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Bio-ethanol production by fermentation of ricotta cheese whey as an effective alternative non-vegetable source

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ABSTRACT

The aim of the present paper is to investigate the feasibility of bio-ethanol production by batch fermentation of ricotta cheese whey (''*Scotta*''), a dairy industry waste characterized by lactose concentration ranging from 4.5% to 5.0% (w/w) and, with respect to traditional (raw) whey, by much lower protein content. *Scotta*, therefore, could represent an effective non-vegetable source for renewable energy production. The microrganism used to carry out the fermentation processes was the yeast *Kluyveromyces marxianus*. Preliminary experiments, performed in aerobic conditions on different volumes of *scotta*, have shown the actual growth of the yeast. The subsequent fermentation experiments were carried out, in anaerobic conditions, on three different substrates: *scotta*, *raw cheese whey* and *deproteinized whey*. The experimental data have demonstrated the process feasibility: *scotta* is an excellent substrate for fermentation and exhibits better performance with respect to both *raw cheese whey* and *deproteinized whey*. Complete lactose consumption, indeed, was observed in the shortest time (13 h) and with the highest ethanol yield (97% of the theoretical value).

1. Introduction

Scotta is a high pollution dairy industry waste characterized by 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, which consists of the following steps: raw cheese whey (the main component of *ricotta cheese*) is initially mixed with acid whey, fresh milk (up to 10%), milk-fats and an acid solution of salts. The so obtained mixture is maintained at high temperature (85–90 °C) for about 25 min to promote the precipitation of most of whey proteins to get the cottage cheese, known as *ricotta*. The liquid solution remaining after cheese separation is actually the so-called *scotta*, which, due to both the severe thermal treatment and the addition of acid salts, has different characteristics with respect to *raw cheese whey*.

Scotta is mainly obtained in Italy but also in other countries belonging to the Mediterranean area. It is estimated that Italian production amounts to about 1.0 Mt per year, thus determining significant environmental problems related to its disposal.

Among all bio-fuels, bio-ethanol is definitely the most common; in 2006 world-wide bio-ethanol production was estimated to be about 40 Mt (70% of which coming from *Brazil* and *USA*), with an always increasing demand as requested, for instance, by the guidelines defined by *Kyoto Protocol*. Nowadays, nearly all bio-ethanol is obtained by fermentation of vegetable biomasses, essentially sugar cane and cereals; thus contributing to the observed increase of foodstuffs price. It is,

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therefore, necessary to identify alternative renewable and non-vegetable sources for bio-fuels production. Scotta could potentially fit this requirement and may potentially represent an interesting fermentation substrate owing to its main characteristics, namely the significant content of fermentable sugar and to its low cost, as determined by the fact that – as a waste – it requires a proper (and costly) treatment, which prevents from serious environmental problems. The relatively high content of lactose does indeed suggest the possibility of bio-conversion into ethanol, according to the following overall reaction:

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

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

In the scientific literature, only few papers dealt with *scotta* and its possible utilization; none of them, however, identified *scotta* 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 marxianus*; a rather low yield, i.e. 11% in 22 h, was, however, achieved [1]. The behavior of the same yeast was investigated to ascertain the effect of operating conditions on batch, fed-batch, and continuous fermentation processes, performed using cheese whey powder solutions as substrate [2–5]. Other researchers [6] 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 [7].

The present study is intended to investigate the possibility of using *scotta* as a source for bio-ethanol production, evidencing the differences existing between *scotta* and other kinds of substrates, namely *raw cheese whey* and *deproteinized whey*, which – in principle – could be used as raw materials to achieve fermentation processes aimed at bio-ethanol production. The batch fermentation experiments were performed by *K. marxianus*, evaluating the time evolutions of lactose, ethanol and biomass concentrations, thus obtaining preliminary indications on the influence of raw materials on system performance.

2. Materials and methods

2.1. Controlled bio-reaction system

A controlled batch bio-reactor, consisting of a 1.5 l autoclavable plexiglass cylinder (*Applikon*, *Holland*), was used to perform the present experimental study. The main operating parameters (pH, O_2 concentration, temperature, agitation and foam level) were monitored by a set of sensors and controlled by means of an *ADI 1030 Shelf – top* controller. The agitation was ensured by an impeller connected to a stirrer speed controller, *ADI 1032*.

Table 1 – Average compositions of *scotta*, *raw cheese whey* and *deproteinized whey*.

Substrate	Proteins (%)	Lactose (%)	Salts (%)	Organic acids (%)
Scotta	0.15–0.22	4.8–5.0	1.0–1.3	0.20–0.25
Cheese whey	0.6–0.8	4.8–5.0	0.5–0.8	0.12–0.18
Cheese whey permeate	0.08–0.12	4.6–4.8	0.4–0.6	0.12–0.18

2.2. Yeast strain

Lactose bio-conversion experiments were performed by a yeast, i.e. *K. marxianus var. marxianus CBS 397*, isolated at the *Centraalbureau voor Schimmercultures, Utrecht, the Netherlands*. This yeast was selected for its capability to produce β-galactosidase that metabolizes lactose [8,9]. The yeast, initially freeze dried, was revived suspending the microorganism by pouring it into a cylinder containing 1–2 mL of sterile water and, then, shaking and storing the suspension at 20 °C for 12 h.

2.3. Maintenance culture

K. marxianus was maintained in a generic yeast medium having the following composition: agar 10 g L^{-1}, lactose 20 g L^{-1}, bactopeptone 10 g L^{-1}, yeast extract 5 g L^{-1}. The culture was sterilized in an autoclave at 121 °C for 30 min, then it was poured on *Petri* dishes for solidification and, eventually, the yeast inoculum was spread on the surface and incubated at 20 °C for 48 h. At completed growth, the dishes were kept at 4 °C.

2.4. 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

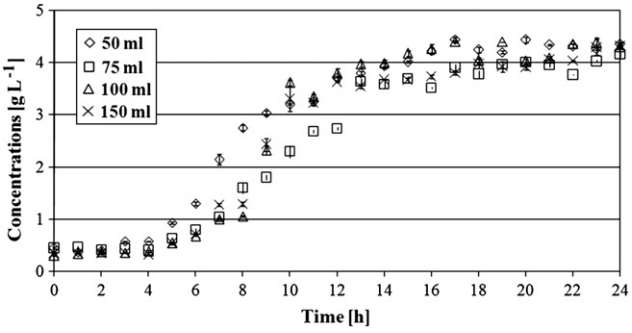

Fig. 1 – Time evolution of average biomass concentrations during aerobic fermentation of *scotta* (T = 37 °C, orbital shaking velocity = 150 rpm).

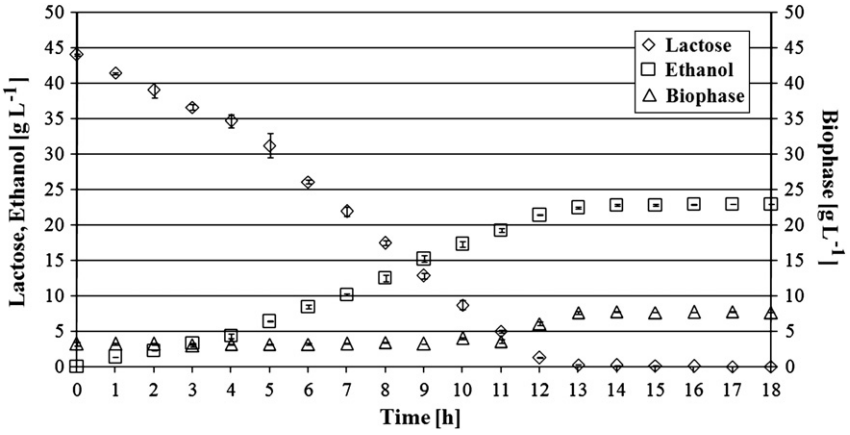

Fig. 2 – Anaerobic fermentation of *scotta*. Time evolution of lactose, ethanol and biomass concentrations ($T = 37\ °C$, orbital shaking velocity = 150 rpm, pH = 5, O_2 = 0–0.2%).

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}.

2.5. *Fermentation medium*

Three kinds of fermentation medium were used, i.e. *scotta*, *raw cheese whey* and *deproteinized whey* to assess the performance of *K. marxianus* and the bio-ethanol yield. All the tested raw materials came from the same lot of cow milk, originally designed to *mozzarella cheese* production; moreover, both the *scotta* and the *raw cheese whey* considered in the present paper represented, respectively, the by-product and the raw material of the same production cycle aimed at *ricotta cheese* obtainment. 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. The UF system was operated at a 200 kPa trans-membrane pressure with a feed flow rate of 2 L min^{-1}. It is worthwhile to remark that each of the comparisons hereafter presented was performed on samples not subjected to any other pre-treatment, but those normally carried out in the production plant. 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.

The average compositions of *scotta*, *raw cheese whey* and *deproteinized whey* are reported in Table 1

2.6. *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* 50 × 4.6 mm pre-column, a *Supelcogel C-610* 300 × 7.8 mm column and a refractive index detector, *Jasco RI 930*, constituted the experimental equipment. Biomass was evaluated by *BactoScan FC*, *Foss Integrator*, 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

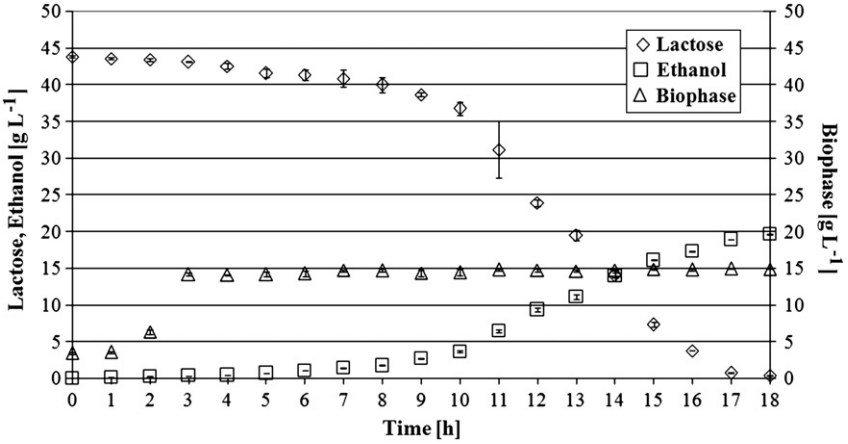

Fig. 3 – Anaerobic fermentation of *raw cheese whey*. Time evolution of lactose, ethanol and biomass concentrations ($T = 37\ °C$, orbital shaking velocity = 150 rpm, pH = 5, O_2 = 0–0.2%).

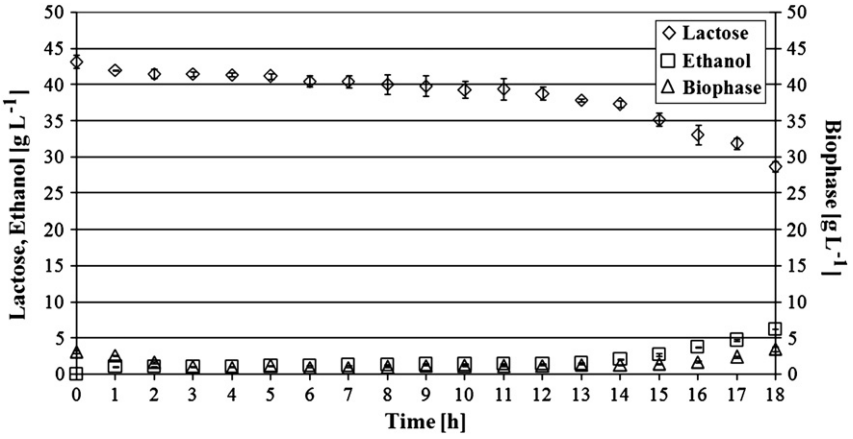

Fig. 4 – Anaerobic fermentation of *cheese whey permeate*. Time evolution of lactose, ethanol and biomass concentrations ($T = 37$ °C, orbital shaking velocity = 150 rpm, pH = 5, O_2 = 0–0.2%).

a mass basis, was obtained by multiplying the cells' concentration by 303 ng per cell [10].

2.7. *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 *K. marxianus* in *scotta*. The microorganism growth experiments were performed withdrawing a single colony from a *K. marxianus* culture, contained in a *Petri* dish, and then inserting this colony in a flask containing a known volume of *scotta*. Four volumes of *scotta* 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 l.

The flasks were placed in a *GRANT OLS 200* 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 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 6 N sodium hydroxide solution. Also in this case, the equipment and the tools were sterilized before each experiment. Two samples of fermentation broth were withdrawn, every hour, during the experiment: a 100 μL sample was destined to the microorganism analysis, as described for growth experiments, 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.

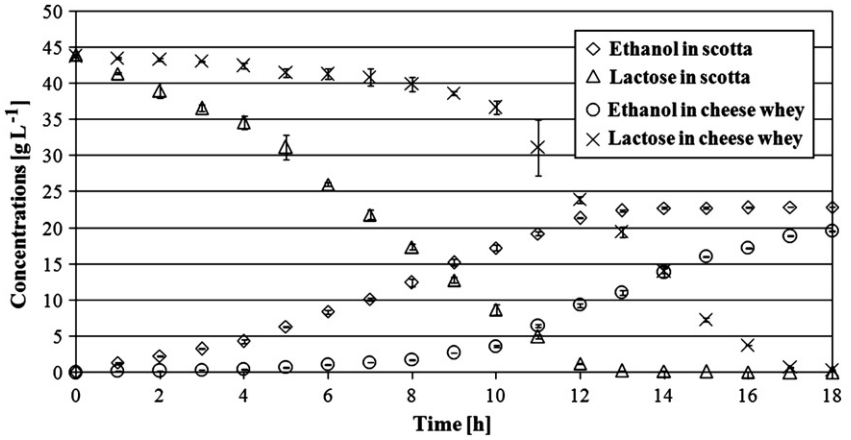

Fig. 5 – Comparison between *scotta* and *raw cheese whey*. ($T = 37$ °C, orbital shaking velocity = 150 rpm, pH = 5, O_2 = 0–0.2%).

3. Results and discussion

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

For each considered volume, the typical growth phases characterizing a batch cultivation are shown; it is worthwhile to observe that after a lag phase of 4 h the linear growth phase takes over.

After 17 h from the beginning of the experiment, the so-called stationary phase starts; the death-phase is not reported in the figure, being of little interest for the purpose of the present work.

These above results are of crucial importance to demonstrate the actual growth of *K. marxianus* in *scotta* 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 *scotta*, *raw cheese whey* and *cheese whey permeate*.

In the case of *scotta* fermentation (Fig. 2), lactose consumption goes to completion within 13 h 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; moreover, ethanol can be detected after 1 h only from the beginning of the experiment. Finally, it is worthwhile to observe the relatively low biomass growth, probably due to the low protein concentration, and, obviously, to the low oxygen concentration.

Fig. 3 shows the behavior of *raw cheese whey* fermentation. As compared with Fig. 2, a higher biomass concentration, related to the existence of an exponential phase starting after 2 h, is achieved; this phenomenon is to be ascribed to the characteristics of *raw cheese whey* that, being richer in nutrients (primarily proteins), allows an improved growthfor 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 h, 5 h later than what it was measured, under the same conditions, with *scotta*; finally, ethanol can be detected after 5 h, thus suggesting that process dynamics is delayed by about 4–5 h. This behavior can be ascribed to several phenomena occurring in the reaction medium; at the beginning, the microorganism follows the respiratory cycle rather than the anaerobic fermentation, at least until oxygen concentration becomes a limiting factor (during the initial stage of reaction, oxygen concentration is in fact relatively high and equal to the equilibrium concentration detectable before yeast addition). When, at this point, 15 g L^{-1} biomass have been formed, the ethanol yield is unavoidably reduced, since a certain amount of lactose has been already consumed to allow the respiratory cycle.

Fig. 4 shows the behavior of *cheese whey permeate* as a fermentation substrate.

Lactose consumption does not occur within the considered time interval; the reason could be somewhat ascribed to the very low protein content (see Table 1) 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 *scotta*; moreover, the two substrates have different concentrations of both salts and organic acids, which might also affect the process performance. A deeper experimental analysis is, however, necessary to better ascertain the reasons of such a different behavior observed during fermentation experiments. As a matter of fact, *cheese whey permeate*, therefore, can be regarded as a poor fermentation substrate, as compared to both *scotta* and *raw cheese whey*.

Fig. 5 shows that ethanol yield, but also the rates of both ethanol formation and lactose consumption, are higher when *scotta* is chosen as the fermentation medium, thus suggesting that it actually may be considered as an effective and promising non-vegetable source for renewable energy production.

4. Conclusions

This study has demonstrated the feasibility of *scotta* fermentation process to produce bio-ethanol by *K. marxianus*. Furthermore, it was showed that *scotta* 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 h for *scotta* as compared to 18 h for *raw cheese whey*. As far as the fermentation process is concerned, *scotta* is to be considered as a substrate completely different from traditional *raw cheese whey* and, also, from *deproteinized whey*, thus representing a valid alternative source to produce bio-ethanol. Moreover, it is worthwhile to remark that *scotta* is an industrial waste that could damage the environment if not properly disposed; this study, instead, identifies a possible reutilization of *scotta*, which would allow a useful supplemental income to many relatively small factories distributed in Italy and in other Mediterranean countries. A more detailed investigation on the influence of fermentation parameters such as temperature, agitation velocity, pH and initial lactose concentration on ethanol yield was beyond the scopes of this preliminary analysis.

It would be, however, advisable to formulate a general kinetic model that could be used to improve *scotta* fermentation performance.

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