Evaluation of the parameters effects on the bio-ethanol production process from Ricotta Cheese Whey

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The work consists of an experimental analysis to evaluate the effects of the variables temperature (T), pH, agitation rate (K) and initial lactose concentration (L) on the batch fermentation process of Ricotta Cheese Whey (RCW) into bio-ethanol by using the yeast *Kluyveromyces marxianus.* A central composite design, constituted by 26 runs, has been carried out, and the effects of the parameters have been evaluated. Eventually, once eliminated the negligible effects, Response Surface Methodology (RSM) has been applied to optimize the four parameters values in RCW fermentation process. After a preliminary experimental analysis, the chosen factors values were 32° and 40°C for *T*, 4 and 6 for *pH,* 100 and 300 rpm for *K*, 40 and 80 g L^{-1} for *L*. Best operating conditions resulted to be *T* = 32.35°C, *pH* = 5.41, *K* = 195.5 rpm and *L* = 40 g L^{-1}.

1. Introduction

RCW is a high pollutant dairy waste (Marwaha and Kennedy, 1988). It comes out as the main by-product in the ricotta cheese production process. A considerable amount of RCW is produced (about 1 Mt per year only in Italy) which represents a serious environmental problem for its disposal (Gonzales-Siso, 1996). Actually, most of RCW is utilized directly as cattle dietary supplement; it could be mistaken with a typical cheese whey but it is rather a by-product of cheese whey instead a particular kind of it. Moreover, the particular composition of RCW, mainly its low protein concentration, makes it useless for all the processes that involve a protein valorization. Nevertheless, high lactose concentration in RCW (about 5%) suggests the chance to convert this sugar content into a high value-added product as bio-ethanol, thus carrying out two goals simultaneously: the valorization and the disposal of RCW (Sansonetti et al., 2009).
Bio-ethanol is nearly totally produced by fermentation of vegetable biomasses causing all the problems linked to both soil availability and soil overexploitation. In this case, RCW would be an alternative non vegetable source for the production of bio-ethanol. This work is aimed to analyze the effects of the main operating conditions on the process and to optimize the fermentation reaction itself through a CCD by using RSM.
Similar processes have been widely considered for other similar substrates such as raw cheese whey (Ghaly and El Tawel, 1994; Zafar Salman and Owais Mohammad, 2005), deproteinized cheese whey (Domingues et al., 2001; Norton et al., 1994) and cheese whey powder solutions (CWP) (Kargi Fikret and Ozmihci Serphil, 2006; Ozmihci

Serphil and Kargi Fikret, 2007a, 2007b). Giuliano Dragone et al. (2008) optimized the operating parameters by using the RSM by considering three factors. The same methodology was also used by Nahit Aktas et al. (2006), by considering four factors, in aerobic fermentation of deproteinized whey.
Anyway, the bio-conversion process of RCW to ethanol has not been considered. Nevertheless, RCW is the actual substrate available in any country that produces ricotta cheese and then, because of its particular chemical and physical characteristics, it deserves particular attention to characterize and optimize this bio-process that seems to be very promising.

2. Materials & Methods

2.1 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*.

2.2 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}.

2.3 Fermentation medium

RCW actually is the fermentation medium. RCW samples have been kindly provided by a local dairy industry, *Agroalimentare Asso.La.C., Calabria, Italy*; it should be remarked that each of the comparisons henceforth presented has been performed on samples coming from the same lot of cow milk. If requested, sterile lactose monohydrate (produced by *Fluka*) has been added to the fermentation medium.

2.4 Analytical methods

Samples have been periodically withdrawn from the bio-reactor in aseptic conditions in order to determine, by HPLC, the time evolution of lactose and ethanol concentrations. Biomass has been evaluated by BactoScan FC instrumentation (*Foss Integrator, Denmark*).

2.5 Experimental protocol

All the experiments have been carried out within 6 h from the RCW production time. Each run has been performed in anaerobic conditions with the same procedure described as follows. Operating conditions have been set at the desired values adding, if requested, the lacking amount of lactose to reach the pre-determined lactose concentration for each run. One-liter fermentation medium has been started by means 100 mL inoculum. Two samples have been withdrawn from the bulk of the started fermentation medium every hour; 1 mL sample has been centrifuged at 5000 rpm for 15 min, filtered through a 0.5 µL filter and analyzed by HPLC instrumentation to assay both lactose and ethanol concentrations. The other sample has been diluted, 100 µL of fermentation broth in 25 mL of 2% sodium citrate solution and eventually analyzed by the BactoScan instrumentation. All the instruments used to perform the experiments have been previously sterilized in autoclave at 121°C for 20 min.

2.6 Experimental design

Four factors have been considered: temperature *T*, *pH*, agitation rate *K* and initial lactose concentration *L*, with two levels for each factor. The chosen factor levels have been 32° and 40°C for *T*, 4 and 6 for *pH*, 100 and 300 rpm for *K*, 40 and 80 g L^{-1} for *L*. Hence, a rotatable central composite design (CCD) has been carried out to evaluate both single and interaction effects. The total number of experiments has been $2^k+2k+2 = 26$ (k = number of factors). Response function (RF) has been defined as RF = (1/theoretical yield)*(g-ethanol formed at t=18h) / (g-initial lactose in the medium). Eventually, once eliminated the negligible effects, data have been optimized through RSM. Analysis has been performed by the software *Statgraphics Plus 5.1.* A 2nd order polynomial model has been used to fit the data and the ANOVA test has been performed to assay the statistical significance of the analysis.

3. Results and discussion

RF values obtained in the CCD have been elaborated and the results are reported as follows. Standardized Pareto chart is shown in *figure 1a*; as it can be seen, every first order effect but *K* is significant, *T* and *L* as a negative effect and *pH* as a positive effect. With regard to the second order effects, *K* is the strongest followed by *pH* and *T*. Other effects, but *K*, actually involved in a second order effect, are negligible and will be omitted in the following analysis. The adjusted R-squared statistic has been 0.8575. After removal of negligible effects, these data have been processed by RSM to optimize the fermentation process with a 2nd order polynomial model; the resulting relation is represented by *equation 2*.

$$RF = -9.3289 + 0.3451T + 1.5442pH + 6.8553\cdot 10^{-3}K - 2.8750\cdot 10^{-3}L + \\ -5.3338\cdot 10^{-3}T^2 - 0.1428pH^2 - 1.7534\cdot 10^{-5}K^2 \qquad (2)$$

Figure 1b shows predicted data versus observed data; a correlation coefficient equal to 0.94 has been obtained.

Response surfaces have been obtained as a function of two factors at a time, while the other two were maintained at intermediate constant values. *Figure 2* shows one of the surfaces in which the RF is reported as a function of *T* and *pH* (the other response surfaces have not been reported for sake of brevity).

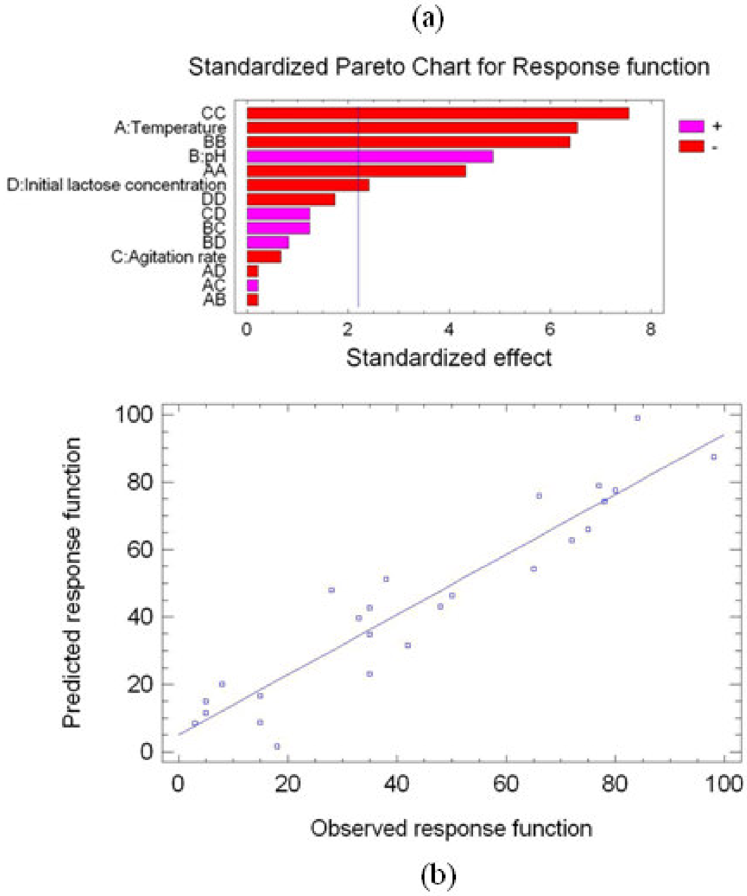

Figure 1 – a) Standardized Pareto chart. b) Predicted response function by RSM versus observed response function

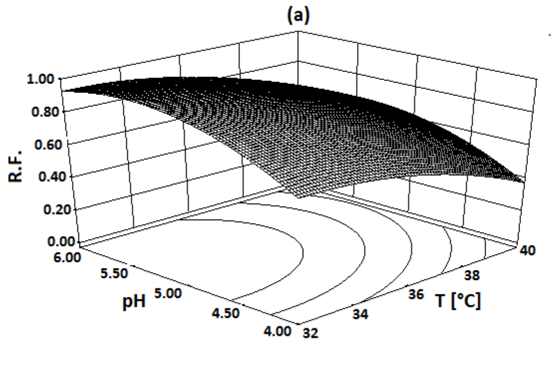

Figure 2 – Response surface as a function of temperature (T) and pH

Figure 2 exhibits a strong response surface dependence on both T and pH; indeed RF changes its value from about 0.35, at 40°C and pH 4, to about 0.60 at the same T and pH 6. A similar behavior was observed passing from 40 to 32°C at the same pH of 4. Moreover, a good response value, i.e. 0.88, was obtained at 32 °C and pH 6.
RF dependence on both *T* and *K* confirmed the strong dependence on *T*, that was capable to determine a variation of the RF from about 0.5 to about 0.75 when T was decreased from at 40°C to 32°C, at the same stirring rate of 100 rpm. As far as the influence of K on the RF was concerned, a relatively weak effect was observed even though an intermediate value, i.e. 200 rpm, resulted to improve the ethanol formation.
Response surface versus *L* and *T* strengthened the conviction that RCW fermentation process is enhanced by relatively low T values. Factor L has a weak effect on the RF, even though better results were achieved with the lowest value of L, i.e. about 40 g L^{-1}.
Response surface versus *pH* and *K* showed that higher pH value helps the fermentation process. The worst conditions were achieved at pH 4 and at a stirring rate of both 100 and 300 rpm..
RF, as a function of the factors *pH* and *L*, showed a relatively weak effect of L and a stronger effect of pH.
The response surface with *L* and *K* as variable factors, confirms that it should be advisable to use intermediate K values and low L values.

4. Conclusions

With this work a central composite design based on the analysis of 26 experiments, has been performed. Effects of the factors temperature, pH, agitation rate and initial lactose concentration have been estimated. As expected, temperature and pH have demonstrated to have strong effects on the RF, nevertheless experimental analysis showed that all the factors but the initial lactose concentration must be considered with both first and second order terms; the remaining factor is involved only with a first order term. Furthermore, experiments have shown that interaction effects between parameters are negligible in anaerobic conditions.
Eventually, RSM has been applied to optimize RCW fermentation process. An empirical model has been used to fit data from CCD; a sufficiently good correlation coefficient, i.e. 0.94, has been obtained. Moreover, ANOVA test has been performed to assay the statistical significance of the analysis finding two fair values, 89.74% and 85.75% for R-squared and adjusted R-squared parameters, respectively. Then, the proposed model resulted to be suitable to simulate the response function in a wide range of the variables. Optimization has shown that best values of the factors are 32.35°C for temperature, 5.41 for pH, 195 rpm for agitation rate and 40 g L^{-1} for initial lactose concentration.
This paper not only deals with a little studied industrial waste, i.e. RCW, but represents also a valid attempt to completely characterize the RCW fermentation process, considering far important parameters, with a novel approach for this kind of dairy by-product, laying the basis for the future development of a more physical process modeling.

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