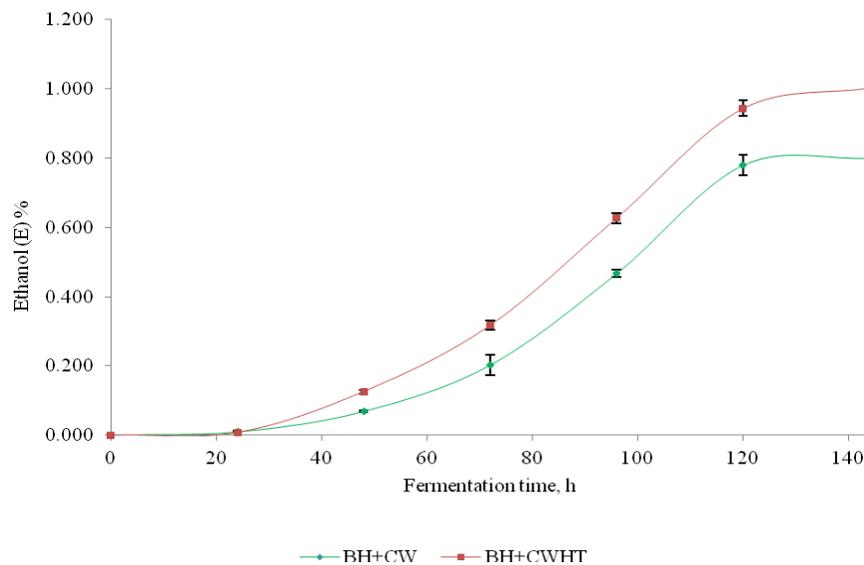
The *Kluyveromyces* KV3 strain shows a long lag period for metabolic adaptability comparing with commercial strain *K. marxianus*, but after 96 h of fermentation, the yield of ethanol is comparable for the both strains (Figure 1). The yield in ethanol of 1.15 g /100 g substrate was obtained with *K. marxianus* strain when the substrate was hydrolyzed and a brewer's spent grains to raw whey ratio of 1:1 was used.



**Figure 1.** The dynamics of the alcoholic fermentation of substrate based on hydrolyzed brewer's spent grains (BH) and raw whey (CW) (1:1)

A comparative fermentation behavior for *Kluyveromyces* KV3 strain by cultivation on media based on hydrolyzed brewer's spent grains (BH) and raw whey (CW) and permeate of cheese whey (CWHT) was studied (Figure 2).

It was observed an increase of the ethanol production with 16% when the heat-treated cheese whey was used. This is due to the organic nitrogen concentration which equilibrates the ratio of carbon content in the fermentation medium. Also, the fermentation progress has a long lag phase, the maximum of fermentation was obtained after 96-120 hours of fermentation.



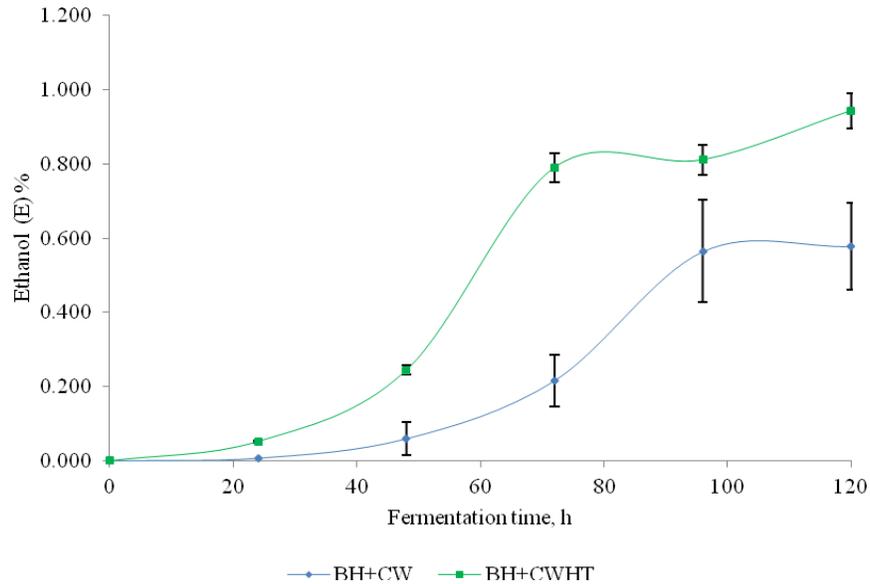
**Figure 2.** The alcoholic fermentation dynamic for *Kluyveromyces* KV3 strain on hydrolyzed brewer's spent grains (BH) mixed in 1:1 ration with raw whey (BH+CW) and heat-treated cheese whey (BH+CWHT)

The fermentation capacity of commercial yeast culture (*K. marxianus*) was analysed. The results are presented in Figure 3.

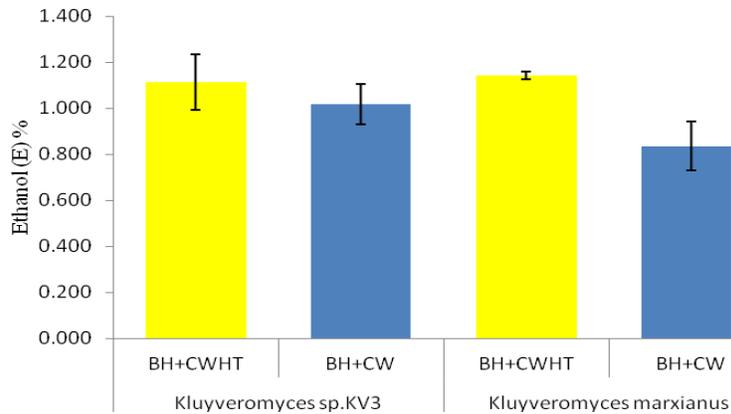
The dynamic of fermentation is similar on both media, with a high fermentation rate and yield, after 72 h, by cultivation on medium based on hydrolysed brewer's spent grains and heat-treated cheese whey in a ratio of 1:1.

The comparative yield of the ethanol production of two yeast strains on tested media based on by-product from brewery and cheese industries demonstrate the positive effect of use of permeate of whey, obtained after raw whey pasteurization and protein precipitate separation, in combination of 1:1 with hydrolyzed brewer's spent grains. The wild strain *Kluyveromyces* KV3 has a good fermentation potential, but requires a long adaptation period under the studied fermentation conditions. Commercial strain *Kluyveromyces marxianus* has an easy adaptation, but the alcoholic fermentation potential is reduced, probably this strain is selected

to ferment preferentially lactose, but complex medium can contain also glucose and maltose (Figure 4).



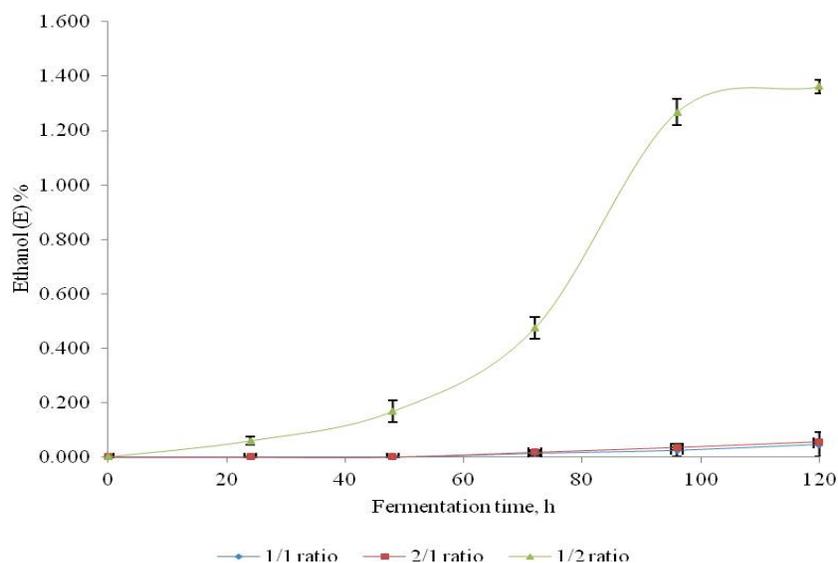
**Figure 3.** Comparative fermentation behavior of commercial *K marxianus* yeast culture on medium based on hydrolyzed brewer's spent grains (BH) and non-treated (CW) and heat-treated cheese whey (CWHT) in 1:1 ratio



**Figure 4.** The ethanol yield in solid state fermentation system, of commercial and wild *Kluveromyces* spp. cultures, cultivated on media based on hydrolyzed brewer's spent grains (BH) mixed in ratio of 1:1 with raw whey (BH+CW) and heat-treated whey (BH+CWHT)

The following study had in view to establish the optimal ratio between whey and hydrolyzed brewer's spent grains, of 1:1, 1:2 and 2:1. In this experiment, the commercial strain *K.marxianus* was used (Figure 5). As can be seen in Figure 5, the increase of the proportion of cheese whey enhances ethanol production. Increasing hydrolyzed brewer's spent grains ration has not a positive effect.

Several authors used raw cheese whey as substrate for ethanol production by selected *K. marxianus* strains. For instance, it was obtained a low fermentation yield, i.e. 11% in 22 h, (Sansonetti *et al.*, 2009, Zafar & Owais, 2006). Aryanti and Hadiyanto (2013) investigated the kinetics of ethanol production from crude whey through fermentation using *K.marxianus*. The yeast was able to metabolize most of the lactose within 16 h and produced 8.64 g/L ethanol and 4.43 g/L biomass; the residual lactose concentration was 3.122 g/L.



**Figure 5.** Dynamics of alcoholic solid state fermentation during *K. marxianus* cultivation on media based hydrolyzed brewer's spent grains and different proportion of heat treated whey (BH+CWHT)

The lactose content of the whey was 4.6% compared to 2% obtained in the present. Sansonetti *et al.* (2011) performed anaerobic batch fermentations of ricotta cheese whey (i.e. containing lactose) under different operating conditions. Ethanol concentrations of about 22 g L<sup>-1</sup> were obtained from whey containing about 44 g L<sup>-1</sup> lactose, which corresponded to up to 95% of the theoretical ethanol yield within 15 h.

The supplementation of the fermentescible sugars content by adding other sources of simple carbohydrates is a good alternative for increasing yield of ethanol and also yeast strains adaptability, fermentation rate and process efficiency.

## Conclusions

The alcoholic fermentation potential of two *Kluyveromyces* strains on media with hydrolyzed brewer's spent grains and whey (raw and permeate after whey pasteurization) was tested. The mix of these two food by-products has the target to obtain a medium with naturally balanced composition in carbon and nitrogen sources and also to increase the carbohydrates content in order to increase the yield of ethanol.

Besides of biotechnological properties of yeast strain, the results can be different from the point of view of adaptability, rate and yield of fermentation.

The results obtained are preliminary, the fermentation parameters have to be optimized in order to obtain a high efficiency of process in simple and cheaper biotechnological condition for agri-food wastes valorization for ethanol production with impact in environmental protection and natural resources utilization.

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