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Feasibility of the batch fermentation process of Ricotta Cheese Whey (RCW)

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The aim of the present work is to investigate the feasibility of bio-ethanol production by batch fermentation of Ricotta Cheese Whey (RCW). RCW is a dairy industry waste characterized by lactose concentration ranging from 4.5% to 5.0% (w/w) and by a lower protein content compared to the raw whey. This relatively high lactose concentration makes RCW a potential effective non-vegetable source for renewable energy production. The microorganism used to carry out the fermentation processes was the yeast *Kluyveromyces marxianus*. Preliminary experiments, performed in aerobic conditions on different volumes of RCW, have shown the actual growth of the yeast. The subsequent fermentation experiments were carried out, in anaerobic conditions, on three different substrates: RCW, raw cheese whey and deproteinized whey. The experimental data have demonstrated that RCW is an excellent substrate for fermentation and exhibits better performance (i.e. ethanol yield) with respect to both raw cheese whey and deproteinized whey. Complete lactose consumption, indeed, was achieved in considerably short time (13 hours) and with the highest ethanol yield (97% of the theoretical value). These results are clearly demonstrate process feasibility at lab-scale, which encourages further evaluation and optimization of the process in pilot and full-scale studies.

Introduction

RCW is a high pollution dairy industry waste characterized by high BOD and COD values of 50 g L^{-1} and 80 g L^{-1}, respectively. Erroneously, it could be regarded as a particular kind of cheese whey but, actually, it is a by-product obtained after Ricotta cheese production, and shows different characteristics with respect to raw cheese whey. It is estimated that Italian RCW production amounts to about 1.0 Mt per year, thus determining significant environmental problems related to its disposal (Gonzales-Siso, 1996).

Among all bio-fuels, bio-ethanol is definitely the most produced liquid biofuel. For example, in 2006 world-wide bio-ethanol production was estimated in about 40 Mt. Nowadays nearly all bio-ethanol is obtained by fermentation of vegetable biomasses,

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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 for bio-fuels production. RCW could potentially fit this requirement thanks to the significant content of sugar (lactose) and to its low cost, indeed – as a waste – it requires a proper (and costly) treatment for its disposal. The relatively high content of lactose (5%) could be converted into ethanol, according to the following overall reaction:

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow{yeast} 4CH_3CH_2OH + 4CO_2 \quad (1)$$

which predicts a theoretical yield equal to 0.538 grams of ethanol per gram of lactose consumed.

In the scientific literature, only few papers dealt with RCW and its possible utilization; none of them, however, identified RCW as a potential source for bio-ethanol production. Several authors actually analyzed raw cheese whey fermentation to ethanol. For instance it was verified that crude whey could be used to obtain bio-ethanol through lactose fermentation by *Kluyveromyces m.*; a rather low yield (Salman and Owais, 2005). The behavior of the same yeast was investigated in batch, fed-batch, and continuous fermentation processes, using cheese whey powder solutions as substrate (Kargi Fikret and Ozmihci Serphil, 2006; Ozmihci Serphil and Kargi Fikret, 2007a, 2007b, 2007c). Other researchers (Ghaly A.E. and El Tawel A.A, 1995) evaluated the effect of micro-aeration on cheese whey fermentation process performed by *Candida pseudotropicalis*. A kinetic study of *Kluyveromyces lactis* fermentation on raw cheese whey was performed to test the Monod equation and to assess the specific growth rate of microorganisms (Barba et al., 2001).

The present study is intended to investigate the feasibility of using RCW as a source for bio-ethanol production at lab-scale conditions. Ultimately it is aimed to reveal the differences existing between RCW and other kinds of substrates, namely raw cheese whey and deproteinized whey, which could be used as raw materials to achieve fermentation processes aimed at bio-ethanol production.

Materials & Methods

1.1 Yeast Strain

Lactose bio-conversion experiments were performed by a yeast, i.e. *Kluyveromyces marxianus. var. marxianus CBS 397*, isolated at the *Centraalbureau voor Schimmercultures, Utrecht, the Netherlands*. The yeast, initially freeze-dried, has been revived, seeded and maintained in a classical solid lactose-based yeast medium in *Petri dish*.

1.2 Inoculum medium

The inoculum medium was prepared with a single colony withdrawn from the *Petri* dishes and incubated in a *GRANT OLS 200* thermostated bath, maintained for 12 h at a temperature of 37°C with an orbital shaking velocity of 150 rpm. In all the experiments 100 mL of medium were poured in a 300 mL sterile flask. Each of the used materials, before performing this stage, was autoclaved at 121°C for 30 min. The inoculum

medium was constituted by lactose, 50 g L^{-1}, bactopeptone, 10 g $L^{-1}$and yeast extract, 5 g L^{-1}.

1.3 Fermentation medium

Three kinds of fermentation medium were used, i.e. RCW, raw cheese whey and deproteinized whey to assess the bio-ethanol yield. All the tested raw materials came from the same lot of cow milk. The deproteinization of raw cheese whey was performed by ultrafiltration (UF) through a cellulose membrane, *Nadir C005 Filtration*, having a nominal molecular weight cut-off of 5000 Da. All the samples, kindly provided by a local dairy industry, *Agroalimentare Asso.La.C., Calabria*, were stored in the fridge at +4°C; each fermentation test, however, was performed within 6 h from the production time.

1.4 Analytical methods

The samples were periodically withdrawn from either the flasks or the bio-reactor in aseptic conditions in order to determine, by HPLC, the time evolution of lactose and ethanol concentrations. A 0.1% v/v phosphoric acid solution was used as mobile phase at a flow rate of 0.5 mL min^{-1}. A *Supelcogel* 50x4.6 *mm* pre-column, a *Supelcogel C-610* 300x7.8 *mm* column and a refractive index detector, *Jasco RI 930*, constituted the experimental equipment. Biomass was evaluated by *BactoScan FC* (*Foss Integrator,Denmark)*, an instrument capable to determine, on the basis of an optical method, the number of cells contained per milliliter of solution. The amount of cells, on a mass basis, was obtained multiplying the cells concentration by 303 ng per cell (Ghaly A.E. and El Tawel A.A., 1994).

1.5 Experimental protocol

Although the inoculum medium was used to start all fermentations, a set of preliminary aerobic tests was carried out in order to assay the actual growth of *Kluyveromyces m.* in RCW. The microorganism growth experiments were performed withdrawing a single colony from a *Kluyveromyces m.* culture, contained in a *Petri* dish, and then inserting this colony in a flask containing a known volume of RCW. Four volumes of RCW were investigated, i.e. 50, 75, 100 and 150 mL. The volume range was chosen according to the widely-accepted consideration that the amount of fermentation starter should be equal to about 10% of the fermentation medium which, on a typical laboratory scale, is in the range 0.5-1.5 liters.

The flasks were placed in a thermostated bath and maintained for 12 h at a temperature of 37°C with an orbital shaking velocity of 150 rpm. A 100 µL sample was collected, every hour, from the bulk and poured in 25 mL of a 2 % sodium citrate solution and eventually analyzed to obtain the amount of biomass formed. The above-described steps were performed in aseptic conditions by instruments and tools previously kept in an autoclave at 121°C for 25 min.

The anaerobic fermentation experiments were performed in a controlled batch bio-reactor (*Fluka, Holland*) and had duration of 24 h and were carried out starting with 1 L fermentation medium in which 100 mL inoculum were dissolved. Each experiment was repeated twice to assess data reproducibility; the average concentrations of lactose, ethanol and biomass were taken into account and reported versus time, together with an "error bar" indicating the maximum variation of each measured point from the

corresponding calculated mean value. The fermentation operating conditions were as follows: temperature 37°C, stirrer velocity 200 rpm, pH 5, dissolved O_2 level ranging between 0 and 0.2%. The pH of reacting mixture was controlled by means of a 6N sodium hydroxide solution. Two samples of fermentation broth were withdrawn, every hour, during the experiment: a 100 µL sample was destined to the microorganism analysis, a 1 mL sample was, instead, centrifuged at 5000 rpm for 15 min, filtered through a 0.45 µm filter and finally sent to the HPLC for assaying the evolution of both lactose and ethanol concentration.

Results and Discussion

Fig.1 shows the biomass concentrations resulting from the growth experiments in RCW for each of the tested flask volumes.

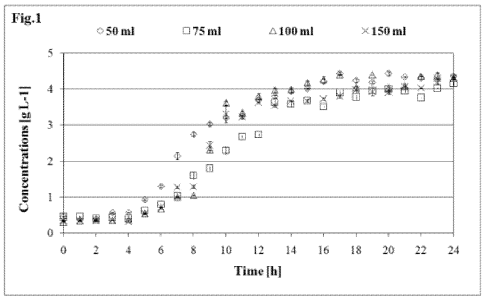

Fig.1 *– Time evolution of average biomass concentrations during aerobic fermentation of RCW (T = 37 °C, orbital shaking velocity=150 rpm).*

It is worthwhile to observe that after a lag phase of 4 hours the linear growth phase takes over. After 17 hours from the beginning of the experiment, the so-called stationary phase starts. These above results are of crucial importance to demonstrate the actual growth of *Kluyveromyces m.* in RCW and to prove that, in the considered range, volume does not affect significantly the system behavior.
Figs. 2-4 show the time evolution of lactose, ethanol and biomass concentrations with reference, respectively, to the fermentation of RCW, raw cheese whey and cheese whey permeate.

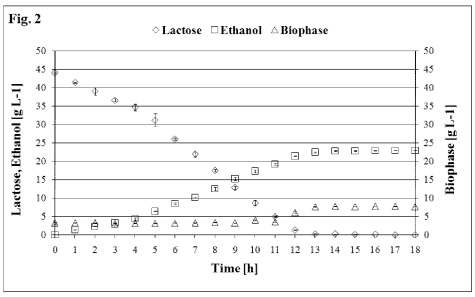

Fig.2 *–Anaerobic fermentation of RCW. Time evolution of lactose, ethanol and biomass concentrations (T = 37 °C, orbital shaking velocity=150 rpm, pH = 5, O_2 = 0 – 0.2%).*

In the case of RCW fermentation *(Fig. 2)*, lactose consumption goes to completion within 13 hours only, i.e. much earlier than it was reported for raw cheese whey fermentation [1]. Another remarkable result is the achieved ethanol concentration, 23 g L^{-1}, corresponding to a final yield equal to 97% of the theoretical one.

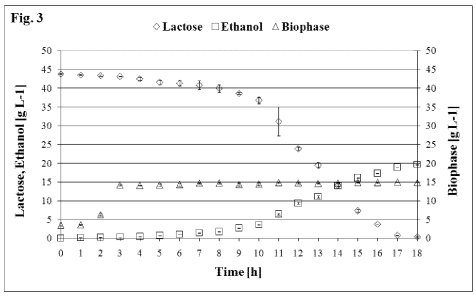

Fig.3 – Anaerobic fermentation of raw cheese whey. Time evolution of lactose, ethanol and biomass concentrations (T = 37 °C, orbital shaking velocity=150 rpm, pH = 5, O_2 = 0 – 0.2%).

Fig.3 shows the behavior of raw cheese whey fermentation. As compared with *Fig.2*, a higher biomass concentration; this phenomenon is to be ascribed to the characteristics of raw cheese whey that, being richer in nutrients (primarily proteins), allows an improved growth-for microorganisms. The higher yeast growth, however, corresponds to a lower ethanol yield, which is equal to about 83% of the theoretical one in the final stage of the experiment. It can be also observed that complete lactose consumption is attained only after 18 hours, 5 h later than what it was measured, in the same conditions, with RCW; finally, ethanol can be detected after 5 h, thus suggesting that process dynamics is delayed of about 4–5 h. *Fig.4* shows the behavior of cheese whey permeate as a fermentation substrate.

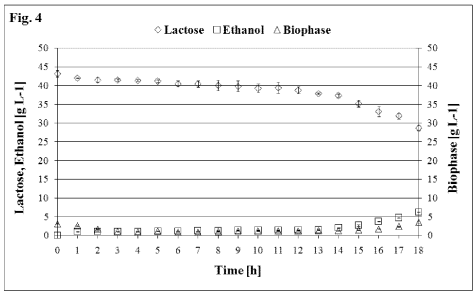

Fig.4 *– Anaerobic fermentation of cheese whey permeate. Time evolution of lactose, ethanol and biomass concentrations (T = 37 °C, orbital shaking velocity=150 rpm, pH = 5, O_2 = 0 – 0.2 %).*

Lactose consumption does not occur within the considered time interval; the reason could be somewhat ascribed to the very low proteins content, that does not allow the microorganisms to produce the molecules actually necessary to perform the fermentation process. The protein concentration in cheese whey permeate is, in fact, about a half of that of RCW; moreover, the two substrates have different concentrations of both salts and organic acids, which might also affect the process performance. As a matter of fact, cheese whey permeate, therefore, can be regarded as a poor fermentation substrate, as compared to both RCW and raw cheese whey.

Conclusions

This study has demonstrated the feasibility of RCW fermentation process to produce bio-ethanol by *Kluyveromyces m.* Furthermore, it was showed that RCW represents an excellent substrate since it allows attaining an ethanol yield of 97 %, very close to the theoretical one. Complete lactose consumption was observed after 13 hours for RCW as compared to 18 hours for raw cheese whey. As far as the fermentation process is concerned, RCW was found a promising alternative source to produce bio-ethanol. Moreover, it is worthwhile to remark that RCW is an industrial waste that could cause wastewater pollution thus harming the environment if not properly treated. This work has pointed out an alternative way to both dispose and valorize this dairy by-product.

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