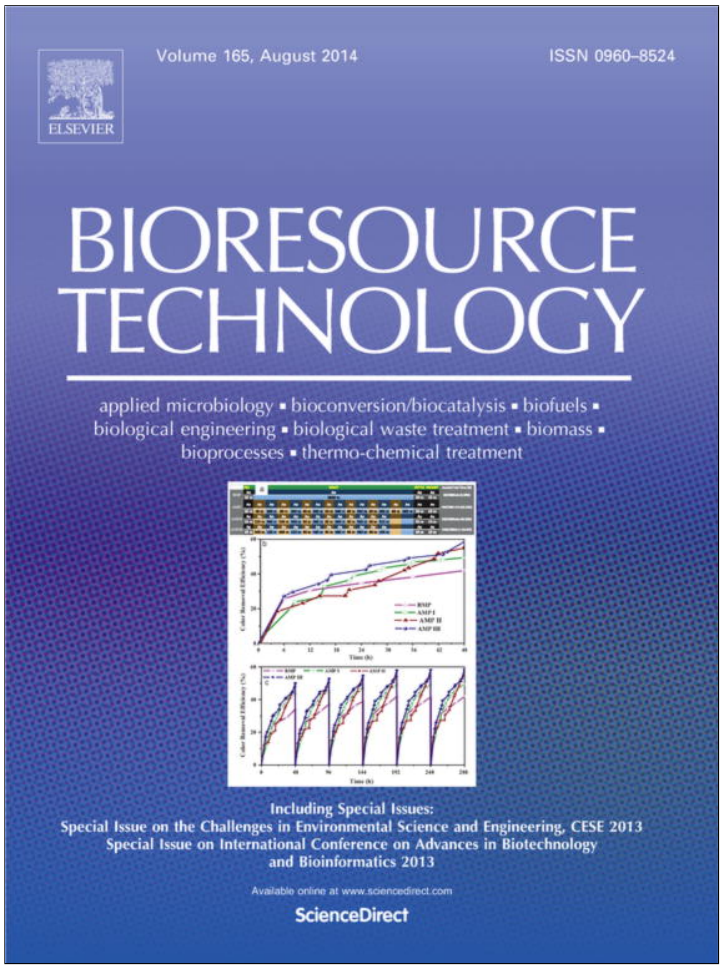

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Development of a hybrid fermentation–enzymatic bioprocess for the production of ethyl lactate from dairy waste

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HIGHLIGHTS

- We propose a hybrid bioprocess for the production of ethyl lactate from whey.
- Ethanol and lactic acid produced in fermentations are esterified to ethyl lactate.
- Toluene is an effective solvent for the enzymatic esterification with lipases.
- Various enzyme concentrations and water contents were tested to enhance production.
- Overall the process is effective in terms of ethyl lactate yield.

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ABSTRACT

This work explores the potential for the development of a hybrid fermentation–enzymatic process for the production of ethyl lactate from dairy waste. Cheese whey was used in *Kluyveromyces marxianus* and *Lactobacillus bulgaricus* batch cultures to produce ethanol and lactic acid respectively. Subsequently, the fermentation products were transferred into an organic phase through liquid–liquid extraction and ethyl lactate was formed in an esterification reaction catalyzed by lipases. The production of ethanol and lactic acid achieved under different conditions was 23 g L^{-1} and 29 g L^{-1}, respectively. Furthermore, the efficiency of various organic solvents for the esterification reaction was evaluated and toluene was chosen for application in the process. The effect of water content was determined aiming to maximize the product yield and 40 mg ml^{-1} was the optimal enzyme concentration. The bioprocess achieved maximum conversion of 33% constituting a valuable alternative to the application of energy demanding chemically derived methods.

1. Introduction

Cheese whey is the main dairy by-product obtained during the coagulation of milk casein in cheese making, which is considered as a residual aqueous solution of lactose containing protein and mineral salts (Guimaraes et al., 2010; Illanes, 2011). Mainly due to the high content in lactose whey exhibits biochemical oxygen demand of 30–50 g L^{-1} and chemical oxygen demand of 60–80 g L^{-1} (Hassan and Nelson, 2012). Therefore, the high polluting load of whey and the tremendous growth of dairy industries worldwide constitute its untreated discharge as a serious environmental problem (Kushwaha et al., 2011). Early disposal methods included release into waterways, the municipal water system, lagoons for oxidation and feeding into ruminants (Kosikowski, 1979). However, the above methods are not satisfactory as the high content of lactose, soluble proteins and lipids and the presence of other essential nutrients for microbial fermentation constitute whey as an important raw material for the biotechnological production of various added-value products (Gonzalez Siso, 1996).

About 50% of the whey produced globally is converted into different food products (Panesar et al., 2007). Furthermore, many microorganisms are capable of utilizing lactose as their main carbon source for the production of added-value products (Adam et al., 2004). Thus, a variety of fermentative applications associated with the valorization of whey have been developed in the dairy and pharmaceutical industries including the production of citric acid, single-cell proteins, fermented beverages, vitamins, biogas, biopolymers, ethanol and lactic acid (Kosseva et al., 2009; Solaiman et al., 2006). However, novel bioprocessing routes for whey utilization still remain unexplored.

The esters of lactic acid are commonly used for the production of food, medicine and cosmetics mainly due to favorable hygroscopic and emulsifying properties (Gao et al., 2011). They are nontoxic and

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biodegradable liquids which have been recently characterized as green solvents, holding the potential to replace the use of toxic solvents for a wide variety of industrial applications (Corma et al., 2007; Pereira et al., 2011). Ethyl lactate belongs to the aforementioned category of compounds and it is traditionally produced by chemical synthesis under drastic reaction conditions with homogenous catalysts such as sulfuric acid, hydrogen chloride and phosphoric acid (Gao et al., 2011). However, the chemical synthesis of ethyl lactate from lactic acid often results in non-specific reaction as α-hydroxy acid has both hydroxyl and carboxy groups which undergo self-polymerization (Hasegawa et al., 2008b). On the other hand, ethyl lactate can be biotechnologically produced through the esterification of lactic acid and ethanol with lipases under mild reaction conditions contributing to a significant environmental objective through the replacement of petroleum based solvents with bio-based derivatives (Gu and Jerome, 2013).

This work tackles the major environmental problem faced by the diary industry through the development of an innovative biotechnological approach for the management of whey. To this end, the potential of a hybrid fermentation–enzymatic process converting the high lactose content of cheese whey into the green solvent ethyl lactate is explored. Microbial fermentations utilizing *Kluyveromyces marxianus* and *Lactobacillus bulgaricus* were first studied for their capacity to convert lactose into ethanol and lactic acid, respectively. Consequently, ethanol and lactic acid produced in bulk were used as substrates in an enzymatic esterification reaction utilizing lipases in organic solvents. The main objective of the work was to determine the feasibility of the hybrid bioprocess and the impact of different process parameters, such as the selection of a suitable solvent and the effect of water and enzyme content on the production of ethyl lactate. The results obtained demonstrate that the development of the proposed bioprocess is feasible based on the high concentration of ethyl lactate achieved, offering a new potential solution to the environmental problem of whey and an alternative route to the common industrial production of ethyl lactate through chemical synthesis.

2. Methods

2.1. Growth conditions

K. marxianus (DSMZ, strain IFO 0288) and *Lactobacillus delbrueckii* spp. *bulgaricus* (DSMZ, strain ATCC 11842) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). *L. bulgaricus* was cultivated at 33 °C in medium containing 51 g L^{-1} MRS broth and 0.1% (v/v) Tween 80. *K. marxianus* was grown at 30 °C in YM medium comprising in w/v 1% glucose, 0.5% peptone, 0.3% malt extract and 0.3% yeast extract. Both strains were harvested by centrifugation at 13,000 rpm for 1 min and added in separate flasks containing a sterilised synthetic medium which comprised w/v concentrations of 5.5% lactose, 0.5% yeast extract, 0.5% $MgSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4 and 0.1% $(NH_4)_2SO_4$. The pH of the medium was adjusted to 5.5 twice a day and experiments were performed under anaerobic conditions and varying temperatures. Lactic acid production was performed in static flasks at 35, 40, 42 and 45 °C, while ethanol fermentations were tested at 28, 30 and 33 °C in flasks stirred at 100 rpm. All chemicals used were obtained from Sigma–Aldrich Company Ltd. (UK) and were of ANALAR grade.

2.2. Batch enzymatic experiments

Enzymatic reactions were performed in 2 ml screw-capped closed vials with reciprocal shaking at 100 rpm in an incubator operated at 30 °C. Commercial lipases (Novozyme 435, immobilized *Candida antarctica* lipase B, Sigma–Aldrich Company Ltd., UK) were used for esterification in concentrations ranging between 10 and 100 mg ml^{-1} according to the requirements of each experiment. Ethanol and lactic acid were either added directly into the solvent that contained the enzyme or they were extracted into the solvent from an aqueous solution of the two substrates. In the latter case, given concentrations of ethanol and lactic acid were added into water and the enzyme was added in the solvent phase. Consequently, the two substrates were continuously extracted from the aqueous phase into the solvent during the experiment and they were converted into ethyl lactate due to the action of the enzyme.

2.3. Analyses (GC, HPLC, UV)

Gas Chromatography (GC) was employed for the determination of ethanol concentration in the samples from *K. marxianus* fermentations. A Shimadzu GC-2014 (Shimadzu, UK) equipped with a flame ionisation detector and a 30 m long Zebron ZB-5 capillary column (Phenomenex, UK) with 0.25 mm internal diameter was used. The mobile phase used was nitrogen, while the stationary phase of the column was 5%-phenyl and 95% dimethylpolysiloxane. Aqueous samples were centrifuged for 5 min at 13,000 rpm and the supernatant was filtered through 0.2 μm filters. Ethanol was extracted into hexane by vigorous vortexing 1 ml of the filtered sample with 2 ml of hexane for 1 min at room temperature. One microliter of the extract was injected into the GC and the temperature of the column was kept constant at 40 °C for 3 min. The concentration of ethanol was calculated interpolating from a previously established ethanol calibration curve and the coefficient of variation for four samples was 4.1% at a concentration level of 1 g L^{-1}.

Determination of ethyl lactate concentration was also performed by GC analysis using the same instrument as for the ethanol concentration measurements. The closed vials used for the enzymatic reaction were placed directly into the GC and 1 μL was injected. The column temperature was kept constant at 110 °C for 3 min and the concentration of ethyl lactate was calculated interpolating from a previously established calibration curve. The coefficient of variation for five samples was 2.4% at a concentration level of 0.1 M ethyl lactate.

Lactic acid concentration was determined using High Pressure Liquid Chromatography (HPLC). A Shimadzu LC-20AD liquid chromatograph (Shimadzu, UK) equipped with a Shimadzu SPD-20A UV/VIS detector, a Shimadzu SIL-20A HT auto sampler and a CTO-10AS VP column oven was used. Samples were eluted with 0.005 N H_2SO_4 at a flow rate of 0.6 ml min^{-1} from an organic acid analysis column (300 × 7.8 mm inside diameter, Rezex-ROA Organic Acid Column, Phenomenex Inc., UK) at 60 °C. Biomedium samples were centrifuged and filtered as described above. Thirty microliters were injected into the HPLC and the concentration of lactic acid was determined interpolating from a previously established lactic acid calibration curve. The coefficient of variation for four samples was 0.9% for a concentration level of 5 M lactic acid.

Biomedium samples from both cultures were measured for absorption at 600 nm on a Perkin Elmer Lambda 25 UV/VIS spectrophotometer (Perkin Elmer Inc., UK). Two previously established dry weight calibration curves were used for the determination of *L. bulgaricus* and *K. marxianus* biomass concentration. The coefficient of variation for four *L. bulgaricus* culture samples was 2.7% at a concentration level of 1.2 $g_{biomass}$ L^{-1} and the coefficient of variation for four *K. marxianus* culture samples was 1.2% at a concentration level of 0.8 $g_{biomass}$ L^{-1}.

3. Results and discussion

3.1. Fermentative production of ethanol and lactic acid

The development of a combined bioprocess converting the lactose content of cheese whey into ethyl lactate involves the

fermentative production of bioethanol and lactic acid in a first step and their subsequent esterification in a second enzymatic step yielding the final product. In order to understand how to size the relevant bioprocess units, the microbial production of ethanol and lactic acid was first investigated in batch experiments utilizing industrially important strains capable of metabolising the lactose content of whey into the desired products at high rates.

The majority of the strains utilized for commercial production of lactic acid belong to the genus of *Lactobacillus*, among which *L. bulgaricus* is commonly used for product formation (Wee et al., 2006). Therefore, preliminary *L. bulgaricus* batch fermentations were conducted in static cultures aiming to investigate the capacity of the bioprocess to produce lactic acid. The biomass dry cell weight and lactic acid concentrations were measured for experiments conducted at temperatures ranging between 35 and 45 °C. The maximum lactic acid concentration achieved was 29 g L^{-1} at 45 °C, while the concentration of lactic acid was 27 g L^{-1} at 42 °C. Additionally, the production of lactic acid was reduced in concentrations below 15 g L^{-1} in cultures performed at 35 and 40 °C. During the experiments presented above, the maximum biomass dry cell weight concentration achieved was 1.7, 2.7, 2.5 and 1.8 g L^{-1} at 35, 40, 42 and 45 °C, respectively. A comparison of the results obtained here with previous studies performed in similar systems confirms that the production of lactic acid was satisfactory (Table 1). Venkatesh et al. (1993) demonstrated that under similar bioprocess conditions to the present study, the yield can reach a maximum of 0.96 $g_{lactic\ acid}\ g^{-1}_{lactose}$. However, the pH used in the process is a crucial factor for the production of lactic acid as for a pH of 4.5 the yield decreased to 0.50 $g_{lactic\ acid}\ g^{-1}_{lactose}$, while when the system was operated without pH control the final concentration of lactic acid was only 8 g L^{-1} and the yield dropped substantially to 0.16 $g_{lactic\ acid}\ g^{-1}_{lactose}$. In the experiments presented here the pH of the biomedium was manually measured and adjusted to 5.5 twice a day. As a result a substantial decrease of the pH occurred overnight, which ranged between 4.0 and 4.8 during the exponential growth phase. Therefore, the pH of the biomedium was not optimal for a significant period of the experiment and the yield obtained (0.53 $g_{lactic\ acid}\ g^{-1}_{lactose}$) was in agreement with literature studies.

Table 1 shows that the temperature employed is also an important parameter affecting product formation and that a decrease in the initial lactose concentration results in substantial increase of lactic acid production. Specifically, Burgos-Rubio et al. (2000) demonstrated that a decrease of the initial lactose concentration from 43 g L^{-1} to 21 g L^{-1} enhanced the yield of lactic acid, which increased from 0.93 $g_{lactic\ acid}\ g^{-1}_{lactose}$ to 1.14 $g_{lactic\ acid}\ g^{-1}_{lactose}$. Increased lactic acid yield was also reported by Ghasemi et al. (2009) during reduction of the initial lactose concentration from 50 g L^{-1} to 25 g L^{-1}. Additionally, apart from *L. bulgaricus* other lactic acid bacterial strains are also capable of obtaining high yields for the conversion of lactose into lactic acid. The yield obtained for *Lactobacillus* sp. RKY2 fermentations with initial lactose concentration of 50 g L^{-1} was 0.99 $g_{lactic\ acid}\ g^{-1}_{lactose}$ (Kim et al., 2006), while *Lactococcus lactis* strain NZ133 performed an average yield of 0.93 $g_{lactic\ acid}\ g^{-1}_{lactose}$ (Boonmee et al., 2003).

K. marxianus is one of the most efficient yeasts in fermenting lactose into ethanol (Mussatto et al., 2010). Thus, *K. marxianus* was chosen for the production of ethanol from whey in preliminary batch fermentations aiming to investigate the formation of the product. The biomass dry cell weight and bioethanol concentrations achieved for cultures performed at temperatures ranging between 28 and 33 °C are presented on Fig. 1. The maximum

Table 1
Production of lactic acid from whey in *Lactobacillus bulgaricus* fermentations.

Initial lactose conc. (g L^{-1})	Temperature (°C)	pH	Final lactic acid conc. (g L^{-1})	Yield ($g_{lactic\ acid}\ g^{-1}_{lactose}$)	Reference
55	45	5.5 (adjusted twice a day)	29	0.53	Present Work
50	45	5.6	48	0.96	Venkatesh et al. (1993)
50	45	5.0	49	0.98	Venkatesh et al. (1993)
50	45	4.5	25	0.50	Venkatesh et al. (1993)
50	45	6 (without pH control)	8	0.16	Venkatesh et al. (1993)
43	42	5.6	40	0.93	Burgos-Rubio et al. (2000)
21	42	5.6	24	1.14	Burgos-Rubio et al. (2000)
25–41	32	6.5	10–21	0.40–0.51	Ghasemi et al. (2009)
50	32	6.5	12	0.24	Ghasemi et al. (2009)

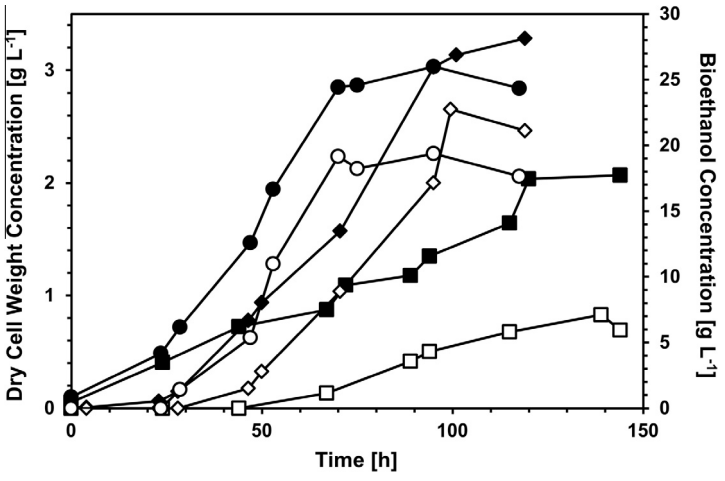

Fig. 1. Bioethanol produced in *K. marxianus* fermentations conducted at different temperatures. Shown are the biomass dry cell weight and bioethanol concentrations for experiments performed at 28, 30 and 33 °C. —■—: dry cell weight concentration (28 °C); —□—: bioethanol concentration (28 °C); —◆—: dry cell weight concentration (30 °C); —◇—: bioethanol concentration (30 °C); —●—: dry cell weight concentration (33 °C); —○—: bioethanol concentration (33 °C).

Table 2
Production of ethanol from whey in *Kluyveromyces marxianus* fermentations.

Initial lactose conc. ($g\ L^{-1}$)	Temperature (°C)	pH	Final ethanol conc. ($g\ L^{-1}$)	Yield ($g_{ethanol}\ g^{-1}_{lactose}$)	Reference
55	30	5.5 (adjusted twice a day)	23	0.42	Present Work
170	30	N/A	80	0.47	Silveira et al. (2005)
50	30	N/A	24	0.48	Silveira et al. (2005)
47	34	5.0	22	0.47	Sansonetti et al. (2011)
48	37	5.0	22	0.46	Sansonetti et al. (2011)
44	33	5.4	23	0.52	Sansonetti et al. (2011)
44	40	6.1	15	0.32	Zoppellari and Bardi (2013)
44	28	6.1	17	0.37	Zoppellari and Bardi (2013)

ethanol concentration measured was 23 $g\ L^{-1}$ for the culture conducted at 30 °C, corresponding to a yield of 0.42 $g_{ethanol}\ g^{-1}_{lactose}$. Furthermore, the concentration of ethanol was significantly reduced when the temperature was lower than 30 °C. The maximum ethanol concentration achieved was in line with the results presented on Table 2, indicating the efficient production of bioethanol in the experiments performed.

3.2. Solvent selection for lactic acid esterification

Following the microbial fermentations of whey, the bulk ethanol and lactic acid formed were used as substrates in an esterification reaction employing lipases for the production of ethyl lactate. Although this reaction can be catalyzed in a variety of different hydrophobic or polar solvents (Findrik et al., 2012), the development of an efficient hybrid bioprocess converting lactose into ethyl lactate would either require the use of purified lactic acid and ethanol in the esterification reaction or the transfer of the two substrates from the aqueous phase into the solvent through liquid–liquid extraction. Therefore, the selection of an appropriate solvent was carefully considered by testing both hydrophobic and polar solvents as shown on Fig. 2.

Toluene, acetone, acetonitrile and chloroform have been previously applied as organic solvents in the biocatalytic process (Hasegawa et al., 2008a; Major et al., 2010). Thus, the above solvents were tested for their efficiency in the esterification reaction, while an additional experiment was performed in water to show that the reaction cannot proceed in aqueous solutions. Fig. 2A presents the production of ethyl lactate during the experiments at different solvents complemented with 10 $mg\ ml^{-1}$ Novozyme 435 and 0.5 M of each substrate. The performance of the reaction was highest when conducted in toluene and acetone, where the final ethyl lactate concentrations reached 0.27 M (54% conversion) and 0.26 M (52% conversion), respectively. The ethyl lactate production was higher than the 35% achieved by Hasegawa et al. (2008a) in acetone containing 1 M of lactic acid and 2 M of ethanol. However, the formation of the product was significantly lower when chloroform and acetonitrile were applied, while the use of water resulted in negligible production of ethyl lactate.

Non-enzymatic production of ethyl lactate was also tested by repeating the experiments described above without the addition of the enzyme. The control experiments (Fig. 2B) showed that acid catalyzed, non-enzymatic esterification can proceed in toluene at the same rate as when 10 $mg\ ml^{-1}$ of enzyme was added, which is demonstrated by the fact that ethyl lactate concentration reached 0.28 M (56% conversion). However, non-enzymatic esterification was completely suppressed in the rest of the solvents.

The purification of lactic acid and ethanol produced from the fermentations and their direct use in the esterification reaction is expected to substantially increase the cost of a potential industrial process. Therefore, in case acetone was chosen for application in the hybrid bioprocess the effect of the water content on the product formation should be tested, since the specific solvent is miscible with water and thus not suitable for liquid–liquid extraction. Fig. 2C presents the effect of the water content and enzyme concentration on the esterification reaction performed in acetone. 0.13 M of product was formed after 93 h when 10 $mg\ ml^{-1}$ of Novozyme 435 was applied. The increase in enzyme concentration substantially enhanced the production of ethyl lactate, since 0.22 M of product was formed after 89 h with the use of 50 $mg\ ml^{-1}$ of Novozyme 435. Furthermore, in the absence of water the concentration of ethyl lactate was 0.19 M at 168 h, while when 2% and 4% of water was added the concentration of ethyl lactate was 0.11 M and 0.06 M at 186 h respectively. The significant reduction of the reaction efficiency in acetone indicates that the water should be removed from the aqueous solution containing bulk ethanol and lactic acid. Therefore, since toluene is immiscible with water and it is an efficient solvent for ethyl lactate production, the use of toluene was proposed for liquid–liquid extraction of ethanol and lactic acid from the aqueous phase and simultaneous esterification due to the presence of lipases in the organic solvent.

3.3. Investigation of different parameters on the feasibility of enzymatic esterification

3.3.1. The effect of substrates concentration on ethyl lactate production

The production of lactic acid and ethanol achieved in this work and in the literature studies of Tables 1 and 2 indicate that the fermentation of cheese way containing 55 $g\ L^{-1}$ lactose can yield a concentration of approximately 0.5 M of each of the products, given that continuous control of the pH is in place. In line with the above, the continuous liquid–liquid extraction of lactic acid and ethanol from the aqueous phase into toluene was first tested using 0.5 M of each compound in the aqueous phase. Although seven different experiments were conducted with various water contents, ranging from 2% (v/v) of aqueous solution and 98% (v/v) of toluene up to 30% (v/v) of aqueous solution and 70% (v/v) of toluene (10 $mg\ ml^{-1}$ Novozyme 435), the production of ethyl lactate remained below the detection level 300 h after the beginning of each experiment. The fact that ethyl lactate was not produced even when 30% (v/v) of aqueous solution was used can be due to the low solubility of ethanol in toluene resulting in very low ethanol concentration in toluene.

The concentration of ethanol and lactic acid were gradually increased in the aqueous phase to determine the required concentration for efficient production of ethyl lactate. Thus, the esterification substrates concentration was increased from 0.5 M to 5 M in experiments were 40 $mg\ ml^{-1}$ Novozyme 435, 15% (v/v) of aqueous solution and 85% (v/v) of toluene was used (Fig. 3). The results show that ethyl lactate was detected for aqueous lactic acid and ethanol concentrations higher than 3 M, while for lower concentrations of substrates ethyl lactate was not detected. The maximum ethyl lactate concentration achieved was 0.07 M and 0.24 M when 3 M and 5 M substrates concentration was used in the aqueous phase, respectively. The produced ethyl lactate concentrations

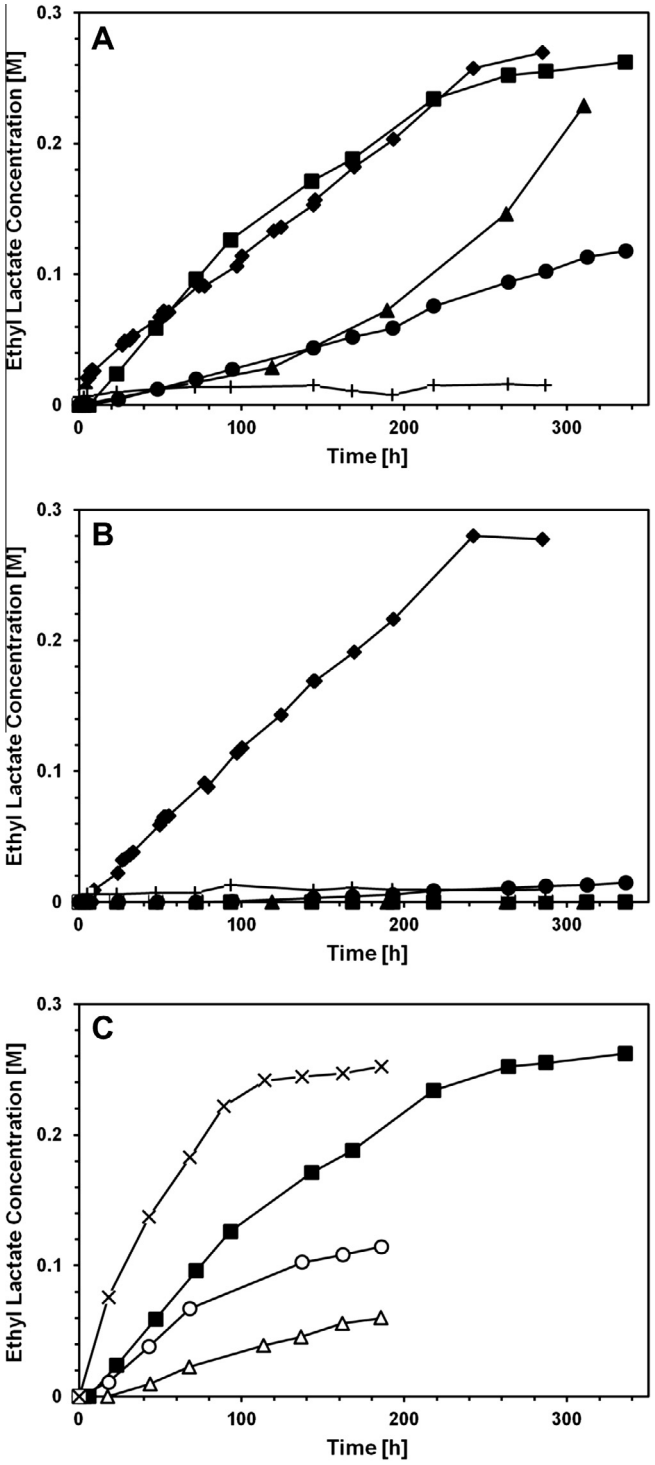

Fig. 2. The effect of different solvents on the esterification of lactic acid with ethanol. All experiments were performed by using 0.5 M of each substrate, at 30 °C and 100 rpm. (A) Ethyl lactate synthesized in different solvents with the addition of 10 mg ml^{-1} Novozyme 435. (B) Ethyl lactate synthesized in different solvents without added enzyme. (C) The effect of enzyme concentration and water content on the esterification reaction performed in acetone. Ethyl lactate produced: in (i) —■—: acetone; (ii) —◆—: toluene; (iii) —▲—: chloroform; (iv) —●—: acetonitrile; (v) —+—: water; (vi) —×—: acetone containing 50 mg ml^{-1} enzyme; (vii) —○—: acetone containing 2% water; (viii) —△—: acetone containing 4% water.

correspond to 13% conversion when the substrates concentration was 3 M and to 27% conversion for the highest concentration tested.

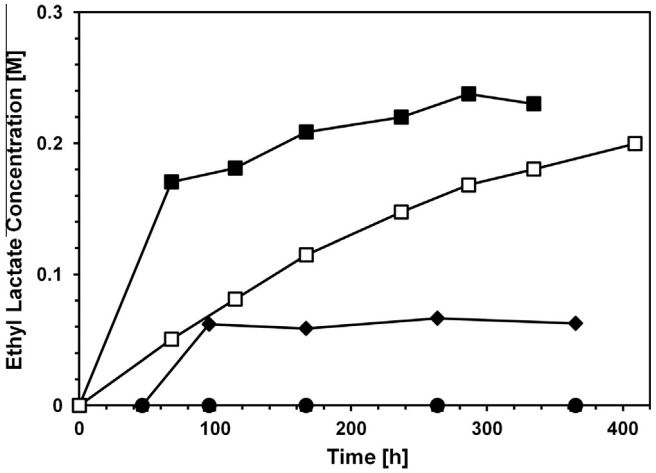

Fig. 3. Ethyl lactate formed in toluene during continuous extraction of ethanol and lactic acid from an aqueous phase. Experiments were performed by using 15% (v/v) of aqueous solution containing different concentrations of substrates and 85% (v/v) of toluene containing 40 mg ml^{-1} Novozyme 435 (apart from the control experiment), at 30 °C and 100 rpm. Symbols correspond to ethyl lactate concentration produced using substrates concentration of: (i) —■—: 5 M lactic acid and ethanol; (ii) —◆—: 3 M lactic acid and ethanol; (iii) —●—: 1 M lactic acid and ethanol; (iv) —▲—: 0.5 M lactic acid and ethanol; (v) —□—: 5 M lactic acid and ethanol (without added enzyme).

3.3.2. The effect of water/toluene volume ratios and enzyme concentration for simultaneous liquid–liquid extraction and esterification

The concentration of ethanol and lactic acid which achieved the highest conversion was applied to test the efficiency of the continuous extraction–esterification process for different water/toluene ratios. Therefore, the volume of the aqueous solution containing 5 M of each substrate was gradually increased from 2% (v/v) to 30% (v/v) in the continuous extraction–esterification process (Fig. 4). The results show that comparing to the control experiments there was no significant difference in the production of ethyl lactate when 10 mg ml^{-1} Novozyme 435 was added. Furthermore, the conversion of the reaction in the experiments reached a maximum of 33% for the lowest water content tested.

The effect of increasing enzyme concentrations on ethyl lactate production was evaluated to determine the optimal lipase concentration required for the process in experiments conducted with 15% (v/v) aqueous phase (containing 5 M of ethanol and lactic acid) and 85% (v/v) toluene. Fig. 5 shows that enzyme concentrations higher than 40 mg ml^{-1} could not further increase the production of ethyl lactate indicating that 40 mg ml^{-1} was the optimal enzyme concentration for the proposed system. Furthermore, the conversion achieved was between 22% and 27%, which is satisfactory for the experiments of the present work conducted aiming to explore the potential of the hybrid bioprocess. Hasegawa et al. (2008a,b) showed that the production of ethyl lactate drops substantially when concentrations of lactic acid higher than 1.5–2.5 M are added in different solvents. However, the conversion of the reaction was not reduced in the experiments of this study conducted with 5 M of lactic acid in the aqueous phase due to the equilibrium established between the two phases. Therefore, the lower concentration of lactic acid formed in the solvent possibly minimized the acid-based inactivation of lipases.

Although different hybrid processes (reaction with integrated pervaporation, batch distillation with reactive distillation, and reaction with vapour permeation and reactive distillation) have been previously explored for the production of ethyl lactate (Mitkowski, 2011), this is the first attempt to our knowledge testing the potential of a hybrid bioprocess for the production of ethyl

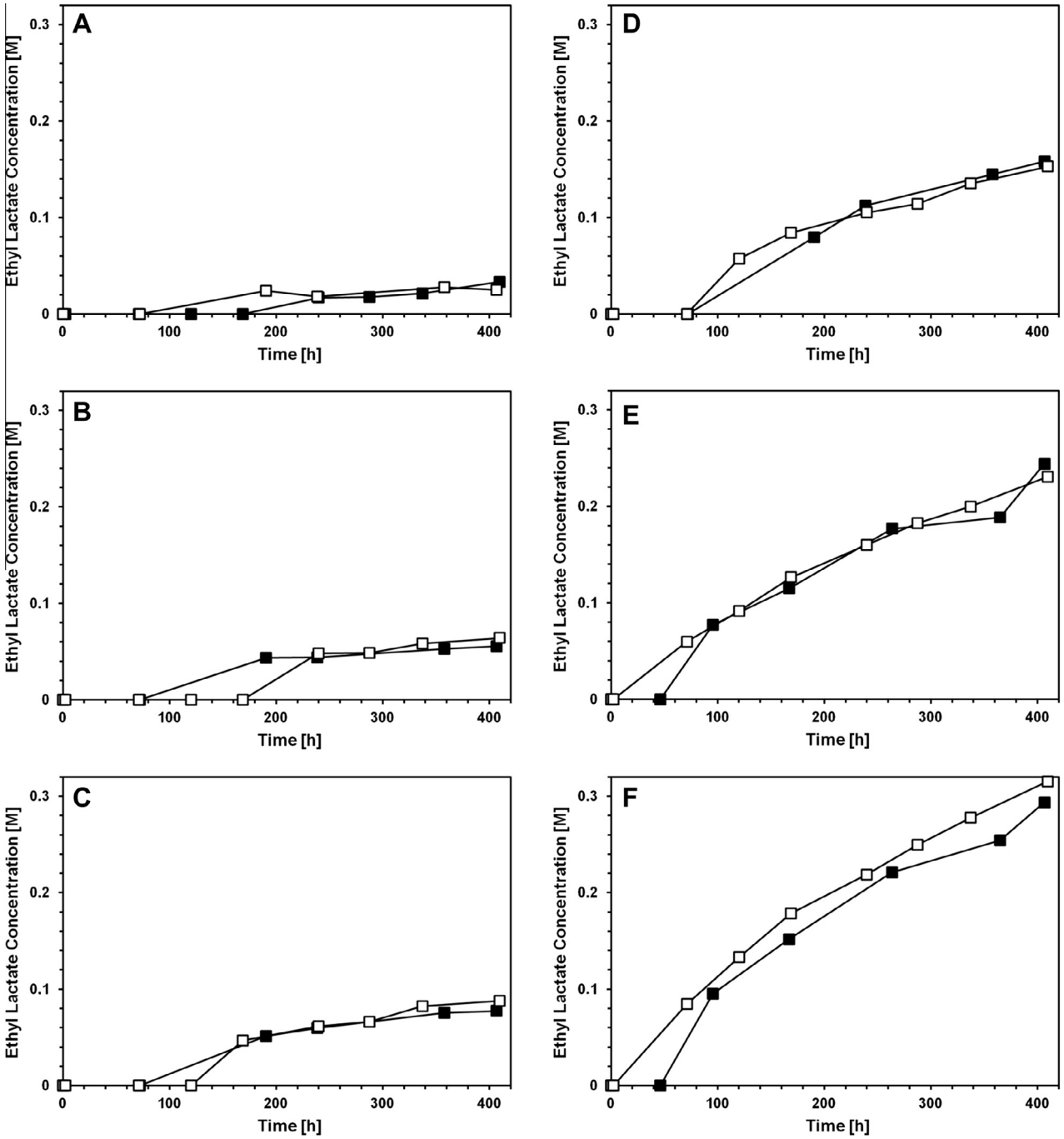

Fig. 4. The effect of different aqueous to solvent solution volume ratios on the esterification of lactic acid with ethanol. Enzymatic reactions were conducted using 10 mg ml^{-1} Novozyme 435, at 30 °C and 100 rpm. Control experiments were performed without the addition of enzyme. The concentration of ethanol and lactic acid in the aqueous phase was 5 M for all experiments. (A) 2% (v/v) of aqueous solution and 98% (v/v) of toluene, (B) 4% (v/v) of aqueous solution and 96% (v/v) of toluene, (C) 6% (v/v) of aqueous solution and 94% (v/v) of toluene, (D) 10% (v/v) of aqueous solution and 90% (v/v) of toluene, (E) 20% (v/v) of aqueous solution and 80% (v/v) of toluene and (F) 30% (v/v) of aqueous solution and 70% (v/v) of toluene. —■—: ethyl lactate produced with enzyme; —□—: ethyl lactate produced without enzyme.

lactate using a food-derived waste as raw material. Research in the future should focus on the improvement of ethyl lactate production. Water is the second product formed in the esterification of lactic acid and ethanol, which has been shown to significantly influence the ester yield in esterification reactions (Major et al., 2010). Therefore, a future direction of the present work could be to enhance the product yield of the combined system with continuous preferential removal of water from the reaction mixture, using pervaporation or desiccants (Delgado et al., 2010; Torres and Otero, 1999), in order to drive the equilibrium of the reaction towards ethyl lactate production. Additionally, the development of a continuous system should be explored to increase the productivity of ethyl lactate and to evaluate any potential operational and design issues. The hybrid bioprocess of this work could be of primary importance from both scientific and commercial standpoints, since the production of lactic acid in microbial fermentation involves extensive purification methods which could be avoided and alternatively performed through esterification with lower alcohols (Ago et al., 2011; Troupe and Dimilla, 1957). Consequently, the production of lactic acid and ethanol conducted from direct fermentation of different food/agro-industrial products and residues (John et al., 2007; Sanchez and Cardona, 2008) could be

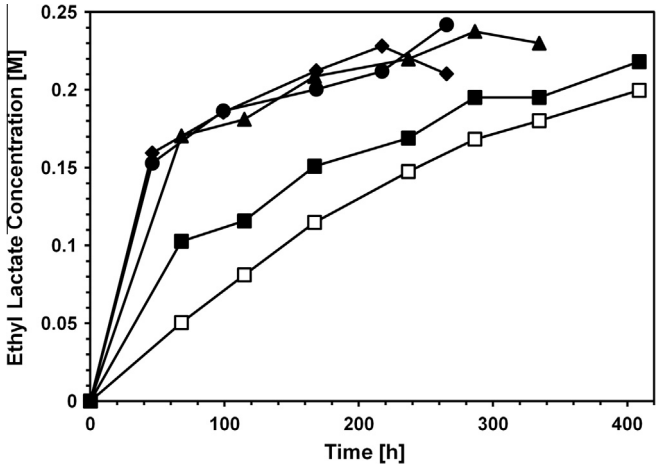

Fig. 5. The effect of different Novozyme 435 concentrations on the production of ethyl lactate. Experiments were performed by using 15% (v/v) of aqueous solution containing 5 M of each substrate and 85% (v/v) of toluene with different Novozyme 435 concentrations. Symbols correspond to the ethyl lactate concentration produced using Novozyme 435 concentrations of: (i) —■—: 10 mg ml^{-1}; (ii) —▲—: 40 mg ml^{-1}; (iii) —●—: 70 mg ml^{-1}; (iv) —◆—: 100 mg ml^{-1}; (v) —□—: without enzyme.

enhanced from an economic point of view through their enzymatic esterification reducing the purification cost.

4. Conclusions

The biotechnological production of ethyl lactate from a naturally derived feedstock is expected to alleviate the dependence on oil supply for the production of other toxic solvents. It has been shown that a fermentation–esterification bioprocess can be efficient for the exploitation of dairy waste to produce ethyl lactate. Furthermore, it was demonstrated that lactic acid and bioethanol can be effectively produced in fermentations and through extraction into hydrophobic solvents containing lipases they can be esterified into ethyl lactate. The process was effective in terms of ethyl lactate yield, which could be further enhanced through the development of a continuous system.

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