Bioresource Technology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

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.

ARTICLE INFO

Article history:
Received 15 January 2014
Received in revised form 7 March 2014
Accepted 12 March 2014
Available online xxxx

Keywords:
Ethyl lactate
Lipase
Cheese whey valorization
Ethanol
Lactic acid

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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http://dx.doi.org/10.1016/j.biortech.2014.03.053
0960-8524/

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

Please cite this article in press as: Koutinas, M., et al. Development of a hybrid fermentation–enzymatic bioprocess for the production of ethyl lactate from dairy waste. Bioresour. Technol. (2014), http://dx.doi.org/10.1016/j.biortech.2014.03.053