



Πανεπιστήμιο Ιωαννίνων

Τμήμα Χημείας, Τομέας Οργανικής
Χημείας & Βιοχημείας
Ιωάννινα, 45110

Δρ. Εμμανουήλ Μ. Παπαμιχαήλ
Ομότιμος Καθηγητής

E-mail: eparamic@cc.uoi.gr,

Tel.: +30 2651 008395, FAX: +30 2651 008977

Ιωάννινα, 25 Απριλίου 2017

ΠΡΟΣ: Τμήμα Δημοσίων Σχέσεων και Εθιμοτυπίας
Θέμα: Διεθνής επιτυχία και αναγνώριση στην Βιοτεχνολογία

Έχουμε την τιμή και την ευχαρίστηση να σας ανακοινώσουμε ότι στο πλαίσιο του Ερευνητικού Προγράμματος «**Συνεργασία 2011**»: «Αγροτοβιομηχανικά υγρά και στερεά απόβλητα ως πρώτες ύλες για την παραγωγή μιας νέας γενιάς βιοκαυσίμου», Ε.Σ.Π.Α. 2007-2013 [11ΣΥΝ_8_839], οι συνεργαζόμενες ερευνητικές ομάδες από τα Πανεπιστήμια Ιωαννίνων και Πατρών¹ ολοκλήρωσαν με μεγάλη επιτυχία και εγκαίρως το έργο τους, το οποίο δημοσιεύτηκε σε διαδοχικές εκδόσεις διεθνούς και έγκριτου περιοδικού Βιοτεχνολογίας. με υψηλό συντελεστή απήχησης (Bioresource Technology: I.F. 4.917/5-ετίας 5.744).

Οι τρεις διαδοχικές δημοσιεύσεις είναι οι ακόλουθες:

- (1) Economic evaluation of technology for a new generation biofuel production using wastes (Οικονομική αξιολόγηση της τεχνολογίας για παραγωγή βιοκαυσίμου νέας γενιάς χρησιμοποιώντας απόβλητα), *Bioresource Technology*, **200** (2016) 178-185.
- (2) Sustainable production of a new generation biofuel by lipase-catalyzed esterification of fatty acids from liquid industrial waste biomass (Βιώσιμη παραγωγή βιοκαυσίμου νέας γενιάς με καταλυόμενη, από λιπάση, εστεροποίηση λιπαρών οξέων από βιομάζα υγρών βιομηχανικών αποβλήτων) *Bioresource Technology*, **238** (2017) 122-128.
- (3) Scale-up for esters production from straw whiskers for biofuel applications (Μεγάλης κλίμακας παραγωγή εστέρων, από μουστάκια άχυρου, για εφαρμογές σε βιοκαύσιμα) *Bioresource Technology*, (2017) *In Press*.

Όπως φαίνεται από τις προαναφερόμενες δημοσιεύσεις, αρχικά προτάθηκε μία ολοκληρωμένη και νεωτεριστική τεχνολογία, για παραγωγή βιοκαυσίμου νέας γενιάς αξιοποιώντας πολλών ειδών βιομηχανικά απόβλητα, η οποία αποδείχθηκε ανταγωνιστική και λόγω απόδοσης και λόγω κόστους επένδυσης, αφού έτσι πραγματοποιήθηκε παραγωγή σημαντικών λιπαρών οξέων και αλκοόλων. Στην συνέχεια επιτεύχθηκε μία ολοκληρωμένη, οικονομικά συμφέρουσα και επιστημονικά προηγμένη μεθοδολογία εργαστηριακής παραγωγής βιοκαυσίμου νέας γενιάς με ενζυμικά καταλυόμενη εστεροποίηση των προαναφερόμενων λιπαρών οξέων και των αλκοολών, που παρήχθησαν από την κατεργασία βιομάζας υγρών βιομηχανικών αποβλήτων (ενός περιβαλλοντολογικού ρυπαντή). Η παραγωγή του βιοκαυσίμου πραγματοποιήθηκε με χρήση σημαντικά μικρότερων ποσοτήτων ενζύμου, από τις ως σήμερα αναφερόμενες, οι οποίες και ανακυκλώθηκαν τουλάχιστον δέκα φορές μηδενίζοντας ουσιαστικά το κόστος τους, ενώ επιτεύχθηκε παραγωγή βιοκαυσίμου σε πρωτοφανείς και πρωτοαναφερόμενες υψηλές συγκεντρώσεις (< 3.35 M) και αποδόσεις (< 97%) υποσχόμενες αντίστοιχες επιδόσεις για την παραγωγή του σε βιομηχανική κλίμακα. Τέλος, ακολούθησε η εστεροποίηση λιπαρών οξέων και αλκοολών, που παρήχθησαν με την εφαρμογή της βιοτεχνολογικής μεθόδου της οξυγένεσης και με βάση την ήδη αναπτυχθείσα μεθοδολογία, σε μεγάλο μέγεθος αντιδραστήρα (40 L) για παραγωγή μεγάλης κλίμακας και με αποδόσεις βιοκαυσίμου ~ 90% και σε συγκεντρώσεις < 2.0 M.

Εμμανουήλ Μ. Παπαμιχαήλ

¹ Ομάδα Βιοτεχνολογίας Τροφίμων (Πανεπιστήμιο Πατρών - Συντονιστής και Επιστ. Υπεύθυνος του Προγράμματος: Αθανάσιος Κουτίνας, Ομότιμος Καθηγητής) και Ομάδα Ενζυμικής Βιοτεχνολογίας και Γενετικής Μηχανικής (Πανεπιστήμιο Ιωαννίνων - Επιστ. Υπεύθυνος του Προγράμματος: Εμμ. Μ. Παπαμιχαήλ, Ομότιμος Καθηγητής)



Economic evaluation of technology for a new generation biofuel production using wastes



Athanasios Koutinas^{a,*}, Maria Kanellaki^a, Argyro Bekatorou^a, Panagiotis Kandylis^a, Katerina Pissaridi^a, Agapi Dima^a, Konstantina Boura^a, Katerina Lappa^a, Panagiota Tsafraikidou^a, Panagiota-Yiolanda Stergiou^b, Athanasios Foukis^b, Olga A. Gkini^b, Emmanuel M. Papamichael^b

^a Food Biotechnology Group, Department of Chemistry, University of Patras, Patras 26500, Greece

^b Group of Enzyme Biotechnology and Genetic Engineering, Department of Chemistry, University of Ioannina, Ioannina 45110, Greece

HIGHLIGHTS

- Economic evaluation of new generation biofuel production using wastes.
- A preliminary investment and cost analysis of the proposed technology is presented.
- The process is based on scale-up results of waste acidogenesis.
- Extraction of the produced organic acids was done with butanol-1.

ARTICLE INFO

Article history:

Received 30 June 2015

Received in revised form 20 September 2015

Accepted 21 September 2015

Available online 3 October 2015

Keywords:

Anaerobic acidogenesis

Ester-based biofuels

Kissiris

γ -Alumina

Scale-up

ABSTRACT

An economic evaluation of an integrated technology for industrial scale new generation biofuel production using whey, vinasse, and lignocellulosic biomass as raw materials is reported. Anaerobic packed-bed bioreactors were used for organic acids production using initially synthetic media and then wastes. Butyric, lactic and acetic acid were predominately produced from vinasse, whey, and cellulose, respectively. Mass balance was calculated for a 16,000 L daily production capacity. Liquid–liquid extraction was applied for recovery of the organic acids using butanol-1 as an effective extraction solvent which serves also as the alcohol for the subsequent enzyme-catalyzed esterification. The investment needed for the installation of the factory was estimated to about 1.7 million € with depreciation excepted at about 3 months. For cellulosics, the installation investment was estimated to be about 7-fold higher with depreciation at about 1.5 years. The proposed technology is an alternative trend in biofuel production.

© 2015 Published by Elsevier Ltd.

1. Introduction

Nowadays there is intense interest in expanding the use of renewable energy sources and therefore the production of biofuels worldwide. Among the proposed biofuels, bioethanol and biodiesel are the most commonly used with numerous applications due to their similar, to some extent, chemical and physical properties with traditional petroleum fuels like diesel and gasoline (Randazzo and Sodre, 2011). These properties allow their use, in combination with traditional fuels, in regular engines after minor modifications (Jayed et al., 2009). In addition, many other types of biofuels have been tested in compression-ignition and spark-ignition engines such as biomethanol (Ogden et al., 1999),

biobutanol (Irimescu, 2012), bioethers (Petre et al., 2011), dimethyl ether (Toulson et al., 2010), Fischer–Tropsch diesel (Gill et al., 2011), biogas (Uusitalo et al., 2013), biohydrogen (Bakonyi et al., 2014) and syngas (Kirillov et al., 2012).

Recently, new generation biofuels have been introduced based on esters of low molecular weight organic acids (OAs). Several studies have been carried out to evaluate the properties of such biofuels (ethyl-, butyl- and pentyl-esters) and to test them in compression-ignition and spark-ignition engines (Contino et al., 2011, 2014; Jenkins et al., 2013). The advantage of these ester-based biofuels is that the OAs and alcohols needed for the esterification can be simultaneously produced by acidogenesis of low-value biomass wastes (Contino et al., 2011). Previous studies have reported the simultaneous production of OAs and ethanol during anaerobic acidogenesis of synthetic media containing glucose (Ren et al., 1997; Syngiridis et al., 2013, 2014). Furthermore,

* Corresponding author. Tel.: +30 2610997104; fax: +30 2610997105.

E-mail address: a.a.koutinas@upatras.gr (A. Koutinas).



Fig. 1. Acidogenic fermentation promoters.

Table 1

Type of fermentation and products for each carbohydrate substrate and promoter used in continuous operation.

Substrate	Initial pH	Type of fermentation	Predominant acid	Other products
<i>γ-Alumina</i>				
Glucose	6.5	Ethanol	Acetic	Butyric, propionic
Glucose	8.9	Butyric acid	Butyric	Acetic, propionic
Sucrose	7.0	Lactic acid	Lactic	Succinic, malic, acetic
Cellobiose	9.0	Succinic acid	Succinic	Lactic, butyric
Lactose	9.0	Lactic acid	Lactic	Succinic, acetic
<i>Kissiris</i>				
Glucose	9.0	Lactic acid	Lactic	Succinic, acetic
Sucrose	8.0	Lactic acid	Lactic	Butyric, succinic
Lactose	9.0	Lactic acid	Lactic	Succinic, butyric, acetic

Table 2

Type of fermentation for each waste substrate and promoter used in continuous operation.

Substrate	Organic acids	Predominant acid	Type of fermentation	Other products
<i>γ-Alumina</i>				
Cellulose	Acetic, butyric	Butyric	Butyric	Lactic, acetic, isobutyric
Vinasse	Butyric	Butyric	Butyric	Succinic, lactic
Whey	Lactic	Lactic	Lactic	Succinic
<i>Kissiris</i>				
Cellulose	Acetic, butyric	Acetic	Acetic	Butyric, isobutyric
Vinasse	Butyric	Butyric	Butyric	Acetic, isobutyric
Whey	Lactic	Lactic	Lactic	Butyric, acetic

very good OAs productivities were obtained during acidogenesis of vinasse, the main liquid waste of the ethanol industry (Lappa et al., 2015a, 2015b).

In previous studies it was also shown that methane, alcoholic and acidogenic fermentations can be promoted in the presence of porous materials such as γ -alumina and kissiris, which acted at the same time as culture immobilization carriers (Fig. 1) and facilitated continuous processing (Koutinas et al., 1991; Galanakis et al., 2012; Syngiridis et al., 2013, 2014). The promoting effect of these carriers was also proved in the acidogenesis of wastes such as vinasse (Lappa et al., 2015a, 2015b). However, the above studies include only published results on acidogenic fermentations of synthetic, simple carbohydrate media as well as of vinasse at laboratory scale (Table 1). Therefore, an integrated technology development using other bulk agroindustrial wastes such as lignocellulosics and whey was necessary (Table 2), including research results on recovery of the produced OAs using organic solvents

which could also serve as the OAs esterification reactant. To prove the feasibility of the proposed technology for industrial application, scale-up experiments and an economic evaluation was also necessary. Hence, the present work presents an economic evaluation of the proposed integrated technology for a new generation biofuel production using agroindustrial liquid and solid wastes as raw materials at industrial scale based on reported results at laboratory and scale-up operations.

2. Methods

2.1. Process principle

The described process is based on scale-up results (80 L bioreactors) of acidogenic fermentations of wastes such as whey, vinasse and lignocellulosic biomass. Furthermore, it was supported on laboratory results obtained for OAs production, recovery and esterification reactions. The technology is based on three unit operations: (i) anaerobic treatment separately of vinasse, whey and lignocellulosic biomass in the presence of γ -alumina or kissiris as promoters, to produce OAs, (ii) OAs recovery from the aqueous solutions, and (iii) esterification of OAs to esters. Extensive research was done for the production of OAs showing that kissiris is a better material to promote the acidogenic fermentations of all the above wastes. Specifically, research on vinasse showed that adaptation was necessary in order to obtain substantial conversion to OAs with high operational stability. Furthermore, it was found that treatment of delignified cellulosic biomass in the presence of kissiris leads to direct production of OAs without pretreatment for cellulose hydrolysis, and therefore it accommodates a one-step process. The produced OAs were recovered from the aqueous solution by extraction with butanol-1 resulting in 80% yield and they were used in the subsequent enzyme-catalyzed esterification reaction. During the acidogenesis of vinasse, whey and lignocellulosic biomass, small amounts of ethanol were also formed.

2.2. Process description

2.2.1. Vinasse

Process flow-sheets were designed and mass balance was calculated taking into account the results of the experimental work that was done at laboratory scale as regards acidogenesis kinetics, preliminary esterification reactions and OAs recovery. In addition, the type of machinery used and unit operations (batch or continuous) were taken into account. Likewise, the machinery cost is given provided that it will be designed by the developers of the technology and produced in mechanical construction companies in Greece. Mass balance was calculated based on a 16,000 L daily production capacity for both vinasse and lignocellulosic biomass. The estimation of investment and production costs was done based on previous experience from research on a biotechnological plant, and in any case the data presented in this investigation are preliminary. The investment cost for vinasse and whey, and cellulose is shown in Tables 3 and 4, respectively. Fig. 2 presents the proposed process flow-sheet with mass balance for the production of the new generation biofuel using vinasse. The process will have to include a set-up for growth of the anaerobic culture (1), and transfer of the culture using a peristaltic pump (2) into a packed-bed bioreactor system (3) containing kissiris distributed in multiple floors. The aqueous solution that contains the produced OAs was pumped to tank (4) for extraction with butanol-1 (Ren et al., 1997; Lappa et al., 2015a, 2015b). The extract containing 80% of the OAs was pumped in reactor (5) for esterification, using lipase produced in bioreactor (7). The solution of esters in butanol-1 was distilled in the solvent recovery column (8) using steam from the boiler (10)

Table 3
Investment cost for vinasse and whey for a daily production capacity of 16,000 L.

Machinery	Vinasse		Whey	
	Capacity	Indirect cost (€)	Capacity	Indirect cost (€)
Liquid waste tank	20 tn	20,000	20 tn	20,000
Peristaltic pump	20 tn/h	20,000	20 tn/h	20,000
Packed-bed continuous bioreactor	250 tn	300,000	250 tn	300,000
Esterification bioreactor	50 tn	50,000	50 tn	50,000
Pump	16 tn/h	10,000	16 tn/h	10,000
Culture production for lipase production	20 tn	20,000	20 tn	20,000
Solvent recovery column	4 tn/h	1,000,000	4 tn/h	1,000,000
Biofuel receiver tank	20 tn	20,000	20 tn	20,000
Boiler	1.4 tn oil/day	200,000	1.4 tn oil/day	200,000
Extraction tank	50 tn	40,000	50 tn	40,000
Pipe lines		10,000		10,000
Royalties		500,000		500,000
Case study		130,000		130,000
Total investment		2,320,000		2,320,000

Table 4
Investment cost for cellulose for a daily production capacity 16,000 L.

Machinery	Capacity	Indirect cost (€)
Liquid waste tank	3 × 100 tn	50,000
Peristaltic pump	2 × 20 tn/h	40,000
Reactor for delignification	2 × 300 m ³	100,000
Packed-bed continuous bioreactor	16 × 250 tn	4,800,000
Esterification bioreactor	8 × 100 tn	4,000,000
Pump	3 × 16 tn/h	300,000
Culture production for lipase production	20 tn	320,000
Solvent recovery column	6 tn/h	1,300,000
Biofuel receiver tank	20 tn	20,000
Boiler	2 tn oil/day	300,000
Extraction tank	50 tn	40,000
Pipe lines		10,000
Royalties		500,000
Case study		880,000
Total investment		12,660,000

and the stream of esters flows to the collection tank (9). The new generation biofuel will mainly consist of butyl butyrate.

2.2.2. Lignocellulosic biomass

The process flow-sheet is presented in Fig. 3. The process design includes about the same unit operations as in the case of vinasse (Fig. 2) with main difference the delignification reactor (3a). However, more bioreactors are necessary for acidogenesis of delignified cellulose and esterification reactions (Tables 3 and 5). The biofuel that will be produced from lignocellulosic biomass will mainly contain a mixture of butyl acetate (predominant) and butyl butyrate.

2.2.3. Whey

The treatment of this effluent needs a similar process as described in the case of vinasse. The biofuel that will be produced from whey will mainly consist of butyl lactate.

3. Results and discussion

3.1. Pilot-plant operation

In the present study anaerobic packed-bed bioreactors (APBBs) were used. APBB have been tested successfully for several anaerobic applications using synthetic media (Ferraz Junior et al., 2014a;

Penteado et al., 2013), and in few cases industrial wastewater, including vinasse and whey (Ferraz Junior et al., 2014b; Perna et al., 2013). The advantages of APBB include the achievement of higher cell concentrations and longer cell retention times. These parameters are essential in bioprocesses that use substrates with high organic load and inhibitory substances.

Three different agroindustrial wastes were used as raw materials for the acidogenesis process: vinasse, whey and wheat straw. Tables 1 and 2 summarize the produced acids and the prevailing type of fermentation for each substrate (carbohydrate or waste) and promoter used.

3.1.1. Acidogenesis of vinasse

3.1.1.1. Description and operation of the bioreactor. The bioreactor was made from stainless steel and had two horizontal screens supported on a vertical axis separating the bioreactor in three separate compartments. The bioreactor had an inlet at the bottom and two outlets, one at each floor and was coated by an external jacket to cool or heat the fermented liquid at the desired temperature. Two glass windows outside each compartment allowed observation of the fermenting liquid and an external glass horizontal cylinder was used to show the liquid level and any gas formation or foaming. The bioreactor was fed by a high accuracy peristaltic pump and was designed to operate as both batch and continuous system.

The middle compartment was packed with 15 kg of mineral kissiris and operated in batch mode for more than 3 months. For the adaptation of the culture, the start-up was done using synthetic media containing sucrose, followed by sucrose–raffinose mixtures and then successively vinasse was added in the mixture increasing its concentration stepwise up to 100%. After the 7th batch the system operated with effluent recirculation at a flow rate of 1 L/h. The experimental apparatus was used for both cell immobilization and acidogenic fermentation with the temperature remaining constant at 37 °C. The bioreactor was filled up to the volume of 60 L with mineral kissiris (15 kg), operating as a two compartment system, it was charged with 30 L sucrose medium and inoculated with 70 g wet weight anaerobic culture. The system was left to ferment for five days at 37 °C with synthetic media containing sucrose and without feeding in order to achieve cell immobilization.

3.1.1.2. Acidogenesis process. The bioreactor was operated in batch mode for more than 3 months. During the first month the system was operating with sucrose and raffinose mixtures. After that vinasse was added in the influent, with successively increasing amounts until it finally reached 100% concentration, as described above, for adaptation of the culture. After the 7th batch the system operated with recirculation (1 L/h). In all experiments the initial pH was adjusted to 8 with NaOH solution. The vinasse used had 4° Be hydrometer density. The operation of the bioreactor was monitored by analysis of samples that were collected at various time intervals and analyzed for OAs, ethanol and residual sugar by GC and HPLC methods.

After 15 days (5 batches) using mixtures of sucrose synthetic media and vinasse, the APBB was fed with vinasse for another 37 days performing 5 batches always using the same immobilized culture. The use of vinasse caused an increase on the total OAs content. Butyric acid accounted for more than 55% of total OAs. Ethanol production was not affected and concentrations up to 1.4 mL/L were obtained.

The produced OAs consisted of butyric, lactic, acetic, propionic, isobutyric and isovaleric acids. During the first day of fermentation all acids were produced at low concentrations, except butyric and lactic acid. As the fermentation proceeded all acid concentrations were increased apart from lactic acid, that was continuously

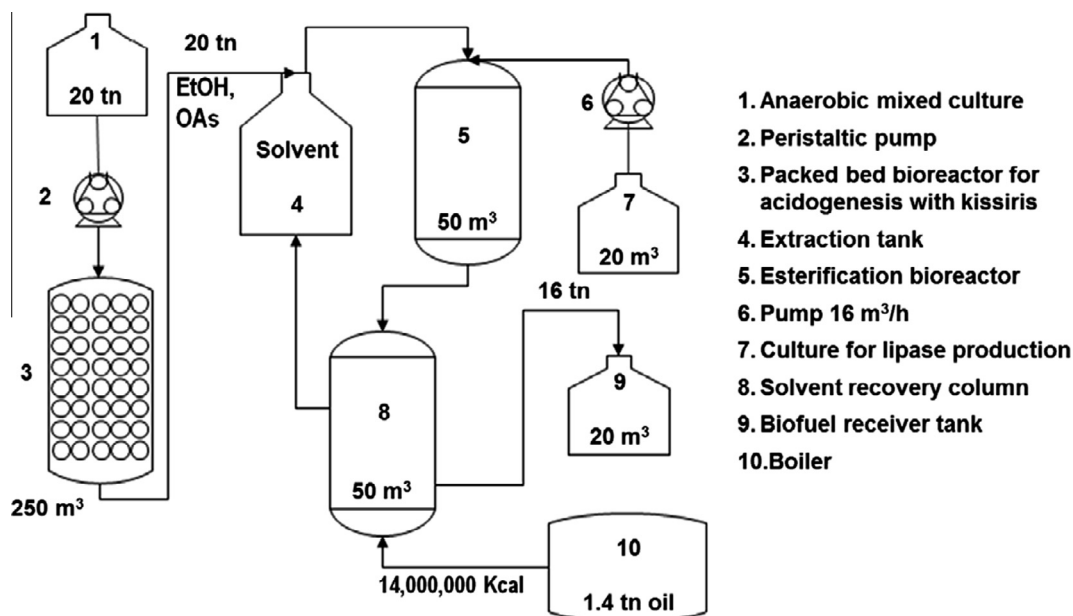


Fig. 2. Process flow-sheet for the production of new generation biofuel from vinasse and whey.

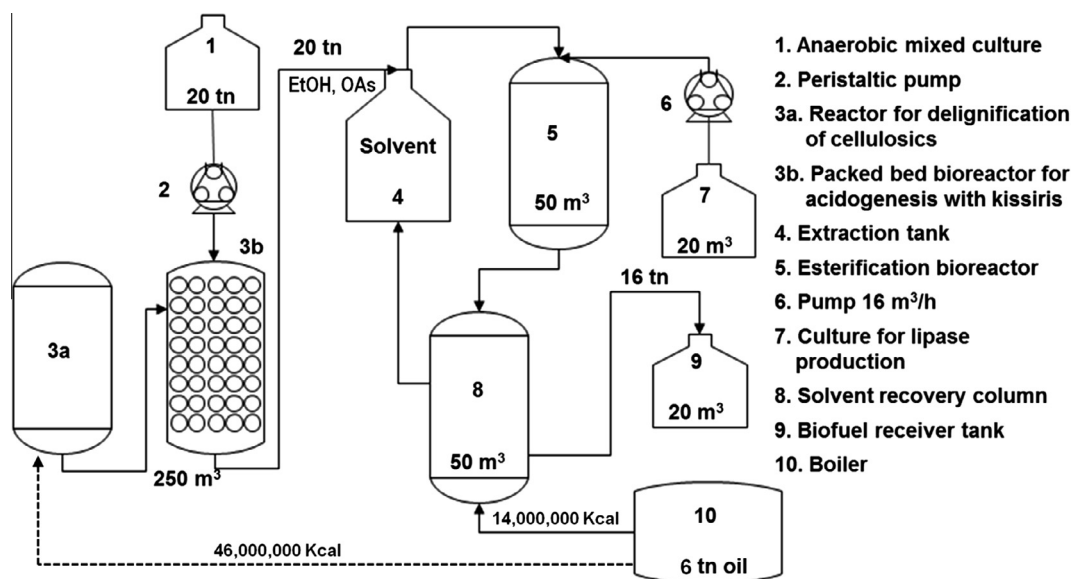


Fig. 3. Process flow-sheet for the production of new generation biofuel from lignocellulosics by direct acidogenesis.

decreasing and eventually stabilized. This progressive lactic acid reduction was mainly attributed to the change of carbohydrate composition of the substrates during the start-up of the process for culture adaptation (starting from 100% sucrose, followed by sucrose–raffinose mixtures and finally vinasse). The higher total OAs concentrations were reached after 7 days. Butyric acid accounted for more than 60% of total OAs.

These results are better compared to those of a continuous laboratory scale system of 1.25 L previously studied (Lappa et al., 2015a). More specifically, the scale-up batch system described in the present study produces an average of 16.5 g/L OAs compared to 12 g/L of the laboratory continuous system. This means an increase of more than 37% in the total OAs concentrations.

3.1.2. Acidogenesis of straw

3.1.2.1. Description and operation of the bioreactor. An APBB similar to that used in the case of vinasse was used for the acidogenesis of

wheat straw. The experimental apparatus used in this body of work consisted of a metal cylindrical tower reactor of 60 L total working volume connected with a peristaltic pump in order to facilitate effluent recirculation. This system was placed in an incubator at 37 °C. An amount of 4.4 kg of the mineral kissiris in coarse form (diameter ~5 cm) was placed inside the bioreactor and another 2.750 kg of mineral kissiris in fine form (diameter ~1 cm) was placed inside a metal basket which was also inserted in the bioreactor.

Delignified straw was used as substrate in all experiments. After delignification the wheat straw was totally dried and cut to smaller pieces of about 1 cm in length. In order for the experiment to commence, 50 g of fermented growth medium containing culture was added in the bioreactor, and 15 L of liquid medium were pumped containing 1.6 mL/L NH₃, 0.32 mL/L of 50% H₃PO₄ solution, 4 g/L yeast extract and 4 g/L NaHCO₃. Delignified straw was added to the desired concentration (30 g/L). Moreover in order to facilitate

Table 5
Cost analysis of vinasse and cellulotics.

	Vinasse and whey	Cellulotics
	<i>Depreciation of the investment</i>	
	16,000 L × 1.6 €/L = 25,600 €/day	16,000 L × 1.6 €/L = 25,600 €/day
	2,320,000 €/25,600 = 91 days	12,660,000 €/25,600 = 495 days
	<i>Production capital/month (€/month)</i>	
Labor cost	40 workers × 1500 € = 60,000	80 workers × 1500 € = 120,000
Energy cost	1.4 tn × 800 €/tn × 30 days = 33,600	6 tn × 800 €/tn × 30 days = 144,000
Electricity and water requirements	30,000	100,000
Raw material cost	154,560	155,000
Maintenance cost	5600	10,400
Total	283,760	529,400
	<i>Added value/month and biofuel production cost</i>	
	480,000 L biofuel × 1.6 = 768,000 €	480,000 L biofuel × 1.6 = 768,000 €
	Production cost 0.59 €/L	Production cost 1.10 €/L

culture adaptation and immobilization on kissiris, 200 g of glucose were also added. The initial pH of the broth was 9. The system was allowed to stand for 24 h. After that the pump was adjusted to allow recirculation of the liquid contained in the bioreactor at a rate of 536 mL/min.

At the end of each batch the fermented liquid was removed, while kissiris and the leftover straw were retained in the bioreactor. For the following batches new straw and nutrients were added, in the same quantities as described above, in addition to 2 L of the previously removed fermented liquid that contained precipitated culture, which was collected after decanting the supernatant liquid.

3.1.2.2. Acidogenesis process. From the results obtained during the startup operation of the bioreactor it can be concluded that the batch acidogenic fermentation in the presence of kissiris resulted in the formation of mainly lactic, acetic, and butyric acids as well as ethanol. As the fermentation progressed it was observed that the lactic acid concentration was decreased when the added glucose was consumed. At the same time the concentration of butyric acid started increasing.

Since the formed OAs during acidogenesis were acetic and butyric, at all the studied fermentation batches, it can be concluded that the use of culture immobilized on kissiris, under the given conditions, promoted the metabolic pathway of Ren et al. (butyric-type fermentation) (Ren et al., 1997). This type of fermentation is favored by insufficient NADH at pH values higher than 6 and lower than 5, due to the consumption of NADH for cellular synthesis or by conversion to NAD⁺ and production of hydrogen (Ren et al., 1997). However, during the described process in the presence of kissiris, little or no formation of gaseous products was observed. Furthermore, the increased free ammonia nitrogen at high pH inhibits the methanogenic bacteria and results in OAs accumulation (Rajagopal et al., 2013).

3.1.3. Acidogenesis of whey

An APBB similar to that used in the case of vinasse was used for the acidogenesis of whey. The main OA produced was lactic acid.

3.2. OAs recovery

The major challenge in the production of OAs by fermentation is their cost effective recovery from the fermentation broths (Alkaya et al., 2009). The contribution of separation and purification on OAs production cost is large. Therefore the effective recovery of these fermentation products has received considerable attention, in order to make their bio-based production feasible. Several

techniques have been applied for the recovery of OAs from fermentation broths, including adsorption, liquid–liquid extraction, precipitation and electrodialysis (Alkaya et al., 2009). Liquid–liquid extraction is one of the oldest methods for the recovery of OAs. However several factors affect their extraction such as the nature of the OAs, the concentration of the extractant, the type of diluent and pH (Alkaya et al., 2009; Singhania et al., 2013). Alcohols, ketones, ethers, aliphatic hydrocarbons and organophosphates can be used as solvents for the extraction of OAs from fermentation broths (Singhania et al., 2013).

Several solvents were evaluated for the recovery of OAs. Solvent alcohols were especially considered in order to facilitate the subsequent direct esterification (either enzymatic or chemical) of the extraction products to produce new generation biofuels. The influence of pH and the extractant concentrations on the recovery of OAs from pure aqueous solutions and fermentation broth were assessed.

A variety of solvents were evaluated. To evaluate the effect of each solvent on the recovery of OAs, standard aqueous solutions were prepared containing the same OAs as those produced during the acidogenic fermentations. The recovery took place at room temperature under magnetic stirring and a solvent/standard solution ratio of 1:1. During 2 h of extraction samples were received from the aqueous phase and were analyzed for the remaining OAs content. Subsequently, the recovery of OAs was evaluated in the fermented broths.

Among the tested solvents, alcohols presented the best extraction performance. Butanol-1 was not only an effective solvent regarding the recovery of OAs (about 90% recovery) but it was also the most cost effective.

3.3. Esterification

For economic, sustainable and safe biotechnological production of biofuels the use of different renewable raw materials, microorganisms and enzymes is recently gaining attention but their application at industrial scale requires the design of sustainable processes (Fortman et al., 2008; Davis et al., 2009). The acidogenesis process was followed by enzyme-catalyzed esterification of the produced OAs. Efforts were made in studying the rate of product formation, the effects of changes in system conditions, and the use of appropriate reactors in order to improve both yield and quality of the lipase-catalyzed ester synthesis (Stergiou et al., 2013) and to improve the process from an economical point of view (optimum alcohol:acid ratio, minimum quantity of the used enzyme, etc.). This new generation biofuel is a mixture of esters

of lower molecular weight that are contained in conventional biodiesel.

3.4. Investment and cost analysis

Tables 3 and 5 contain the economic data of the new generation biofuel production using vinasse and have been calculated for a 16,000 L production capacity. The investment needed for the installation of the factory is about 1.7 million €, with depreciation of the investment expected in about three months. The created added value is close to 0.76 million €/month, with a monthly capital of 283,760 € to operate the industrial unit. The production cost of the new generation biofuel is estimated to about 0.59 €/L. For cellulose, the investment for the installation is about 7-fold higher because more bioreactors are needed (Table 4). The depreciation of the investment will be achieved at about 1.5 years and about 3-fold capital is needed to operate the industrial unit. The production cost of the new generation biofuel using cellulose is about 2-fold higher as compared with vinasse.

Regarding the process flow-sheets of the different raw materials (lignocellulosics, vinasse, whey), their differences are more than the obvious requirement for a delignification reactor, justifying the big difference of investment and production cost for lignocellulosics. This is due to the increased complexity of the plant that uses lignocellulosics as raw material in comparison with vinasse and whey. The increased number of bioreactors increases investment cost, labor cost and energy related costs. The last is due to kinetic reasons related with the rate of the acidogenesis process.

3.5. Aims of the proposed technology

The main aims of the proposed technology are (i) to create an integrated process for the production of a new generation biofuel suitable for most of the bulk liquid wastes of the agri-food industry, and (ii) to offer an alternative for the production of biofuels from lignocellulosic biomass, versus bioethanol production. Most of the bulk wastes have been extensively examined as raw materials for fuel grade bioethanol production during at least the last three decades. However, although bioethanol is a promising biofuel (Vohra et al., 2014), its production from lignocellulosic biomass is limited by the low rates of cellulose hydrolysis, the low glucose and therefore alcohol yield, and the high energy demand in the alcohol production plant; problems that still remain unsolved (Sanchez and Cardona, 2008; Cheng and Timilsina, 2011). By the proposed technology these problems can be overcome through the low energy demand for distillation of low volume solutions of esters in an organic solvent such as butanol-1, compared to the high energy demand for bulk aqueous solutions of bioethanol. The technology is based on the anaerobic treatment of different kinds of wastes using a mixed culture, leading to formation of C₂–C₅ OAs followed by their esterification with butanol-1 and the bioethanol simultaneously formed during the process. Butanol-1 is preferred compared to bioethanol because it forms an upper layer with water and facilitates, without energy demand, the extraction of OAs and subsequent esterification using lipases. Furthermore, butanol-1 can be also produced by fermentation from raw materials of low cost (Becerra et al., 2015).

3.6. Cost, investment, and added value

The production cost of the new generation biofuel using bulk liquid wastes such as vinasse and whey, is promising and it is the half of that of lignocellulosic biomass. Using ethanol for the esterification reaction, the cost is about 20% lower in the case of vinasse and whey, and 10% for cellulose. However, using bioethanol has the disadvantage of higher energy demand, and feasibility

of the process can be obtained using butanol-1. The investment cost is very low for vinasse and whey and although it is much higher for cellulose, it still is affordable for the investor. Using lignocellulosic biomass from wood, straw, municipal wastes and agricultural residues, EU can produce annually a bulk volume of esters that can be used as a new generation biofuel (Table 6). The annual added value for Greece, EU and Developed Countries, is estimated to 5.5, 275, and 690 billion \$, respectively.

3.7. Comparison of the three waste substrates

Regarding the culture immobilization carriers, kissiris is superior compared to γ -alumina due to its lower cost and higher availability in nature. Furthermore, its presence promoted the direct acidogenesis of cellulose without prior hydrolysis required. This why kissiris was further evaluated regarding its effect on the acidogenic fermentation of three different wastes. Cellulose produced more than 50% of acetic acid and 25–30% butyric acid. During treatment of vinasse, butyric acid predominated with acetic acid also produced at lower concentrations. Whey gave about 70% lactic acid with smaller amounts of butyric acid (Table 2). The suitable solvent for OAs recovery was butanol-1, which could also serve as the subsequent OAs esterification alcohol to produce esters suitable as a new generation biofuel. The concentration of total OAs produced during the scale-up experiments for cellulose, vinasse and whey, was about 16 g/L. For that reason the liquid wastes vinasse and whey have similar mass and energy balance processes, investment and production costs. The increased investment and production costs of cellulose, compared to whey and vinasse, are attributed to the lower cellulose bioconversion rate, which increases the number of required bioreactors for the same production capacity. Finally, the new generation biofuel will consist of acetate and butyrate esters in the case of cellulose and vinasse, and lactate and butyrate esters in the case of whey.

3.8. Technological consideration

Esters of C₂–C₅ OAs have been recently examined for their suitability as biofuels and were found to be promising for use in automobiles (Contino et al., 2011). The described extensive research was carried out at the University of Patras, the University of Ioannina, and three companies in the frame of a Grant co-funded by the Greek Government and EU with 1.2 million €. It has been shown that using kissiris promotes the anaerobic acidogenic fermentations, and wastes such as vinasse, whey and lignocellulosics as raw materials leads to mainly production of butyric acid, lactic acid, and acetic acid, respectively (Table 2). Comparing the butanol esters of the aforementioned acids with bioethanol, the proportion of carbon and oxygen atoms is about 3–4 versus 2, respectively. For the corresponding ethanol esters, the proportion is 2–3. This indicates that esters will have the same advantages (Vohra et al., 2014) with ethanol as biofuel in automobiles. The acidogenesis of simple

Table 6
New generation biofuel annual production and raw material availability.

Cellulosics	Amount (tn)	Biofuel in Greece (m ³)	EU biofuel annually (m ³)
Straw	750,000	600,000	30,000,000
Wood	750,000	600,000	30,000,000
Municipal wastes	500,000	400,000	20,000,000
Whey	500,000	25,000	1,250,000
Vinasse	360,000	10,000	500,000
Other agricultural residues	750,000	600,000	30,000,000
Total	3,610,000	2,235,000	111,750,000

carbohydrates resulted to different OAs formation (Table 1), as compared with the waste raw materials. The research proved that the acid formation depends on the carbohydrate or raw material substrates, on the carrier used to promote the fermentation and on pH (Tables 1 and 2). Here, the substantial simultaneous ethanol formation along with OAs in the case of acidogenesis of pure carbohydrates in the presence of promoters, also has to be highlighted (Syngiridis et al., 2013; Lappa et al., 2015a, 2015b). However, ethanol formation in the case of the raw materials (vinasse, whey and cellulose) was very low.

Comparing the research results obtained in the lab-scale bioreactors of 1.5 L and the scale-up bioreactors of 50–100 L, an improvement of the rate of acidogenesis and OAs concentration was observed for the scale-up process. This improvement can be attributed to the recirculation of the effluent, which increased the contact time with the kissiris supported culture. The reduction of ethanol concentration when the waste substrates were used compared to the synthetic media, may be attributed to changes in the culture microflora and prevailing fermentation type (Ren et al., 1997); from ethanol-type to acetate, butyrate and lactate type.

It could also be stressed here that the use of whey and vinasse has fewer technical obstacles than cellulosic biomass and the technology is more feasible. Furthermore, there are no risks for the use of esters as a biofuel in automobiles. The esterification using lipases, in the process to be industrially feasible, and the only technical risks remaining are (i) the use of kissiris as culture immobilization carrier and fermentation promoter, and (ii) the pretreatment of cellulosic substrates. Specifically, these risks are associated with the handling of big amounts of kissiris in the bioreactor and the reduction of particle size of cellulose for more efficient conversion. The first has been proved feasible in the case of molasses fermentation (Bakoyianis and Koutinas, 1996). Likewise, the reduction of cellulose particle size is also feasible due to existing cutting machineries.

Regarding raw materials availability for a production capacity of 16,000 L/day, this is obvious for lignocellulosic biomass as shown in Table 6. In the case of whey, for this biofuel production capacity, a dairy company that treats 400 tn milk/day, like those existing in Europe, is adequate. Finally for vinasse, an alcohol distillery of 25,000 L daily ethanol production is necessary, which is also common in the EU region and elsewhere.

4. Conclusions

An integrated, innovative technology for new generation biofuel production from various wastes was proposed, with competitive production and investment costs. Kissiris (culture immobilization carrier) better promoted the acidogenic fermentations compared to γ -alumina. Butyric, lactic and acetic acids were predominantly produced from vinasse, whey, and cellulose, respectively. Ethanol was formed when synthetic media were used, and was lower than 3 mL/L in the case of wastes. Butanol-1 found as an efficient solvent for OAs recovery, which could be serve as reagent for their subsequent esterification. The proposed technology is an alternative to biofuel production from lignocellulosics, with lower production cost than that of gasoline.

Acknowledgements

Research project co-financed by the European Union (European Regional Development Fund – ERDF) and Greek national funds through the Operational Program “Competitiveness and Entrepreneurship” of the National Strategic Reference Framework (NSRF) 2007–2013 – National Action “Cooperation 2011: Partnerships of Production and Research Institutions in Focused Research

and Technology Sectors” of General Secretariat for Research and Technology – Greece (Contract No. 11SYN-8-839).

References

- Alkaya, E., Kaptan, S., Ozkan, L., Uludag-Demirer, S., Demirer, N.G., 2009. Recovery of acids from anaerobic acidification broth by liquid–liquid extraction. *Chemosphere* 77, 51–59.
- Bakonyi, P., Nemestothy, N., Simon, V., Belafi-Bako, K., 2014. Review on the start-up experiences of continuous fermentative hydrogen producing bioreactors. *Renewable Sustainable Energy Rev.* 40, 806–813.
- Bakoyianis, V., Koutinas, A.A., 1996. A catalytic multistage fixed-bed tower bioreactor in an industrial-scale pilot plant for alcohol production. *Biotechnol. Bioeng.* 49, 197–203.
- Becerra, M., Cerdan, M.E., Gonzalez-Siso, M.I., 2015. Biobutanol from cheese whey. *Microb. Cell Fact.* 14, 27.
- Cheng, J.J., Timilsina, G.R., 2011. Status and barriers of advanced biofuel technologies: a review. *Renewable Energy* 36, 3541–3549.
- Contino, F., Foucher, F., Mounaim-Rousselle, C., Jeanmart, H., 2011. Combustion characteristics of tricomponent fuel blends of ethyl acetate, ethyl propionate, and ethyl butyrate in homogeneous charge compression ignition (HCCI). *Energy Fuel* 25, 1497–1503.
- Contino, F., Dagaut, P., Dayma, G., Halter, F., Foucher, F., Mounaim-Rousselle, C., 2014. Combustion and emissions characteristics of valeric biofuels in a compression ignition engine. *J. Energy Eng.* 140, SI: Innovative Technologies on Combustion of Biofuels in Engines: Issues and Challenges, A4014013.
- Davis, S.C., Anderson-Teixeira, K.J., DeLucia, E.H., 2009. Life-cycle analysis and the ecology of biofuels. *Trends Plant Sci.* 14, 140–146.
- Ferraz Junior, A.D.N., Wenzel, J., Etchebehere, C., Zaiat, M., 2014a. Effect of organic loading rate on hydrogen production from sugarcane vinasse in thermophilic acidogenic packed bed reactors. *Int. J. Hydrogen Energy* 39, 16852–16862.
- Ferraz Junior, A.D.N., Zaiat, M., Gupta, M., Elbeshbishy, E., Hafez, H., Nakhla, G., 2014b. Impact of organic loading rate on biohydrogen production in an up-flow anaerobic packed bed reactor (UANPBR). *Bioresour. Technol.* 164, 371–379.
- Fortman, J.L., Chhabra, S., Mukhopadhyay, A., Chou, H., Lee, T.S., Steen, E., Keasling, J. D., 2008. Biofuel alternatives to ethanol: pumping the microbial well. *Trends Biotechnol.* 26, 375–381.
- Galanakis, C.M., Kordulis, C., Kanellaki, M., Koutinas, A.A., Bekatorou, A., Lycourghiotis, A., 2012. Effect of pressure and temperature on alcoholic fermentation by *Saccharomyces cerevisiae* immobilized on γ -alumina pellets. *Bioresour. Technol.* 114, 492–498.
- Gill, S.S., Tsolakis, A., Dearn, K.D., Rodriguez-Fernandez, J., 2011. Combustion characteristics and emissions of Fischer–Tropsch diesel fuels in IC engines. *Prog. Energy Combust. Sci.* 37, 503–523.
- Irimescu, A., 2012. Performance and fuel conversion efficiency of a spark ignition engine fueled with iso-butanol. *Appl. Energy* 96, 477–483.
- Jayed, M.H., Masjuki, H.H., Saidur, R., Kalam, M.A., Jahirul, M.I., 2009. Environmental aspects and challenges of oilseed produced biodiesel in Southeast Asia. *Renewable Sustainable Energy Rev.* 13, 2452–2462.
- Jenkins, R.W., Munro, M., Nash, S., Chuck, C.J., 2013. Potential renewable oxygenated biofuels for the aviation and road transport sectors. *Fuel* 103, 593–599.
- Kirillov, V.A., Sobyagina, V.A., Kuzin, N.A., Brizitski, O.F., Terentiev, V.Y., 2012. Synthesis gas generation on-board a vehicle: development and results of testing. *Int. J. Hydrogen Energy* 37, 16359–16366.
- Koutinas, A.A., Toutoutzidakis, G., Kana, K., Kouinis, I., 1991. Methane fermentation promoted by gamma-alumina pellets. *J. Ferment. Bioeng.* 72, 64–67.
- Lappa, K., Kandyliis, P., Bekatorou, A., Bastas, N., Klaoudatos, S., Athanasopoulos, N., Kanellaki, M., Koutinas, A.A., 2015a. Continuous acidogenesis of sucrose, raffinose and vinasse using mineral kissiris as promoter. *Bioresour. Technol.* 188, 43–48.
- Lappa, K., Kandyliis, P., Bastas, N., Klaoudatos, S., Athanasopoulos, N., Bekatorou, A., Kanellaki, M., Koutinas, A.A., 2015b. New generation biofuel: continuous acidogenesis of sucrose–raffinose mixture simulating vinasse is promoted by γ -alumina pellets. *Biotechnol. Biofuels* 8, 74.
- Ogden, J.M., Steinbugler, M.M., Kreutz, T.G., 1999. A comparison of hydrogen, methanol and gasoline as fuels for fuel cell vehicles: implications for vehicle design and infrastructure development. *J. Power Sources* 79, 143–168.
- Penteado, E.D., Lazaro, C.Z., Sakamoto, I.K., Zaiat, M., 2013. Influence of seed sludge and pretreatment method on hydrogen production in packed-bed anaerobic reactors. *Int. J. Hydrogen Energy* 38 (14), 6137–6145.
- Perna, V., Castello, E., Wenzel, J., Zampol, C., Fontes Lima, D.M., Borzacconi, L., et al., 2013. Hydrogen production in an upflow anaerobic packed bed reactor used to treat cheese whey. *Int. J. Hydrogen Energy* 38 (1), 54–62.
- Petre, M.N., Rosca, P., Dragomir, R., 2011. The effect of bio-ethers on the volatility properties of oxygenated gasoline. *Rev. Chim.* 62, 567–574.
- Rajagopal, R., Masse, D.I., Singh, G., 2013. A critical review on inhibition of anaerobic digestion process by excess ammonia. *Bioresour. Technol.* 143, 632–641.
- Randazzo, M.L., Sodre, J.R., 2011. Exhaust emissions from a diesel powered vehicle fuelled by soybean biodiesel blends (B3–B20) with ethanol as an additive (B20E2–B20E5). *Fuel* 90, 98–103.
- Ren, N.Q., Wang, B.Z., Huang, J.C., 1997. Ethanol-type fermentation from carbohydrate in high rate acidogenic reactor. *Biotechnol. Bioeng.* 54, 428–433.
- Sanchez, O.J., Cardona, C.A., 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresour. Technol.* 99, 5270–5295.

- Singhania, R.R., Kumar Patel, A., Christophe, G., Fontanille, P., Larroche, C., 2013. Biological upgrading of volatile fatty acids, key intermediates for the valorization of biowaste through dark anaerobic fermentation. *Bioresour. Technol.* 145, 166–174.
- Stergiou, P.-Y., Foukis, A., Filippou, M., Koukouritaki, M., Parapouli, M., Theodorou, L. G., Hatziloukas, E., Afendra, A., Pandey, A., Papamichael, E.M., 2013. Advances in lipase-catalyzed esterification reactions. *Biotechnol. Adv.* 31, 1846–1859.
- Syngiridis, K., Bekatorou, A., Kallis, M., Kandyli, P., Kanellaki, M., Koutinas, A.A., 2013. Γ -Alumina as a process advancing tool for a new generation biofuel. *Bioresour. Technol.* 132, 45–48.
- Syngiridis, K., Bekatorou, A., Kandyli, P., Larroche, C., Kanellaki, M., Koutinas, A.A., 2014. Favouring butyrate production for a new generation biofuel by acidogenic glucose fermentation using cells immobilised on γ -alumina. *Bioresour. Technol.* 161, 118–123.
- Toulson, E., Allen, C.M., Miller, D.J., Schock, H.J., Lee, T., 2010. Optimization of a multi-step model for the auto-ignition of dimethyl ether in a rapid compression machine. *Energy Fuel* 24, 3510–3516.
- Uusitalo, V., Soukka, R., Horttanainen, M., Niskanen, A., Havukainen, J., 2013. Economics and greenhouse gas balance of biogas use systems in the Finnish transportation sector. *Renewable Energy* 51, 132–140.
- Vohra, M., Manwar, J., Manmode, R., Padgilwar, S., Patil, S., 2014. Bioethanol production: feedstock and current technologies. *J. Environ. Chem. Eng.* 2, 573–584.



Contents lists available at ScienceDirect

Bioresource Technology

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



Sustainable production of a new generation biofuel by lipase-catalyzed esterification of fatty acids from liquid industrial waste biomass



Athanasios Foukis^a, Olga A. Gkini^a, Panagiota-Yiolanda Stergiou^a, Vasilios A. Sakkas^a, Agapi Dima^b, Konstantina Boura^b, Athanasios Koutinas^b, Emmanuel M. Papamichael^{a,*}

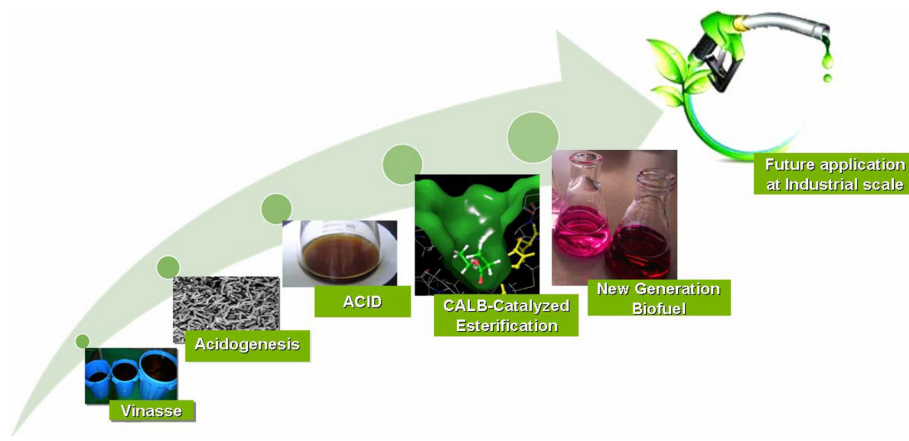
^aEnzyme Biotechnology & Genetic Engineering Group, University of Ioannina, Department of Chemistry, Ioannina 45110, Greece

^bFood Biotechnology Group, University of Patras, Department of Chemistry, Patras 26500, Greece

HIGHLIGHTS

- Lipase-catalyzed esterification of fatty acids from industrial wastes' acidogenesis.
- Production of a new generation biofuel at very high yield and concentration.
- Optimal reaction conditions were achieved by factorial central composite designs.
- CALB was significantly stable and recovered after ten cycles of esterification.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 6 March 2017

Received in revised form 3 April 2017

Accepted 6 April 2017

Available online 11 April 2017

Keywords:

Biofuel

Lipase-catalyzed esterification

Acidogenesis

Liquid industrial waste biomass

Process optimization

ABSTRACT

In this work we suggest a methodology comprising the design and use of cost-effective, sustainable, and environmentally friendly process for biofuel production compatible with the market demands. A new generation biofuel is produced using fatty acids, which were generated from acidogenesis of industrial wastes of bioethanol distilleries, and esterified with selected alcohols by immobilized *Candida antarctica* Lipase-B. Suitable reactors with significant parameters and conditions were studied through experimental design, and novel esterification processes were suggested; among others, the continuous removal of the produced water was provided. Finally, economically sustainable biofuel production was achieved providing high ester yield (<97%) along with augmented concentration (3.35 M) in the reaction mixtures at relatively short esterification times, whereas the immobilized lipase maintained over 90% of its initial esterifying ability after reused for ten cycles.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

Currently, the interest to substitute fossil fuels by biofuels is increased worldwide; alternatively, synthetic esters of fatty acids are used in mixtures with gasoline as extending similar physical properties (Stergiou et al., 2013; Koutinas et al., 2016). The

* Corresponding author.

E-mail address: epapamic@cc.uoi.gr (E.M. Papamichael).

lipase-catalyzed synthesis of low molecular weight esters seems advantageous as mostly is based on fatty acids which can be formed during acidogenesis of low-value liquid industrial waste biomass, e.g. vinasse; this latter concept overcomes successfully the dilemma food vs. fuel (Contino et al., 2011; Patel et al., 2014), and it is carried out using fermentation promoters which are acting simultaneously as immobilization carriers, like the γ -alumina and/or kissiris (Syngiridis et al., 2014).

The lipase-catalyzed esterification, in non-aqueous organic solvents, has received increasing interest in the last years given both the recovering of pure products and the industrial and biotechnological significance of organic esters. Long ago, the drawback that lipases may be insufficiently active in non-aqueous organic reaction media has been resolved by their immobilization on inert matrices (Ranganathan et al., 2008; Stergiou et al., 2013); depending on the immobilization method, the active structures of lipases seems to be thermally stabilized as compared to those of free enzymes (Barros et al., 2014). Nevertheless, how effectively lipases catalyze esterification depends strongly on the thermodynamics of the operated reaction system; an example is to keep the activity of H_2O as low as required to facilitate esterification to compete the backward hydrolysis reaction (Stergiou et al., 2013). Conversely, transesterification reactions are slow and in order to shift the equilibrium to the products require huge excess of the reacting ester; generally, this latter is expensive either being an edible oily compound and/or derived from processed liquid waste biomass, and it could not be alternative to the esterification (Degueil-Castaing et al., 1987; Zhanga et al., 2003).

In this work are studied factors and conditions which drive options in estimating the advantages of lipase-catalyzed esterification of fatty acids, formed by acidogenesis of industrial liquid waste biomass, and alcohols; these procedures were performed through the combined effects of important parameters affecting the esterification reaction, as well as the minimization of the necessary experimental work (Shieh et al., 2003). In more details, experimental design followed by RSM analysis was implemented focusing on maximizing the responses percentage ester yield, and concentration of the formed ester ([Ester]) within the reaction mixture, as well as on minimizing the reacting quantity of the applied immobilized biocatalyst along with its maximum number of reusing cycles; additionally, they were considered as significant the minimum reaction time, the highest starting concentration of the used fatty acids and the reduction of the final cost. Furthermore, it was studied the effect of H_2O content on the stability and reuse of the applied immobilized lipase, during esterification.

2. Materials and methods

2.1. Fatty acids, reagents and enzyme

The mixtures of fatty acids, which were formed from acidogenesis of low-value liquid industrial wastes of bioethanol distilleries (vinasse) were provided by the Food Biotechnology Group, Department of Chemistry, University of Patras, Patras 26500, Greece (Syngiridis et al., 2014; Lappa et al., 2015 and references therein); these mixtures, with a mean percentage composition per acid ($PCA_i = v_i/v_{total}$): propionic 0.14, butyric 0.74 and valeric 0.12, always they were dried before their use firstly over anhydrous Na_2SO_4 and then over molecular sieves activated at 250 °C, and will be referred to hereinafter as ACID. All reagents of analytical grade, i.e. Na_2SO_4 , granular $CaCl_2$ (desiccant), absolute Ethanol, esters-standards for Gas chromatography (GC) analyses (i.e. Ethyl and Butyl propionate, Ethyl and Butyl butyrate, Ethyl and Butyl valerate) and activated molecular sieves (3 Å), were purchased from Sigma-Aldrich (St. Louis, USA). Anhydrous n-Butanol and n-hexane were

purchased from Alfa Aesar (Thermo Fisher, Germany). Absolute Ethanol, n-Butanol and n-hexane were dried and reserved over molecular sieves activated at 250 °C. The pervaporation membranes 4100–342 were purchased from SULZER (Switzerland). Immobilized lipase-B from *Candida antarctica* (CALB) on acrylic resin (recombinant, expressed in *Aspergillus niger* – Novozyme 435), was purchased from Sigma-Aldrich (Code L4777, St. Louis, USA).

2.2. Ester synthesis

All esterification reactions were carried out in anhydrous n-hexane, under continuous stirring (150 rpm), in a 150 mL jacketed filter reactor (ACE GLASS INCORPORATED Vineland, NJ, USA) thermostated (40 °C), and equipped with: (a) vertical mechanical stirrer (IKA RW 20 digital), (b) reflux condenser capped with drying tube filled with granular $CaCl_2$, (c) fluid recirculation device consisting of a water retention trap, filled with molecular sieves (15 g) activated at 250 °C, and a peristaltic pump, (d) heated/refrigerated circulating bath (PolyScience, ILL-USA), and (e) diaphragm vacuum pump (BUCHI v-700, Switzerland) coupled with liquid nitrogen trap, used when the produced water was removed by pervaporation under vacuum of 10 mbar (Fig. 1); esterification by the use of pervaporation for continuous removal of the produced water was performed under the achieved optimal conditions, and it has been elaborated as being more friendly in industrially scale applications.

During the ester synthesis, the product-water was either maintained in the reaction mixture, or continuously removed through the provided retention trap and/or by pervaporation. Preliminary esterification experiments showed that, within customary limits,

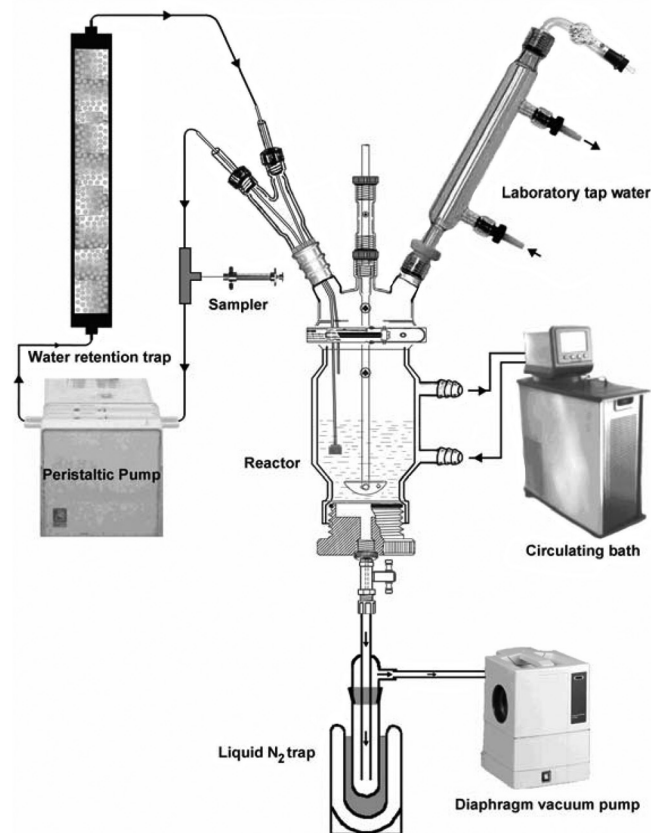


Fig. 1. Representation of the reactor scheme utilized to produce a new generation biofuel by lipase-catalyzed esterification of fatty acids. All peripheral parts and their use are described in the text.

stirring rate, temperature and flow-rate of recirculation of the reaction mixture (10 ml/min) were found as insignificant, and thus they were kept constant. In all cases, suitable volumes of (a) ACID, (b) alcohols, and (c) anhydrous n-hexane were added in the reactor so that to maintain a 90 mL total reaction volume, while the reaction was initiated by the addition of a sufficient quantity of immobilized *Candida antarctica* lipase-B. Subsequently, the following esters were synthesized: ethyl and butyl propionate, ethyl and butyl butyrate, and ethyl and butyl valerate.

2.3. Analysis and calculations

The progression of all lipase-catalyzed esterifications was monitored by assaying the percentage (%) of the formed esters in aliquots withdrawn from the reaction mixtures at specific time intervals, by GC-chromatography; a DANI MASTER GC Fast Flame ionization detector (FID) was used, equipped with a DB-5 column (Agilent Technologies, 30 m length, 0.32 mm internal diameter, 1 μ m film thickness). Helium (carrier gas), air, and hydrogen, at flow rates 6 mL/min, 300 mL/min and 60 mL/min were used, respectively; both the injector and detector temperatures were set at 250 °C, whereas a temperature gradient from 80 °C – 110 °C (2 °C/min for 15 min and isocratic for more 5 min at 110 °C) was applied for the column oven. Samples (aliquots) of 50 μ L, properly diluted in n-hexane, were injected in the GC-FID apparatus; the retention times of the injected esters varied between 7 and 14 min. The mean molecular weight (\overline{MW}) of the fatty acids mixtures, from acidogenesis of liquid industrial waste biomass, was calculated 87.1168, as based on their aforementioned mean PCA_i , as well as on Eq. (1) (Shrestha and van Gerpen, 2010):

$$\overline{MW} = \frac{\sum_{i=1}^3 [\text{density}_i \text{ (g/mL)} \times PCA_i]}{\sum_{i=1}^3 \frac{[\text{density}_i \text{ (g/mL)} \times PCA_i]}{MW_i}} \quad (1)$$

The % yields of the synthesized esters, as well as their concentrations, were estimated by based on the initial amount of the corresponding fatty acid, according to Eqs. (2) and (3), respectively (Knežević-Jugović et al., 2008), where the integrated areas of the GC-profiles were used:

$$\% \text{ Ester Yield} = \frac{\sum_{i=1}^3 (\text{Peak area of ester}_i)_i \times PCA_i \times 100}{(\text{Peak area of ester}_i)_i + (\text{Peak area of acid}_i)_i} \quad (2)$$

$$[\text{Ester}] = [\text{ACID}]_{\text{initial}} \times (\% \text{ Ester Yield})/100 \quad (3)$$

In all cases, and at the end of each esterification reaction, the water contents in the reaction mixtures were determined by means of Karl Fischer coulometric titration in triplicates (apparatus Metrohm 851 Titrando/Tiamo); the sample (1 mL) was cooled before measurement.

2.4. Experimental design and optimization

Four user defined 2^4 factorial central composite designs (CCD) of 16 experimental points were applied, including additional design points i.e. the center of edges (32 points), plane centroids (8 points), and overall centroid (4 points), to obtain a second order model based on a sum of 60 points; these factorial designs, which were performed using the software package Design Expert® (Stat-Ease, 2012), are referred to procedures, where either ethanol or n-butanol were used as esterifying alcohols, and they were performed either by maintaining the water produced during esterification within the reaction mixture or by continuous removal more than 99.90% of this water. The studied variables and their levels for the designs, using ethanol as the reacting alcohol, were

the: (a) total [ACID] (1.75 M – 2.75 M), (b) ratio [Ethanol]/[ACID] (1.00–3.00), (c) contained CALB (0.09 g – 0.27 g), and (d) reaction time (8–16 h); in the case where n-butanol was the reacting alcohol the corresponding variable and levels were the: (a) total [ACID] (2.5 M – 3.5 M), (b) ratio [n-Butanol]/[ACID] (1.00–2.00), (c) contained CALB as in above (c), and (d) the reaction time (6–12 h). As responses, were considered the percentage (%) ester yield, and the [Ester].

The RSM methodology was used to consider the influence of input variables on particular responses, which should be optimized over the specified region of interest. The quadratic Eq. (4) was used to fit the obtained data for both responses aiming to plot surfaces for all variables.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad (4)$$

In Eq. (4) Y and β_0 are the response variable and a constant term, respectively, while β_i , β_{ij} , β_{ii} are the coefficients for the linear, interaction, and quadratic effects, respectively, and x_i and x_j are the independent variables (factors); herein, this latter model equation has been simplified occasionally by subtracting terms which found not statistically significant ($p > 0.05$) by ANOVA. Subsequently, and through the same software package Design Expert®, a multiple-criteria decision analysis (MCDA) was applied in order to optimize simultaneously all responses; then, optimal agreements between all responses were achieved and expressed by means of the overall desirability function (Murphy et al., 2005, and references therein).

2.5. Reuse of CALB (ester synthesis in successive batches)

The initial % ester yield was used as a measure to investigate the stability and recoverability of the CALB, in repeated batches of esterification and under the optimal conditions (Sections 2.4 and 2.4). After the completion of the esterification, as described above, the reaction mixture was filtered under medium vacuum to separate the immobilized enzyme, which was washed three times with anhydrous n-hexane, dried, collected, weighed on a tared weighing-bottle, and it reused in a successive cycle.

2.6. Statistical analysis

All experimental designs, as well as the fitting of quadratic models to the independent variables, were achieved by means of the Design-Expert® Software (Stat-Ease Inc., Minneapolis, USA); the significance of the regression coefficients and their associated probabilities, were determined. Two-way ANOVA were used in order to evaluate the developed models, whose significance was determined, in all cases, by the Fisher's test value, while the proportion of variance explained by each model was given through the multiple coefficient of determination, R^2 -value; all models were assembled as 3D- response surface curves, followed by their corresponding contour and ramp plots, in order to illustrate the interactive effects of the independent and the dependent variables.

3. Results and discussion

3.1. Optimal model parameters of the esterification reactions

From the implementation of the abovementioned four procedures the experimental % yield along with the concentration of the synthesized esters were obtained, for the 60 experiments per factorial design; the results of experiments which were carried out for the development of the models are depicted in the [Supplementary material](#) for simplicity purposes, and they were based on the studied factors, i.e. [ACID], [ALCOHOL]/[ACID] (i.e. either ethanol or n-butanol), amount of CALB and reaction time. Furthermore,

Table 1
Experimental design (ANOVA of Response Surfaces Reduced Quadratic Models): Effects and significance of the ACID esterification parameters.^{a,b}

Source	H ₂ O was maintained in the reactor				H ₂ O was continually removed from reactor			
	SUM OF SQUARES							
	With Ethanol		With n-Butanol		With Ethanol		With n-Butanol	
	% Yield	[Ester]	% Yield	[Ester]	% Yield	[Ester]	% Yield	[Ester]
Model	5726.92	6.71	2779.00	8.32	8881.44	10.80	8006.54	12.15
A-Acid	392.29	4.06	60.29	6.82	697.27	2.85	173.12	4.69
B-Ratio	1572.72	0.70	19.15	3.72E-3	491.25	0.46	502.18	0.47
C-Enzyme	1926.07	0.98	1079.30	0.57	4118.20	3.89	3813.14	3.62
D-Time	732.92	0.39	1345.04	0.69	2631.66	2.38	2422.73	2.10
AB	150.38	0.01	73.63	0.03		0.030		0.04
AC		0.04	12.75	0.048	122.38	0.38	125.45	
AD		0.03		0.04		0.06	37.17	
BC			43.50	0.03	296.58	0.29	278.48	0.27
BD	74.76	0.04		3.79E-3				
CD	17.21	9.88E-3	41.40	0.02	96.60	0.09	94.33	0.08
A ²		9.61E-3						
B ²	860.58	0.38	41.29	0.02	42.19	0.07	82.74	0.08
C ²					209.76	0.26	410.56	0.37
D ²			41.20	0.024	14.99			
Residual	234.75	0.12	134.61	0.064	269.32	0.27	291.07	0.08
Lack of Fit	232.53	0.11	131.49	0.06	265.01	0.26	281.10	0.37
Pure Error	2.22	1.12E-3	3.12	1.58E-3	4.30	3.87E-3	9.97	0.08
Corr Total	5961.67	6.82	2913.61	8.38	9153.55	11.07	8299.75	0.37

^a In all cases, the Prob > F p-values were calculated as significant, while those of Lack-of-fit as no significant.

^b The mean values of sources as Adjusted R², and Predicted R², were calculated as 0.97 and 0.96, respectively, as well as they calculated Adeq Precision values >4, in all cases.

Table 2
Calculated values of regression coefficients of the considered quadratic models (esterification of ACID).^a

Factor	H ₂ O was maintained in the reactor				H ₂ O was continually removed from reactor			
	With Ethanol		With n-Butanol		With Ethanol		With n-Butanol	
	% Yield	[Ester]	% Yield	[Ester]	% Yield	[Ester]	% Yield	[Ester]
Intercept	10.04945	-0.98897	31.54398	-0.44689	55.14603	0.37567	29.59382	-0.97520
A-Acid	2.55887	0.86875	1.14631	0.56324	-15.97155	0.00237	-5.51360	0.45211
B-Ratio	44.34659	0.82697	12.56935	0.22065	5.64324	0.72013	13.50577	0.85914
C-Enzyme	99.69130	0.48395	36.76984	-0.44464	139.73350	1.43520	198.59391	2.69227
D-Time	0.64685	-0.02114	4.75553	0.066975	6.66164	0.02498	5.83187	0.10869
AB	-4.33562	-0.03896	-3.03375	-0.063041		0.12191		-0.14229
AC		0.79957	14.02778	0.86472	43.45833	2.40670	44.00000	2.38799
AD		0.01628		0.017760		0.029796	-0.71854	
BC			12.95486	0.32301	67.65278	2.10017	65.55556	2.03021
BD	0.38211	0.00863		0.00272				
CD	-2.03733	-0.04881	-3.15972	-0.07108	-6.43519	-0.19421	-6.35880	-0.18948
A ²		-0.11291						
B ²	-8.26437	-0.17837	-1.85001	-0.04005	-8.22025	-0.31372	-10.74040	-0.33665
C ²					-565.68663	-18.53938	-738.41029	-22.04530
D ²			-0.11550	-0.00281	-0.15915			

^a The standard errors of the factors were calculated relatively small (0.1% ≤ st. error ≤ 7%), in most cases; correlation coefficients: R² > 0.93 in all cases.

the effects and the significance of the esterification parameters of mixture ACID were evaluated by a two-way ANOVA (Table 1). Obviously, and for all cases appeared in Table 1, the p-values of all parameters (sources) were estimated as significant, including those referred to the Models, which indicate that their selected terms (within A, B, C, D, AB, AC, AD, BC, BD, CD, A², B², C², D²) are significant model terms; besides, the estimated values of Adjusted-R² and Predicted-R² are in full agreement, with the required Adequate Precision values (>4), in all cases (Saravanan and Muthuvelayudham, 2016). Subsequently, the regression coefficients of the considered quadratic models were calculated and used to assemble the corresponding equations, whose the general form is Eq. (4); these latter data are presented in Table 2 (all, plots of predicted vs. actual data, 3D-response surface curves, contours and ramp-plots are shown in the Supplementary material). Additionally, within each of the four abovementioned factorial designs,

and by means of simultaneously optimization of all responses, the overall desirability functions were calculated (Fig. 2); lastly, the four corresponding solutions were obtained (Table 3).

3.2. Esterification processes (production of a new generation biofuel)

Our results, which are depicted in Table 3, give clear evidence that the produced H₂O, during all the particular four CALB-catalyzed esterifications, significantly affected both the % yield and the final concentration of the synthesized esters ([Ester]). Additionally, the following significant issues were emerged: (a) the increased hydrophobicity of n-butanol, as well as the declined hydrophilic nature of ethanol, when the produced water was continuously removed from the reaction mixture, (b) the remarkable reduction of the [Ethanol] required for esterification under continuous abstraction of water vs. its presence, and (c) the use

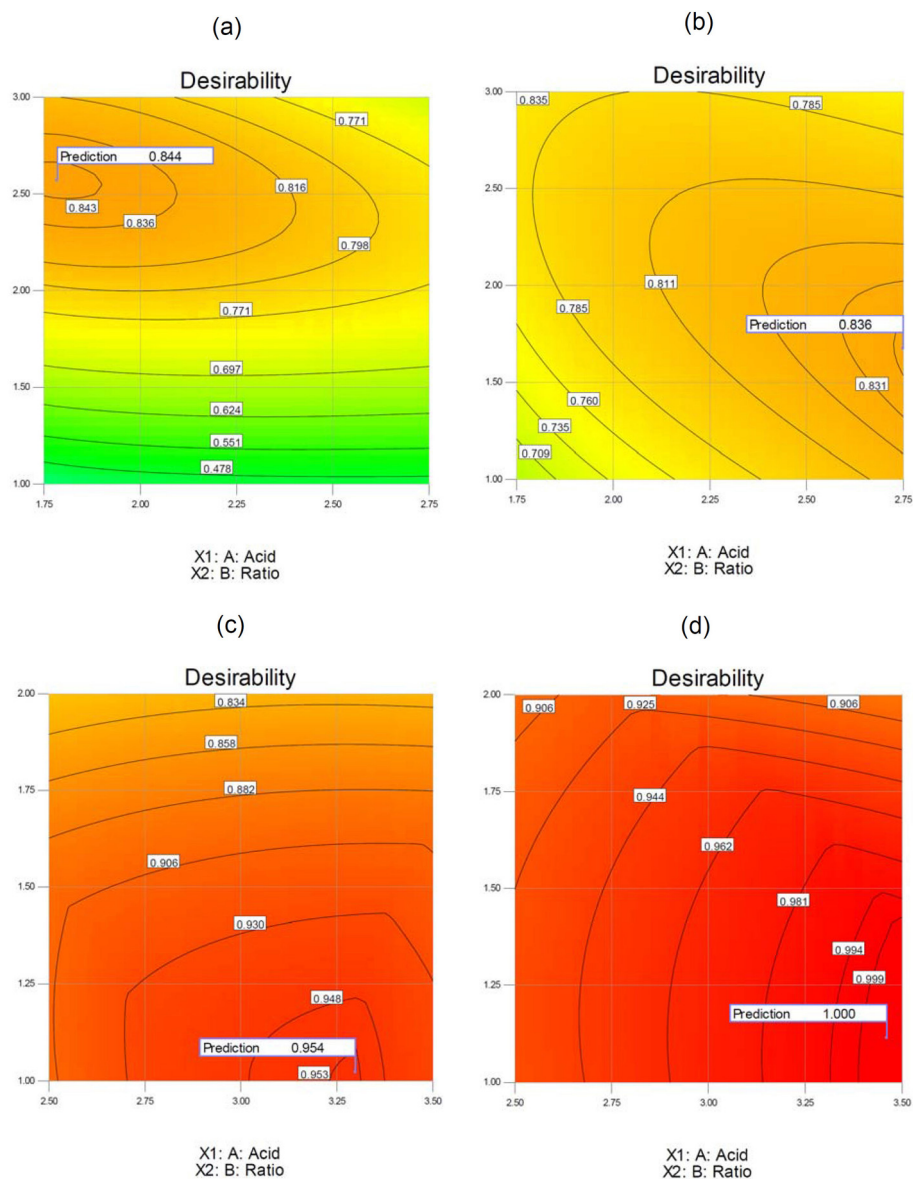


Fig. 2. Contours of the estimated values of desirability function, in the cases of esterification by: (a) Ethanol while the produced water was maintained in the reactor, (b) Ethanol while the produced water was continually removed from reactor, (c) Butanol while the produced water was maintained in the reactor, (d) Butanol while the produced water was continually removed from reactor.

Table 3

Calculated values of the parameters which affect the CALB-catalyzed esterification reactions.^a

Parameter	H ₂ O was maintained		H ₂ O was continually removed	
	Esterifying alcohols			
	Ethanol	n-Butanol	Ethanol	n-Butanol
[ACID] (M)	1.780	3.300	2.750	3.460
% ester yield	90.240	90.150	90.080	97.260
[Ester] ^b (M)	1.620	3.200	2.480	3.350
Desirability f-value	0.844	0.954	0.836	1.000

^a All esterification reactions were performed during a time period of 16:00 h, using 0.15 g of CALB and at 40 °C.

^b The final ester concentration in the reaction mixture.

of only few mg of the CALB to perform the described esterifications; all the latter three strongly contributed in the findings of this work. In previous works, and although they have reported high esterification percentages, however both the concentrations of

employed fatty acids and of the produced esters were reported as very low vs. the reported herein (Manjón et al., 1991; Santos and de Castro, 2006; Pires-Cabral et al., 2007; Friedrich et al., 2013; Martins et al., 2013a,b).

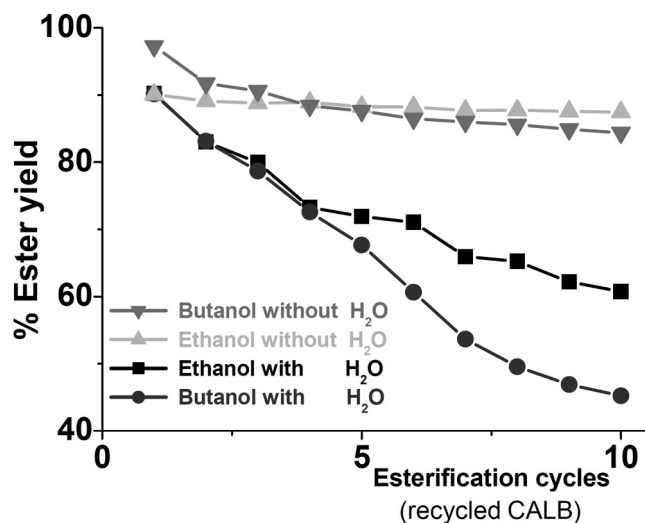


Fig. 3. Graphical illustration of the significant stability and recoverability exhibited by the CALB, after ten cycles of esterification, under the reaction conditions described in the text, and by using both Ethanol and Butanol as esterifying alcohols; an emphasis should be given on the finding that by continuous removal of the produced water from the esterification mixtures both higher [ACID] were utilized, and [Ester] were achieved.

3.2.1. Comparing ethyl ester vs. butyl ester synthesis

Once the esterification was progressively performed in an environment of increased polarity, as the produced water was maintained in the reaction mixture, the largest fraction of hydrophilic ethanol should be located in the aqueous phase, whereas and under similar conditions, the opposite seems more likely for the hydrophobic n-butanol i.e. to be located largely in the solvent (n-hexane) phase; then, it was found that in the former case (ethanol as esterifying alcohol) lower [ACID], in presence of higher [Ethanol], was required to deactivate the CALB, than in the latter one (n-butanol as esterifying alcohol). On the contrary and when the hydrophobicity of the reaction mixture was increased due to the continuous removal of produced water, higher [ACID] was required to deactivate the CALB, although in presence of lower [Alcohol] (much lower in case of n-butanol), in both cases of used alcohols; thus, the achieved much higher [ACID], which react with an almost equimolar [n-butanol] when the produced water was continuous removed from the reaction mixture, is a reasonable result based more likely onto n-butanol hydrophobicity (Table 3). These results clearly show that by increasing the [ACID] (although at higher [Alcohol]), and in reaction environments of lower hydrophobicity (presence of the produced water, and/or by ethanol as the esterifying alcohol), it promotes the CALB inactivation, which is reflected to both the calculated % ester yield as well as the [Ester], more likely due to a dramatic decrease of the active pH value in the microenvironment of the catalytic site of the used immobilized lipase. An opposite event is more likely to occur in reaction environments of higher hydrophobicity (removal of the produced water, and/or by n-butanol as the esterifying alcohol). Besides, the observed decrease of ratio [Alcohol]/[ACID] in more hydrophobic environments (especially for [Ethanol]) has a dual meaning: (a) it denotes decreased needs for alcohol which is accompanied by increased consumption of [ACID], and (b) it signifies an increased degree of esterification due to that alcohols leave the water phase, move in the solvent (n-hexane) phase, and thus contribute in the protection of the catalytic site of CALB. Although it is not clearly shown, however these results could brought into play the influence of the pH-value, in the employed reaction media of this work, and by using immobilized lipases; it seems more likely now that

substrates as fatty acids and alcohols, and within the referred, herein, reaction conditions, contribute in configuring the pH-value and its impacts on the nucleophilic status of the catalytic residues in the microenvironment of the used enzymes (Himmel et al., 2010, and references therein). Finally, it is noticeable that the discussed results in this section are more likely due to the thermodynamic properties of the used alcohols (ethanol and n-butanol) which in turn influence their binding mode and status onto CALB, and thus induce either positive or negative effects on the esterification results.

3.3. Cycles of reuse of the CALB

It is not uncommon to consider the product cost of synthetic reactions especially of the enzymatic ones, as enzymes are generally expensive reagents and particularly their immobilized forms; thus, the reuse of the CALB in this work, will contribute considerably in the reduction of cost of the suggested production of new generation biofuel. The CALB showed significant stability and recoverability after ten cycles of esterification, under anhydrous conditions; ten successive esterifications were performed using the same CALB, either with ethanol or n-butanol as the esterifying alcohol, in both cases by maintaining the produced water in the reaction mixture, as well as by continuously removing it through the provided devices (Section 2.2), as it is depicted in Fig. 3; it should be pointed out, and by taking into account also the results of the reuse of CALB, that the continuous removal of the water from the esterification mixtures is an important factor for achieving increased [Ester].

4. Conclusions

This work reports an integrated cost-effective and advanced methodology of new generation biofuel production, by CALB-catalyzed esterification of fatty acids, from acidogenesis of liquid industrial waste biomass, an environmental pollutant. Minimal CALB quantities, which were recycled ten times along with the higher ever reported concentrations of utilized fatty acids and produced esters, using pervaporation to remove the produced water, were achieved at relatively short esterification times; these findings are reported herein for first time. Given these advantageous approaches and results, their application at industrial scale is in progress through a large scale experimental and feasibility study.

Acknowledgements

This article is in line of a research project co-financed by the European Union (European Regional Development Fund – ERDF), through the operational programs for “competitiveness and entrepreneurship” and regions in transition “Cooperation 2011 – Partnerships of Production and Research Institutions in Focused Research and Technology Sectors”, of the National Strategic Reference Framework (NSRF) 2007–2013, and the Hellenic Ministry of Education, Lifelong Learning and Religious Affairs – General Secretariat for Research and Technology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2017.04.028>.

References

- Barros, D.P.C., Pinto, F., Fonseca, L.P., Cabral, J.M.S., Lemo, F., 2014. Kinetic model for the esterification of ethyl caproate for reaction optimization. *J. Mol. Catal. B Enzym.* 101, 16–22.
- Contino, F., Foucher, F., Mounaim-Rousselle, C., Jeanmart, H., 2011. Combustion characteristics of tricomponent fuel blends of ethyl acetate, ethyl propionate, and ethyl butyrate in homogeneous charge compression ignition (HCCI). *Energy Fuel* 25, 1497–1503.
- Degueil-Castaing, M., De Jeso, B., Drouillard, S., Maillard, B., 1987. Enzymatic reactions in organic synthesis: 2-ester interchange of vinyl esters. *Tetrahedron Lett.* 28, 953–954.
- Friedrich, J.L.R., Pena, F.P., Garcia-Galan, C., Fernandez-Lafuente, R., Ayub, M.A.Z., Rodrigues, R.C., 2013. Effect of immobilization protocol on optimal conditions of ethyl butyrate synthesis catalyzed by lipase B from *Candida Antarctica*. *J. Chem. Technol. Biotechnol.* 88, 1089–1095.
- Himmel, D., Goll, S.K., Leito, I., Ingo Krossing, I., 2010. A unified pH scale for all phases. *Angew. Chem. Int. Ed.* 49, 6885–6888.
- Knežević-Jugović, Z., Bezbradica, D., Jakovljević, Ž., Branković-Dimitrijević, S., Mijin, D., 2008. Lipase catalyzed synthesis of flavor esters in non-aqueous media: optimization of the yield of pentyl 2-methylpropanoate by statistical analysis. *J. Serb. Chem. Soc.* 73, 1139–1151.
- Koutinas, A., Kanellaki, M., Bekatorou, A., Kandyliis, P., Pissaridi, K., Dima, A., Boura, K., Lappa, K., Tsafrakidou, P., Stergiou, P.-Y., Foukis, A., Gkini, O.A., Papamichael, E.M., 2016. Economic evaluation of technology for a new generation biofuel production using wastes. *Bioresour. Technol.* 200, 178–185.
- Lappa, K., Kandyliis, K., Bastas, N., Klaoudatos, S., Athanasopoulos, N., Bekatorou, A., Kanellaki, M., Koutinas, A., 2015. New generation biofuel: continuous acidogenesis of sucrose-raffinose mixture simulating vinasse is promoted by γ -alumina pellets. *Biotechnol. Biofuels* 8, 74. <http://dx.doi.org/10.1186/s13068-015-0255-6>.
- Manjón, A., Iborra, J.L., Arocas, A., 1991. Short-chain flavour ester synthesis by immobilized lipase in organic media. *Biotechnol. Lett.* 13, 339–344.
- Martins, A.B., Friedrich, J.L.R., Cavalheiro, J.C., Garcia-Galan, C., Barbosa, O., Ayub, M. A.Z., Fernandez-Lafuente, R., Rodrigues, R.C., 2013a. Improved production of butyl butyrate with lipase from *Thermomyces lanuginosus* immobilized on styrene-divinylbenzene beads. *Bioresour. Technol.* 134, 417–422.
- Martins, A.B., Friedrich, J.L.R., Cavalheiro, J.C., Rodrigues, R.C., Garcia-Galan, C., Fernandez-Lafuente, R., Ayub, M.A.Z., 2013b. Optimized butyl butyrate synthesis catalyzed by *Thermomyces lanuginosus* lipase. *Biotechnol. Prog.* 29, 1416–1421.
- Murphy, T.E., Tsui, K.L., Allen, J.K., 2005. A review of robust design methods for multiple responses. *Res. Eng. Des.* 16, 118–132.
- Patel, A.K., Singhania, R.S., Pandey, A., 2014. Biofuels from biomass. In: Agarwal, A. K., Pandey, A., Gupta, A.K., Aggarwal, S.K., Kushari, A. (Eds.), *Novel Combustion Concepts for Sustainable Energy Development*. Springer, New Delhi, India, pp. 25–44.
- Pires-Cabral, P., da Fonseca, M.M.R., Ferreira-Dias, S., 2007. Modelling the production of ethyl butyrate catalyzed by *Candida rugosa* lipase immobilised in polyurethane foams. *Biochem. Eng. J.* 33, 148–158.
- Ranganathan, S.V., Narasimhan, S.L., Muthukumar, K., 2008. An overview of enzymatic production of biodiesel. *Bioresour. Technol.* 99, 3975–3981.
- Santos, J.C., de Castro, H.F., 2006. Optimization of lipase-catalysed synthesis of butyl butyrate using a factorial design. *World J. Microbiol. Biotechnol.* 22, 1007–1011.
- Saravanan, P., Muthuvelayudham, R., 2016. Enhancing the bioproduction of cellulase by *Aspergillus nidulan* via medium optimization. In: Prasanna, B.D., Gummadi, S.N., Vadlani, P.V. (Eds.), *Biotechnology and Biochemical Engineering – Select Proceedings of ICACE 2015*. Springer Science & Business, Singapore, pp. 65–72.
- Shieh, C.J., Liao, H.F., Lee, C.C., 2003. Optimization of lipase-catalyzed biodiesel by response surface methodology. *Bioresour. Technol.* 88, 103–106.
- Shrestha, D., van Gerpen, J., 2010. Biodiesel from oilseed crops. In: Singh, B.P. (Ed.), *Industrial Crops and Uses*. CABI, Oxfordshire, UK, pp. 140–156.
- Stergiou, P.-Y., Foukis, A., Filippou, M., Koukouritaki, M., Parapouli, M., Theodorou, L. G., Hatziloukas, E., Afendra, A., Pandey, A., Papamichael, E.M., 2013. Advances in lipase-catalyzed esterification reactions. *Biotechnol. Adv.* 31, 1846–1859.
- Syngiridis, K., Bekatorou, A., Kandyliis, P., Larroche, C., Kanellaki, M., Koutinas, A., 2014. Favouring butyrate production for a new generation biofuel by acidogenic glucose fermentation using cells immobilised on γ -alumina. *Bioresour. Technol.* 161, 118–123.
- Zhanga, Y., Dubéa, M.A., McLeana, D.D., Katesb, M., 2003. Biodiesel production from waste cooking oil: 1. Process design and technological assessment. *Bioresour. Technol.* 89, 1–16.



Contents lists available at ScienceDirect

Bioresource Technology

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

Scale-up for esters production from straw whiskers for biofuel applications

Agapi Dima^a, Konstantina Boura^a, Argyro Bekatorou^a, Panagiota-Yirolanda Stergiou^b, Athanasios Foukis^b, Olga A. Gkini^b, Panagiotis Kandylis^a, Katerina Pissaridi^a, Maria Kanellaki^a, Emmanuel M. Papamichael^b, Athanasios A. Koutinas^{a,*}

^a Food Biotechnology Group, Department of Chemistry, University of Patras, 26500 Patras, Greece

^b Group of Enzyme Biotechnology and Genetic Engineering, Department of Chemistry, University of Ioannina, Ioannina 45110, Greece

HIGHLIGHTS

- Scale-up of anaerobic acidogenesis of wheat straw by a UASB culture.
- The process was promoted by culture immobilization on kissiris.
- Organic acids were produced from straw without a separate hydrolysis step.
- 1-Butanol was the best solvent for acids recovery from the fermentation broth.
- Enzymatic esterification of the acids with 1-butanol resulted to 90% yield.

ARTICLE INFO

Article history:

Received 31 January 2017

Received in revised form 3 April 2017

Accepted 6 April 2017

Available online xxx

Keywords:

Anaerobic acidogenesis

Wheat straw

Immobilization

Scale-up

Enzymatic esterification

ABSTRACT

Delignified wheat straw was fermented by a mixed bacterial anaerobic culture obtained from a UASB reactor to produce organic acids (OAs). Kissiris was used as immobilization carrier in a 2-compartment 82 L bioreactor filled with 17 L of fermentation broth for the first 7 fermentation batches and up to 40 L for the subsequent batches. The amount of straw used was 30 g/L and the temperature was set at 37 °C for all experiments. The total OAs reached concentrations up to 17.53 g/L and the produced ethanol ranged from 0.3 to 1 mL/L. The main OAs produced was acetic acid (6–8 g/L) and butyric acid (3–8 g/L). The OAs were recovered from the fermentation broth by a downstream process using 1-butanol, which was the solvent with the best recovery yields and also served as the esterification alcohol. The enzymatic esterification of OAs resulted to 90% yield.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

Nowadays there is an enormous interest on renewable resources such as waste lignocellulosic biomass for chemicals and biofuels production in order to overcome the ethical and economic issues related to arable land use. Lignocellulosics are the most abundant source of biomass, consisting mainly of cellulose (40–50%), hemicelluloses (25–30%), and lignin (15–20%) (Deng et al., 2014; Menon and Rao, 2012). In this respect, the anaerobic digestion of waste biomass is a promising perspective for energy production. Anaerobic digestion involves four main steps: (1) hydrolytic breakdown of organic compounds into soluble oligomers, (2) acidogenesis and (3) acetogenesis, which include both

hydrolysis and fermentation into organic acids (OAs), H₂ and CO₂, and finally (4) methanogenesis to produce CH₄ and CO₂ (Tomei et al., 2009).

In previous studies it was shown that fermentation processes can be promoted in the presence of porous materials such as kissiris and γ -alumina, which acted as culture immobilization carriers and facilitated continuous processing (Galanakis et al., 2012; Lappa et al., 2015; Koutinas et al., 2016). Specifically, the acidogenic fermentation of various sugars and waste resources, promoted by culture immobilization techniques for OAs production has been reported, in some cases with simultaneous production of ethanol (Syngiridis et al., 2014, 2013; Lappa et al., 2015; Koutinas et al., 2016). OAs and ethanol can be used as the reagents of an esterification reaction (enzymatic or chemical) to produce new generation biofuels similar to biodiesel. A limiting factor for such processes is the recovery of the OAs from the fermented broths. A liquid-liquid

* Corresponding author.

E-mail address: a.a.koutinas@upatras.gr (A.A. Koutinas).

downstream OAs extraction process using various alcohols that could also serve as esterification reagents was previously reported (Bekatorou et al., 2016). Lipase-catalyzed esterification of the recovered OAs from a fermentation broth was subsequently studied, evaluating the effect of process conditions and bioreactor design on the yield and quality of ester synthesis (Stergiou et al., 2013).

Based on the above studies that involved small scale laboratory experiments, the scale-up of anaerobic acidogenesis of wheat straw, promoted by kissiris as immobilization carrier, followed by a scale-up enzymatic esterification of the recovered OAs in a suitable alcohol solvent, were examined in this study.

2. Materials and methods

2.1. Culture and materials

A mixed bacterial anaerobic culture was obtained from a UASB reactor and grown at 37 °C in a medium containing: 50 g/L glucose, NH₃ and 50% H₃PO₄ solution of 100:5:1 COD:N:P ratio, 4 g/L NaHCO₃, and 4 g/L yeast extract. The media were sterilized by autoclaving at 120 °C for 15 min. The culture was immobilized on kissiris (Lappa et al., 2015). Delignified wheat straw was used as substrate in all experiments. Delignification took place by boiling 300 g of wheat straw for 3 h in 3 L of 1% w/v NaOH solution (Koutinas et al., 2012). The lignin removal percentage was 62.3% as determined previously (Tsafraikidou et al., 2014). The delignified straw was dried and cut into 1 cm pieces.

2.2. Scale-up acidogenesis of wheat straw

The experimental apparatus consisted of a stainless steel cylindrical tower bioreactor of 82 L total working volume connected with a peristaltic pump in order to achieve effluent recycling (Fig. 1). The bioreactor consisted of two compartments, each made of a metal net basket filled with kissiris. The bioreactor was placed in a incubator set at 37 °C and was also equipped with two sample receivers as well as a vertical transparent cylinder to allow observation of the broth level. An amount of 2.75 kg of kissiris (1 cm particles) was placed in the 1st basket compartment, while 4.4 kg of kissiris (5 cm pieces) were placed in the bioreactor, outside the basket. To start-up the experiment, 2 L of fermented growth

medium with 50 g of suspended culture were added, 15 L of fresh liquid were pumped, and 510 g of delignified straw were added. The final composition of the fermenting liquid was delignified straw 30 g/L, 27.2 mL NH₃ and 5.44 mL 50% H₃PO₄, 68 g/L NaHCO₃, and 68 g/L yeast extract. Moreover, for immobilization of the culture on kissiris as well as for adaptation in the cellulosic fermentation medium, 200 g of glucose were also added in the bioreactor. The initial pH of the broth was 9. The system was allowed to ferment for 24 h, and then the pump was connected to recycle the fermenting liquid at a rate of 536 mL/min. At the end of each batch (about 10 days duration, when the formed OAs concentration was starting to decline), the liquid was removed, while the leftover straw was maintained in the bioreactor. For the following fermentation batches, additional straw and nutrients were added as above in addition to 2 L of the previously fermented liquid that contained the culture and served as the inoculate. Six batches were performed with this configuration. Subsequently, the 2nd compartment was also loaded with kissiris (2.75 kg; 1 cm particles), and fermentations were performed using only the culture mass that was immobilized on the kissiris of the 1st compartment. Two more fermentation batches were carried out at 40 L total liquid volume. For these fermentations, the liquid medium contained (per litre) 1.6 mL NH₃ and 0.32 mL 50% H₃PO₄, 4 g yeast extract, 4 g NaHCO₃ and 30 g delignified straw.

2.3. Recovery of OAs from a fermented broth

The solvents used for OAs recovery were HPLC grade 2-methyl-1-propanol, 1-butanol, 3-methyl-1-butanol, 2-pentanol, as well as fusel oil (obtained from the alcohol distillery B.G. Spiliopoulos S.A., Patras, Greece). Recovery took place for 30 min at 20 °C under stirring and a solvent/solution ratio of 1:1. The fermented broth used for the study of OAs recovery contained 6 g/L acetic acid, 1.1 g/L propionic acid, 1.1 g/L isobutyric acid, and 8 g/L butyric acid. The recovered OAs were analyzed by HPLC. All experiments were carried out in triplicate.

2.4. Bioreactor and process for scale-up esterification

The esterification of the recovered OAs with 1-butanol was performed using immobilized *Candida antarctica* lipase-B recombinant from *Aspergillus oryzae*. The experimental apparatus consisted of a

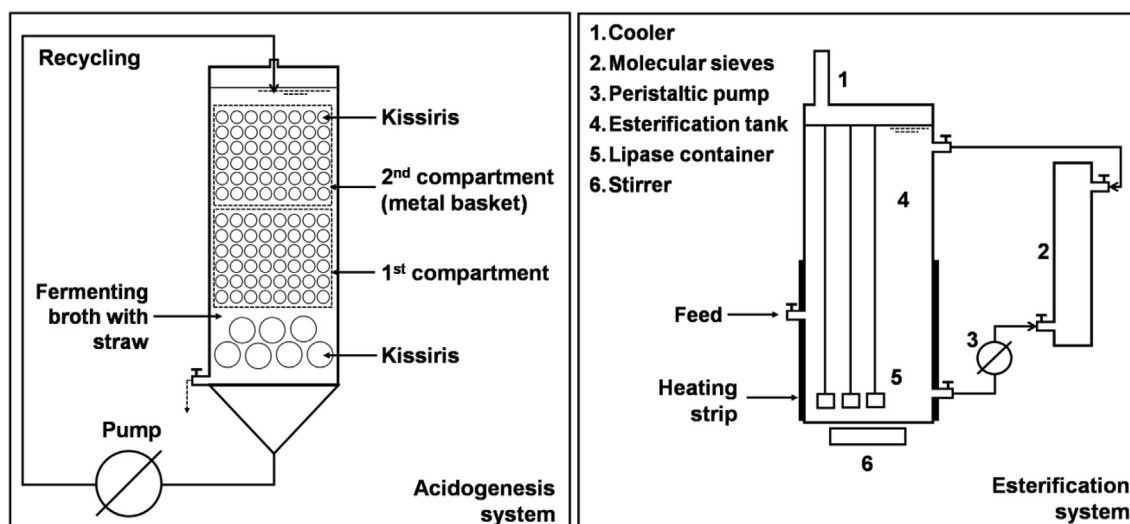


Fig. 1. Bioreactor designs for scale-up acidogenic fermentation of delignified wheat straw using a UASB culture immobilized on kissiris (left), and for enzymatic esterification of the recovered OAs (in 1-butanol) (right).

Table 1

OAs and ethanol formation during scale-up of acidogenic fermentation of delignified wheat straw using a UASB culture immobilized on kissiris.

Batch	Ethanol (mL/L)	Organic acids (g/L)						Total
		Succinic	Lactic	Acetic	Propionic	Isobutyric	Butyric	
<i>One bioreactor compartment operation</i>								
1	1.14	1.02	6.82	1.6	0	0	3.14	12.58
2	0.31	0	0	6.51	0	1.61	3.71	11.83
3	0.39	0	0	6.55	0.86	1.68	4.57	13.66
4	0.52	0	0	8.09	1.15	1.95	4.28	15.47
5	0.36	0	0	8.18	1.06	2.17	4.16	15.57
6	0.27	0	0	8.01	1.06	1.86	5.69	16.62
7	0.25	0	0	7.34	0.98	4.23	4.98	17.53
<i>Two bioreactor compartments operation</i>								
1	0.37	0	0	6.49	0.96	1.78	7.97	17.2
2	0.35	0	0	6.43	1.28	1.94	7.53	17.18

Table 2

OAs recovery from the acidogenic fermentation broths after 30 min extraction.

pH	Solvent	OAs recovery (%)					Total OAs
		Acetic	Propionic	Isobutyric	Butyric	Total OAs	
2.5	2-Methyl-1-propanol	40.2 ± 0.9	61.2 ± 0.6	73.8 ± 0.3	69.2 ± 0.8	61.1 ± 0.7	
	1-Butanol	48.9 ± 0.9	66.1 ± 0.3	76.1 ± 0.4	76.4 ± 1.1	66.9 ± 0.7	
	3-Methyl-1-butanol	31.5 ± 0.6	59.1 ± 0.8	71.3 ± 1.1	67.6 ± 1.1	57.4 ± 0.9	
	2-Pentanol	34.2 ± 0.8	52.0 ± 1.2	69.2 ± 1.3	71.2 ± 0.7	56.65 ± 1.0	
	Fusel oil	22.1 ± 2.3	34.9 ± 1.8	57.9 ± 0.7	73.7 ± 1.3	47.2 ± 1.5	
1.5	2-Methyl-1-propanol	49.1 ± 0.3	69.5 ± 0.3	78.3 ± 0.4	74.8 ± 0.5	67.9 ± 0.4	
	1-Butanol	59.1 ± 0.7	73.4 ± 0.7	81.1 ± 0.8	76.1 ± 0.5	72.4 ± 0.7	
	3-Methyl-1-butanol	37.4 ± 0.4	59.5 ± 0.9	74.3 ± 1.3	78.2 ± 0.5	62.4 ± 0.8	
	2-Pentanol	38.2 ± 0.1	64.5 ± 0.2	71.0 ± 0.4	84.0 ± 0.1	64.4 ± 0.2	
	Fusel oil	25.9 ± 2.2	38.9 ± 1.1	72.2 ± 0.4	75.0 ± 0.4	53 ± 1.0	
1.0	2-Methyl-1-propanol	55.6 ± 0.9	75.1 ± 0.6	78.9 ± 0.7	82.1 ± 0.7	72.9 ± 0.7	
	1-Butanol	65.4 ± 0.6	80.4 ± 0.4	86.5 ± 0.3	87.1 ± 0.4	79.9 ± 0.4	
	3-Methyl-1-butanol	42.6 ± 0.9	71.5 ± 0.8	78.6 ± 0.8	85.4 ± 0.6	69.5 ± 0.8	
	2-Pentanol	44.6 ± 0.7	68.4 ± 0.4	79.6 ± 0.7	91.6 ± 0.4	71.1 ± 0.6	
	Fusel oil	34.9 ± 2.7	45.9 ± 1.1	69.4 ± 0.8	78.1 ± 0.9	57.1 ± 1.4	

cylindrical tower reactor of 40 L total working volume connected with a peristaltic pump to achieve recycling (Fig. 1). The bioreactor was heated at 37 °C by a surrounding heating coat. The produced water from the esterification reaction was continuously removed by circulating the reaction mixture through a column packed with a molecular sieve. Aliquot samples were collected at specified time intervals to determine the amount of the formed ester.

2.5. Analytical methods

The OAs (acetic, propionic, butyric, citric, tartaric, lactic, and succinic) were analyzed by HPLC on a Jasco LC-2000 Series HPLC system (Jasco Inc., Japan) equipped with Bio-rad Aminex HPX-87H column (300 × 7.8 mm i.d., 9 μm particle size), CO-2060 PLUS column oven (50 °C), PU-2089 pump, AS 2050 PLUS autosampler and MD-2018 photodiode array detector (210 nm). Isocratic separation was performed with 0.008 N H₂SO₄ as mobile phase (0.6 mL/min). The samples were filtered through 0.20 μm Chromafil cellulose acetate filters. Determinations were done by means of standard curves.

Residual sugar was determined by HPLC on a Shimadzu system equipped with a Nucleogel Ion 300 OA column, LC-9A pump, CTO-10A oven (70 °C), and RID-6A refractive index detector. The mobile phase was 0.008 N H₂SO₄ (0.4 mL/min), and 1-butanol was used as internal standard. Amounts of 0.5 mL of sample and 2.5 mL of 1% v/v 1-butanol solution were diluted to 50 mL. The samples were filtered with 0.20 μm Chromafil filters and 80 μL of the final solution were injected into the column. Calculation were done using standard curves.

Ethanol was determined on a Shimadzu GC-8A system with HayeSep Q 80/100 column (130 °C), C-R6A Chromatopack integrator, He as carrier gas (40 mL/min) and FID detector. The injection port and FID temperature was 210 °C. Samples of 2 μL were directly injected and determinations were performed by means of standard curves.

Finally, esters were determined on a Dani Master GC Fast (with FID) system equipped with DB-5 column (Agilent Technologies, 30 m length, 0.32 mm i.d., 1 μm film thickness) (temperature 80–110 °C; 2 °C/min; maintained at 110 °C for 5 min). Helium (carrier gas), air, and hydrogen, at flow rates of 6, 300, and 60 mL/min, respectively, were used. The injector and detector temperatures were both 250 °C. Samples of 5 μL, properly diluted in n-hexane, were injected. The retention times of the eluted esters were between 7 and 14 min. All determinations were performed by means of standard curves.

3. Results and discussion

3.1. Scale-up of wheat straw acidogenic fermentation

Delignification of wheat straw was carried out to facilitate contact with the culture cells immobilized on the porous kissiris. Also, to facilitate scale-up and easier handling of kissiris and circulation of the liquid contents, a 2-compartment bioreactor was designed. Due to the fact that cellulose is very difficult to convert, an adaptation of cells in the presence of glucose in the substrate was initially done, while the bioreactor was equipped with an effluent recycling pump. In Table 1, an increase in butyric and acetic acid formation

can be observed as the fermentation progressed, with acetic acid reaching a maximum (8.18 g/L) at the 5th fermentation batch and butyric acid (5.69 g/L) at the 6th batch when only one bioreactor compartment was used. The total OAs reached their highest concentration (17.53 g/L) during the 7th batch. When the 2nd compartment was added for the next 2 batches the concentration of acetic acid was about 6.5 g/L, while butyric acid was increased (7.5–8 g/L) and the total OAs concentration was stabilised at about 17.2 g/L (Table 1). Taking into account that 40 L of fermented liquid and the 1.2 kg of cellulosic whiskers were used, the yield of the process was 0.57 g OAs/g cellulose.

The results indicate that the proposed system favoured a butyric-type fermentation (Ren et al., 1997), which occurs under insufficient NADH at pH higher than 6 and lower than 5, due to consumption for cellular synthesis or by conversion to NAD⁺ and production of hydrogen. However, little to no formation of gaseous products was observed. Furthermore, the increased free ammonia nitrogen at high pH inhibits the methanogenic bacteria and results in OAs accumulation (Rajagopal et al., 2013). The results obtained from the proposed scale-up system were very promising compared to the lab-scale acidogenesis of delignified straw whiskers that presented serious inhibition problems (data not shown). The system efficiency was improved as the fermentation batches succeeded as well as by increasing the bioreactor volume. The direct bioconversion of cellulose to OAs using anaerobic UASB culture immobilized on kissiris is therefore a promising alternative method for biofuels or chemicals production based on OAs.

3.2. Recovery of organic acids from fermentation broth

The solvent that gave the best results (79.9% OAs recovery; pH 1) was 1-butanol at all the studied pH values (Table 2). All solvents performed better at pH 1. Butyric and isobutyric acid were the most easily extracted (70–80%), while propionic showed much lower extraction rates (50–60% in most cases). Finally, acetic acid presented low recovery rates that in most cases did not exceed 50%.

3.3. Scale-up esterification

The produced esters from this experiment were butyl acetate, butyl butyrate, butyl propionate and butyl isobutyrate. The esterification process gave about 75% esters yield at 15 h with a maximum 90% yield reached at 24 h. The final yield was determined at about 1.8 mol ester/L when 17 g/L OAs were recovered from the fermentation broth. The optimum OA: alcohol molar ratio for maximum yield and ester productivity was 1:4.5. The highest% yield of esters was obtained at 2.0 M total OAs concentration. The 90% yield of enzymatic esterification from this scale-up experiment indicates suitability of the process for industrial application.

4. Conclusions

The proposed scale-up system for acidogenic fermentation of straw, using a UASB culture immobilized on kissiris, is promising for new generation ester-based biofuel technology development from cellulosic biomass. The acidogenesis system presented good

operational stability. The predominant OAs formed were butyric and acetic, with lower amounts of isobutyric and propionic. The ethanol that was also formed was at levels enough to esterify about 5% of the OAs. For OAs recovery from the fermentation broth, 1-butanol was the best solvent, with butyric and isobutyric being the most easily recovered acids. The subsequent esterification process resulted in high butyl ester yields (90%).

Acknowledgements

This research has been co-financed by the European Union (European Regional Development Fund – ERDF) and Greek national funds through the Operational Program “Competitiveness and Entrepreneurship” of the National Strategic Reference Framework (Greece) 2007–2013 – National Action “Cooperation 2011: Partnerships of Production and Research Institutions in Focused Research and Technology Sectors” of General Secretariat for Research and Technology (Contract Nr. 11SYN_8_839).

References

- Bekatorou, A., Dima, A., Tsafrakidou, P., Boura, K., Lappa, K., Kandyli, P., Pissaridi, K., Kanellaki, M., Koutinas, A.A., 2016. Downstream extraction process development for recovery of organic acids from a fermentation broth. *Bioresour. Technol.* 220, 34–37.
- Deng, W., Zhang, Q., Wang, Y., 2014. Catalytic transformations of cellulose and cellulose-derived carbohydrates into organic acids. *Catal. Today* 234, 31–41.
- Galanakis, C.M., Kordulis, C., Kanellaki, M., Koutinas, A.A., Bekatorou, A., Lycourghiotis, A., 2012. Effect of pressure and temperature on alcoholic fermentation by *Saccharomyces cerevisiae* immobilized on γ -alumina pellets. *Bioresour. Technol.* 114, 492–498.
- Koutinas, A.A., Sypas, V., Kandyli, P., Michelis, A., Bekatorou, A., Kourkoutas, Y., Kordulis, C., Lycourghiotis, A., Banat, I.M., Nigam, P., Marchant, R., Giannouli, M., Yianoulis, P., 2012. Nano-tubular cellulose for bioprocess technology development. *PLoS One* 7, e34350.
- Koutinas, A.A., Kanellaki, M., Bekatorou, A., Kandyli, P., Pissaridi, K., Dima, A., Boura, K., Lappa, K., Tsafrakidou, P., Stergiou, P.-Y., Foukis, A., Gkini, O.A., Papamichael, E.M., 2016. Economic evaluation of technology for a new generation biofuel production using wastes. *Bioresour. Technol.* 200, 178–185.
- Lappa, K., Kandyli, P., Bekatorou, A., Bastas, N., Klaoudatos, S., Athanasopoulos, N., Kanellaki, M., Koutinas, A.A., 2015. Continuous acidogenesis of sucrose, raffinose and vinasse using mineral kissiris as promoter. *Bioresour. Technol.* 188, 43–48.
- Menon, V., Rao, M., 2012. Trends in bioconversion of lignocellulose: biofuels, platform chemicals and biorefinery concept. *Prog. Energy Combust. Sci.* 38, 522–550.
- Rajagopal, R., Massé, D.I., Singh, G., 2013. A critical review on inhibition of anaerobic digestion process by excess ammonia. *Bioresour. Technol.* 143, 632–641.
- Ren, N., Wang, B., Huang, J.C., 1997. Ethanol-type fermentation from carbohydrate in high rate acidogenic reactor. *Biotechnol. Bioeng.* 54, 428–433.
- Stergiou, P.Y., Foukis, A., Filippou, M., Koukouritaki, M., Parapouli, M., Theodorou, L. G., Hatziloukas, E., Afendra, A., Pandey, A., Papamichael, E.M., 2013. Advances in lipase-catalyzed esterification reactions. *Biotechnol. Adv.* 31, 1846–1859.
- Syngiridis, K., Bekatorou, A., Kallis, M., Kandyli, P., Kanellaki, M., Koutinas, A.A., 2013. Γ -Alumina as a process advancing tool for a new generation biofuel. *Bioresour. Technol.* 132, 45–48.
- Syngiridis, K., Bekatorou, A., Kandyli, P., Larroche, C., Kanellaki, M., Koutinas, A.A., 2014. Favouring butyrate production for a new generation biofuel by acidogenic glucose fermentation using cells immobilized on γ -alumina. *Bioresour. Technol.* 161, 118–123.
- Tomei, M.C., Braguglia, C.M., Cento, G., Mininni, G., 2009. Modeling of anaerobic digestion of sludge. *Crit. Rev. Environ. Sci. Technol.* 39 (12), 1003–1051.
- Tsafrakidou, P., Pissaridi, K., Bekatorou, A., Koutinas, A.A., Kanellaki, M. One-step process acidogenesis of lignocellulosics using anaerobic mixed culture immobilized on γ -alumina. In: International Conference on Emerging Trends in Biotechnology, ICETB 2014, November 6–9, New Delhi, India.