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ORIGINAL PAPER

A comparative study of an intensive malolactic transformation of cider using *Lactobacillus brevis* and *Oenococcus oeni* in a membrane bioreactor

I. S. Jung · R. W. Lovitt

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Abstract The aim of this study was to investigate the secondary fermentation of alcoholic green cider by *Lactobacillus brevis* and *Oenococcus oeni* in a membrane bioreactor so as to compare the performance of the two organisms to rapidly carry out the malolactic fermentation (MLF), an important step in reducing acidity and enhancing the flavor characteristics of the beverages. First, the growth of both organisms was intensified by using perfusion culture in a membrane bioreactor (MBR). *O. oeni* and *L. brevis* were grown up to 12.8 g dry cell weight (DCW) l^{-1} and 15.5 g DCW l^{-1} in the MBR. Secondly, the resultant cells were then used for the malolactic transformation of green cider in the MBR. The influences of the residence time in the MBR and the ethanol concentration of the green cider on the organic acid transformation were investigated. Both organisms showed a good tolerance against the acidic conditions (pH 3.0–4.0) and ethanol (90 g l^{-1}). Good levels of malate removal in the MBR were achieved by both organisms but *O. oeni* was more tolerant to high ethanol concentrations and was capable of growth and malate removal in 130 g ethanol l^{-1} green cider. *L. brevis* malate removal was significantly inhibited above 110 g ethanol l^{-1}. The MBR allowed the development of high concentrations of active cells capable of rapid MLF and could be achieved over a prolonged period and over a wide range of conditions thus allowing the control of malate transformation rate. Organism selection for the transformation will be governed by the desired beverage characteristics. There is considerable scope to optimize the process further both with the choice of organisms and the design and operation of the reactor. Rapid beverage maturation on a commercial scale may be possible using MBR and pure cultures of MLF lactic acid bacteria.

Keywords Biotransformation · Bioprocessing · Beverages *Lactobacillus brevis* · *Oenococcus oeni*

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Introduction

Lactobacillus brevis and *Oenococcus oeni* are commonly associated with secondary fermentation of cider and wine [18–20, 44, 45]. *L. brevis* found as a contaminant in beer is hop-resistant and has a good tolerance against stresses caused by acids and alcohols [33, 34]. This organism is also commonly found in English cider maturation systems. *O. oeni* is commonly found in wine, can survive at pH 3.0 and tolerate alcohol concentrations over 130 g l^{-1} [7, 10, 31, 42]. Within cider or wine, these organisms can make significant contribution to the quality of the maturation process in either high alcoholic cider or wine by performing a malolactic fermentation to reduce the acidity and transforming many flavor-related compounds that significantly change the flavor of the beverage.

The malolactic fermentation (MLF) is a microbiologically mediated de-acidifying process typically associated with alcoholic beverages such as wine, cider, and beer. It has a significant influence on the flavor and maturation of these beverages. Alcohols, aldehydes, esters, and various organic acids formed during the alcoholic fermentation by

yeast contribute to a strong flavor in the post-alcoholic fermented beverages prior to maturation. These astringent tastes in wine and cider can be reduced by the maturation process, which comprises not only MLF, but also by a series of reductases that catalyze transformation and condensation of a wide range of flavor compounds such as alcohols, aldehydes, ketones, and esters [17, 36]. During the MLF, malic acid can be degraded into lactic acid and CO_2. Three different degradation pathways of malic acid as intracellular processing of the MLF organisms is involved in overall MLF [40, 41] and the results of the malolactic transformations are dependent on environmental conditions and the organisms involved.

Malate loses one carboxyl group to form lactic acid with liberation of CO_2. The process is also thought to yield some ATP [40, 41]. This de-acidification results in an increase pH during the MLF. Citric acid metabolism by *O. oeni* is another significant catabolic pathway to affect wine acidity and also interacts with MLF. Almost all citric acid is metabolized into acetic acid and C_4 compounds, resulting in accumulation of diacetyl, acetoin, and acetic acid in wine. Usually more acetic acid is produced from the given amounts of citric acid during the MLF [3, 16, 32, 24, 26, 27, 18].

Lactobacillus brevis, a heterolactic fermentative bacterium producing lactic acid, acetic acid, and ethanol with liberation of CO_2 is also found in wines and beer. *L. brevis* has been used in the maturing process of cider and has a good ability to degrade malic acid to lactic acid. *L. brevis* produces 2 mol of mannitol, 1 mol each of lactate and acetic acid with liberation of CO_2 at the expense of 3 mol of fructose in green cider [29, 23]. However, the proportion of the products can vary with the substrate and the culture conditions. The amounts of ethanol produced by *L. brevis* during the cultivation vary with the amount of fructose. *L. brevis* produces mannitol by mannitol dehydrogenase using fructose in green cider rather than ethanol from acetaldehyde to regenerate NAD^+ exhausted during the catabolism of glucose [23]. *L. brevis* can also regenerate NAD^+ through a branched pathway toward ethanol when no fructose is present in green cider. *L. brevis* also has the capability to degrade lactate into acetate, 1,2-propanediol, and ethanol, producing ATP for survival and maintenance in the acidic conditions below pH 4.0 [6].

Traditional MLF is plagued by its slow and inefficient performance. A series of strategies have been developed to overcome these problems. In order for cider or wine producers to cope with rapid changes and fluctuations of beverage market, intensive and rapid maturation processes have many attractions for the timely and economical production of wine or cider. The use of membrane bioreactors (MBR) seems well suited for this, having proven to be capable of efficient high cell density systems in anaerobic conditions [1, 4, 13, 14, 37]. High cell density lactic acid bacteria obtained in MBRs have been used to enhance biological processing in areas such as waste treatment processes [12, 15, 25, 30], lactic acid production [5, 28, 46], and biomass production [37, 38].

Most attempts to improve MLF efficiency on the laboratory scale have included the integrated use of starter culture in the green cider. However, the main disadvantage of using starter cultures is in providing sufficient concentration of MLF organisms; over 10^6 cfu ml^{-1} active MLF organisms are required to instigate a rapid, reproducible MLF in the extreme environment associated with green cider or grape must (pH < 3.5, > 80 g ethanol l^{-1}) for growth of lactic acid bacteria [18].

Published work on the MLF of wines using high cell concentrations of free cells is limited but it has been carried out by using *Oenococcus oeni* [8, 9, 19, 20]. Studies of MLF in systems have only been carried out on the laboratory scale using a 300-ml system; one study achieved rapid MLF for a prolonged period (125 h) in a small MBR system [9].

Although *L. brevis* and *O. oeni* have been used for maturation of grape must or green cider, the contributions made by both the organisms during the MLF differ as MLF performance is based on different physiology, biochemistry, and alcohol tolerance of these organisms. This investigation compares the MLF efficiency of two organisms using green cider as substrate in an MBR on a pilot scale (36.0 l) over prolonged periods of operation (250 h), investigating the effects of alcohol concentration and the hydrolytic retention time. This system was also employed to produce the high concentrations of LAB required to study MLF in the extreme environment of green cider [2, 21, 39]. We also report the potential limitations of the MBR system used in the study.

Materials and methods

Strains, growth media, and reagents

Oenococcus oeni NCIB 11648 was purchased from NCIMB Ltd (Aberdeen, UK) and *Lactobacillus brevis* was originally obtained from HP Bulmer Ltd (Hereford, UK). *L. brevis* was grown in MRS agar medium (Difco. Lab., Detroit, USA) and was selected by morphological difference of the colony on agar plates. All strains were preserved in glycerol 240 ml l^{-1} (from Merck Co.) at −70°C and thawed at 30°C before use.

MLM-I medium containing yeast extract (20.0 g l^{-1}), soy peptone (17.0 g l^{-1}), KH_2PO_4 (4.0 g l^{-1}), Tween-80 (0.5 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (0.5 g l^{-1}), and $MnSO_4{\cdot}4H_2O$ (0.005 g l^{-1}) was used for propagation and pre-culture of

O. oeni and L. brevis. Growth medium for intensive propagation of *L. brevis* in the MBR contains 10 g l^{-1} glucose, 25 g l^{-1} fructose, 3.9 g l^{-1} KH_2PO_4, 15 g l^{-1} yeast extract, 0.18 g l^{-1} $MnSO_4{\cdot}4H_2O$, and 1 g l^{-1} Tween-80, and pH was 6.0 after sterilization. Growth medium for *O. oeni* in the MBR contains 25 g l^{-1} glucose, 10 g l^{-1} fructose, 3.9 g l^{-1} KH_2PO_4, 15 g l^{-1} yeast extract, 0.05 g l^{-1} $MnSO_4{\cdot}4H_2O$, 1 g l^{-1}, 0.5 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.5 g l^{-1} $(NH_4)_2SO_4$, 8.8 g l^{-1} tri-sodium citrate, and 1 g l^{-1} Tween-80, and pH was 5.8 after sterilization. All media were prepared by using distilled water and autoclaved at 121°C for 20 min.

All other chemicals were of analytical grade and purchased from Fisher Scientific Ltd (UK). Green cider as substrate of the MLF was obtained from HP Bulmer Ltd (Hereford, UK).

Preparation of pre-culture for intensive propagation of two malolactic organisms in MBR

Intensive propagation of *O. oeni* and *L. brevis* in the MBR was started with inoculation of pre-culture scaled up in a series of culture bottles. All pre-cultures of two malolactic organisms were prepared in MLM-1 medium. The frozen vial suspensions (> 10^8 cfu ml^{-1}) were removed from an ultra-freezer (−70°C), thawed in a 30°C water bath, and then immediately inoculated into a 50-ml serum vial containing 30 ml MLM-1 medium.

Pre-cultures of *L. brevis* were grown in a 50-ml serum vial and scaled up to three 20.0-l culture bottles of 18.0-l working volume (Fig. 1, no. 10) using MLM-1 medium, via three culture bottle of 200-ml working volume with 5% inoculation volume. *O. oeni* was scaled up in a similar manner using 10% inoculation volumes. *L. brevis* and *O. oeni* were grown in 28°C for 15 and 20 h, respectively, in unagitated conditions. Ultimately, 54.0 l pre-culture in three 20.0-l culture bottles was loaded into the sterile MBR by the peristaltic pump (Fig. 1, no. 28; 323U, Watson Marlow Ltd, UK) purging with oxygen-free nitrogen gas (Fig. 1, no. 7) to maintain anoxic conditions within the MBR.

Growth of both organisms in the stirred tank reactor

Both *O. oeni* and *L. brevis* were grown in a 5.0-l stirred tank reactor (STR) to investigate growth conditions and

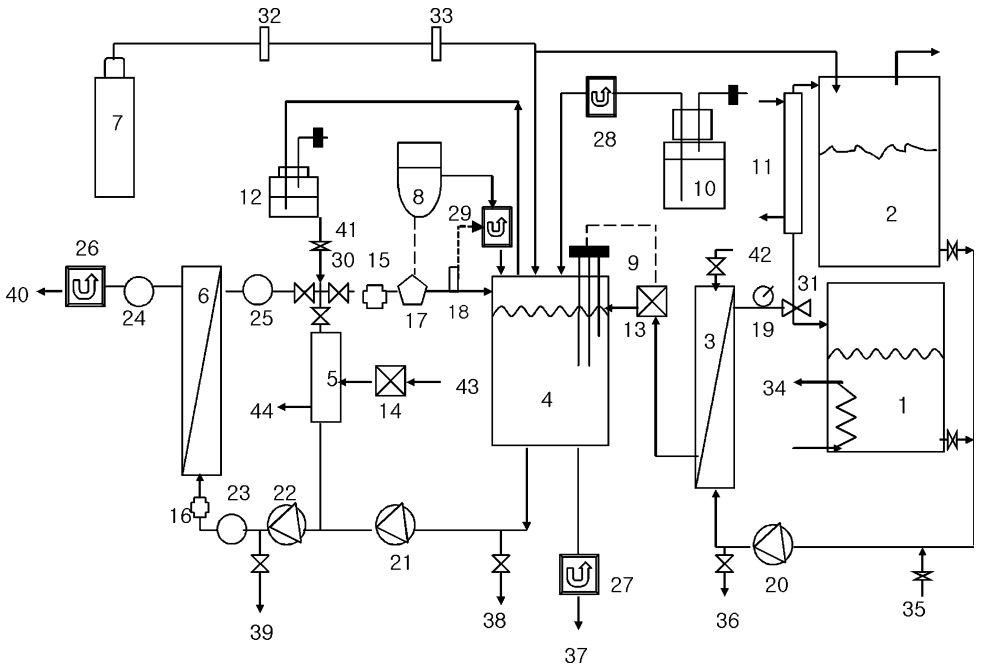

Fig. 1 A schematic diagram of a membrane bioreactor. *1* Feed tank (100 l), *2* feed cider tank (400 l), *3* two feed membranes (total 0.4 m^2), *4* bioreactor (26 l), *5* heat exchanger, *6* product membrane (1 m^2), *7* nitrogen gas cylinder, *8* alkali tank (6 l), *9* level indicator, *10* starter culture (20 l), *11* heat exchanger, *12* bubble trap, *13* pinch valve, *14* solenoid valve, *15* flow meter for reactor/transmitter, *16* flow meter for recycling/transmitter, *17* load cell/transmitter, *18* pH indicator/transmitter, *19* pressure gauge, *20* centrifugal pump/programmable logic control (PLC), *21* magnetic pump for reactor fluid/PLC, *22* magnetic pump for recycling fluid/PLC, *23–25* pressure gauges, *26* production pump/PLC, *27* bleeding and sampling pump/PLC, *28* inoculating pump, *29* alkali pump/PLC, *30*, *31* diaphragm valves, *32* manometer, *33* air filter, *34* heat exchanger, *35* original cider in, *36* drain out, *37* bleeding and sampling, *38* drain out of bioreactor, *39* drain out of production membrane, *40* product out, *41*, *42* steam in *43*, *44* cooling water in and out. Total volume of fluids to circulate through the whole system was 36 l

performance. Biomass yields ($Y_{x/s}$), volumetric biomass production rates ($P_{x/t}$), and growth rates (μ) obtained during the batch culture in the STR were analyzed for comparison with those obtained during the perfusion culture in the MBR (Table 1). In the STR, temperature was controlled at 28°C as described above. The pH was automatically controlled in pH 4.8 for growth of *O. oeni* and pH 5.2 for growth of *L. brevis* with 2 N NaOH and alkali consumption for pH control was measured in every 3 h.

Membrane bioreactor (MBR) design

A membrane bioreactor (MBR) was required for propagation of lactic acid bacteria and the intensive MLF of green cider as shown in Fig. 1. The MBR (36.0 l) was constructed from a stainless steel vessel (SUS 316) and connecting pipe work, ceramic membranes, and magnetically coupled centrifugal pumps and clamp fittings using silicon rubber seals. Growth medium or green cider were formulated at the stainless steel reservoir tanks (Fig. 1, no. 1, 100.0 l; no. 2, 400.0 l) before being automatically fed into the main vessel (Fig. 1, no. 4, 26.0 l) via the feed ceramic membrane (Fig. 1, no. 3; effective area, 0.2 m^2; pore size, 0.2 micron). The liquid level in the main vessel was controlled by the level controller (Fig. 1, no. 9) inside the vessel and the on/off valve (Fig. 1, no. 13) fitted between the feed membrane (Fig. 1, no. 3) and the vessel. Growth medium and green cider were circulated in the MBR by two magnetically coupled stainless steel pumps (Fig. 1, nos. 21 and 22, Q-Max series Caster Pump, Italy) and then released through the product membrane (Fig. 1, no. 6; effective area, 1 m^2; pore size, 0.2 micron). The operating pressure of the product membrane was regulated by controlling the flow rates of the fluids with two magnetically coupled stainless steel pumps and the diaphragm valve. Typically the product membrane and the feed membrane were operated under high pressure (50–100 kPa) and the bioreactor part including the main vessel, heat exchanger, and pumps were at ambient pressure during the bacterial growth and the MLF. Ceramic membrane (Fig. 1, no. 6) for filtered production of cider or culture broth was externally fitted next to a heat exchanger (Fig. 1, no. 5) in the MBR. Permeate flow from the product membrane was controlled by the peristaltic pump fitted in the permeate line. Temperature was controlled by the heat exchanger with the solenoid valve (Fig. 1, no. 14) under control of a PLC (Fig. 1, no. 43). The pH was controlled at pH 5.2 for growth of *L. brevis* and at pH 4.8 for growth of *O. oeni* to support the maximum growth. The pH was not controlled during the MLF using both organisms, but only measured. Alkali consumption was measured with the load cell (Fig. 1, no. 8) to measure the weight of the alkali reserve tank (Fig. 1 no. 8, 6.0 l). Alkaline solution (2 N NaOH) was fed into the main vessel by the peristaltic pump (Fig. 1, no. 29) as needed for pH control (Fig. 1, no. 17). The MBR was purged with oxygen-free nitrogen gas to ensure anoxic conditions at the beginning of the culture.

The membrane bioreactor was sterilized at 103°C for 20 min by circulating steam through the MBR system. The feed membrane, the product membrane, and the main vessel were sequentially sterilized by circulating steam through the MBR system via a series of butterfly valves. The feed membrane was first sterilized for 20 min and then the product membrane and the main vessel were sterilized. Temperature of the product membrane and the heat exchanger was increased to 103°C by circulating steam and then the steam was pushed into the main vessel. Once the temperature of whole system reached 103°C, the whole system was maintained at 103°C for 30 min by adding more steam. After sterilization of the whole system for 30 min, all valves were closed and the whole system was cooled to be ambient temperature.

Table 1 A comparison of the performance of the growth of *L. brevis* and *O. oeni* in the MBR and the STR

Strains	Culture	Kinetics		Product yields (g g^{-1})					$P_{x/t}$
		μ	X	$Y_{x/s}$	$Y_{l/s}$	$Y_{a/s}$	$Y_{e/s}$	$Y_{m/s}$	
L. brevis	STR	0.20	2.33	0.07	0.4	0.08	0.22	0.03	0.07
	MBR	0.05	15.5	0.08	0.9	0.02	ND	0.01	2.02
	MBR to STR ratio[a]	0.25	6.65	1.14	2.25	0.25	ND	0.33	28.86
O. oeni	STR	0.11	0.54	0.02	0.2	0.27	0	0.43	0.02
	MBR	0.07	12.8	0.02	0.18	0.29	0.03	0.4	0.19
	MBR to STR ratio[a]	0.64	23.7	1.0	0.9	1.07	0.03	0.93	9.5

μ (h^{-1}), growth rate; X (DCW g l^{-1}), cell mass; $Y_{x/s}$, cell product yield; $Y_{l/s}$, lactate product yield; $Y_{a/s}$, acetate product yields; $Y_{e/s}$, ethanol product yields; $Y_{m/s}$, mannitol product yields; $P_{x/t}$ (DCW g l^{-1} h^{-1}), volumetric cell production rate; ND, not determined

[a] These figures are the ratio of the specified parameter between that observed for the MBR and that for the STR. All experiments were carried out in triplcate and have an error range of ± 5.0%

Feed of green cider and growth media to MBR

Green cider was fed into the MBR via a sterile ceramic membrane filter (Fig. 1, no. 3) to remove the insoluble particles such as bacteria, yeast, and other particles (apple pulp). Membrane flux of green cider through the product membrane was a key factor as it was used to control the hydraulic retention time. The hydraulic retention time had a strong influence on de-acidifying and formation of flavors during the MLF.

Intensive propagation of the malolactic organisms in MBR

Pre-culture inoculated into the MBR was washed with three culture volumes of fresh growth medium and then the organisms were grown in the MBR. The circulation of growth medium in the MBR was driven by two magnetically coupled stainless steel pumps (Fig. 1, nos. 21 and 22, Q-Max series Caster Pump, Italy) during the cultivation. Growth medium was fed into the main vessel via the feed membrane (Fig. 1, no. 3) under control of the level controller (Fig. 1, no. 9) and an on/off valve (Fig. 1, no. 13). At the same time, to maintain the volume of the culture, broth was simultaneously filtered out of the MBR through the product membrane (Fig. 1, no. 6) as a permeate, the flux being controlled by a peristaltic pump on the permeate line. The feeding rate of growth medium was controlled to maintain the concentration of carbon source above 15 g l^{-1} and achieved by measuring the concentration of the residual carbohydrate in every 3 h during the cultivation. Temperature was controlled at 28°C for *L. brevis* and 25°C for *O. oeni*.

Intensive malolactic transformation by MLF organisms in MBR

Once culture reached an appropriate density for the MLF process, green cider was continuously fed into the MBR via the feed membrane (Fig. 1, no. 3). As the ethanol concentration of green cider supplied from HP Bulmer Ltd was above 130 g l^{-1}, it was diluted to an appropriate ethanol concentration (90.0–130.0 g l^{-1}) in the reservoir tank (Fig. 1, no. 2).

The MLF of green cider using organisms was first investigated with different hydraulic retention times. The hydraulic retention time of the cider was controlled from 3 to 12 h and ethanol concentration was 90.0 g l^{-1}. The investigation was carried out for a total of 250 h. Secondly, the influence of ethanol concentration on the MLF performance was investigated. A range of ethanol concentrations from 30.0 to 130.0 g l^{-1} and a hydraulic retention time of 9.0 h were used and investigated over a total of 200 h.

In both investigations, the pH, organic acids and biomass concentrations were monitored to assess performance. The temperature was controlled at 22°C.

Measurement of cell mass

Cell mass was measured to investigate tolerance of the MLF organisms to the acidity and the alcoholic stresses of green cider during the MLF. Cell mass of samples were measured at an absorbance of 660 nm through 1.0-cm light path using a spectrophotometer (Phillips). Samples of culture fluids from the MBR were diluted with distilled water prior to optical density measurement and then these data were correlated to dry cell weight (g l^{-1}) using a standard curve.

Analysis of organic acids and carbohydrates

Organic acids and carbohydrates in culture media and cider were measured with HPLC (Varian Ltd) fitted with an electrochemical detector (model ED40, Dionex Corporation, USA) and ethanol concentrations were also measured with gas chromatography (GC) system (Varian Ltd) fitted with a flame ionization detector (FID). Concentrations of organic acids, carbohydrates, and ethanol were regularly measured to investigate their variation in relation to MLF activity and culture conditions.

During the propagation of both organisms, glucose, fructose, and mannitol were measured by HPLC fitted with CarboPac™ MA-1 (4 × 50 mm) as the guard column and CarboPac™ MA-1 (4 × 250 mm) as the main column (Dionex Corporation, USA). NaOH (480 mmol l^{-1}) was used for the mobile phase in the isocratic mode.

HPLC was also used for measurement of organic acids during the propagation of both organisms and MLF. HPLC was fitted with Ion Pac® ATC-1 trap column (9 × 2.4 mm) between the pump and the injection valve. ATC-1 trap column was used to strip anionic contaminants such as carbonate in the hydroxide effluent [ATC-1 manual; Doc No. 032697-06]. IonPac® ATC-1 (4 × 50 mm) as the guard column was placed between the injection valve and the main column. Ion-Pac AS11 HC anion exchange column (4 × 250 mm) as the main column was fitted in the heating chamber (Waters, Millipore) for temperature control at 35°C. An ED40 electrochemical detector was used wired to a DS3 detector stabilizer (DS3-1, Dionex Corp). ASRS-Ultra 4 mm (P/N 53946), the pressure controller (Dionex Corp, USA), was placed in the next to DS-3 detector stabilizer. HPLC analysis of organic acids was performed in the gradient module using NaOH solution from 0.05 to 50 mmol l^{-1}.

Ethanol was measured with a GC system fitted with a ChromPac capillary column (WCOT fused SILICA, 25 M × 0.32 mm ID, coating CO-WAX 57CB, DF = 0.2) from Varian, Canada. Oxygen-free nitrogen gas and air were used for the mobile phase. The rear FID was controlled at 220°C. The injector (200°C) was purchased from Dominic Hunter (UHP-20H). For ethanol analysis, column oven temperature was controlled at 30°C for the first 2 min, and the column temperature was increased at the rate of 40°C min^{-1} to 100°C and held at 100°C for 0.5 min.

Results

Intensive propagation of *L. brevis* and *O. oeni* in MBR

Lactobacillus brevis was grown to 15.5 DCW g l^{-1} in 20 h and *Oenococcus oeni* was grow to 12.8 DCW g l^{-1} in 68 h using the MBR (Fig. 2).

Total biomass (X) and volumetric biomass production rate ($P_{x/t}$) were greatly improved as compared with the STR (Table 1). Biomass concentrations (X) increase 6.65 times and by 28.86 times in volumetric biomass production rate ($P_{x/t}$) as compared with the STR for *L. brevis*. Lactic acid, the main fermentation product, was maintained between 200 and 350 mmol l^{-1} during the culture in the MBR. During the perfusion culture in the MBR, the dilution rate could not increase over 0.5 h^{-1} due to a serious membrane fouling in the product membrane (Fig. 2) while the initial growth rate (μ) of 0.2 h^{-1} decreased to 0.05 h^{-1} by the time the culture was terminated. Fructose in the growth medium was converted to mannitol but ultimately this was not produced by the end of the perfusion culture.

For *O. eoni,* the biomass concentration (X) was increased 23.7 times and by 9.5 times in volumetric biomass production rate ($P_{x/t}$) as compared with the STR cultures. Dilution rate of feeding medium could not increase over 0.5 h^{-1} due to a serious membrane fouling in both of the product membranes and the feed membrane (Fig. 2).

During the growth of both MLF organisms in the MBR, fouling of the product membrane caused by accumulation of end products was high enough to cause a serious

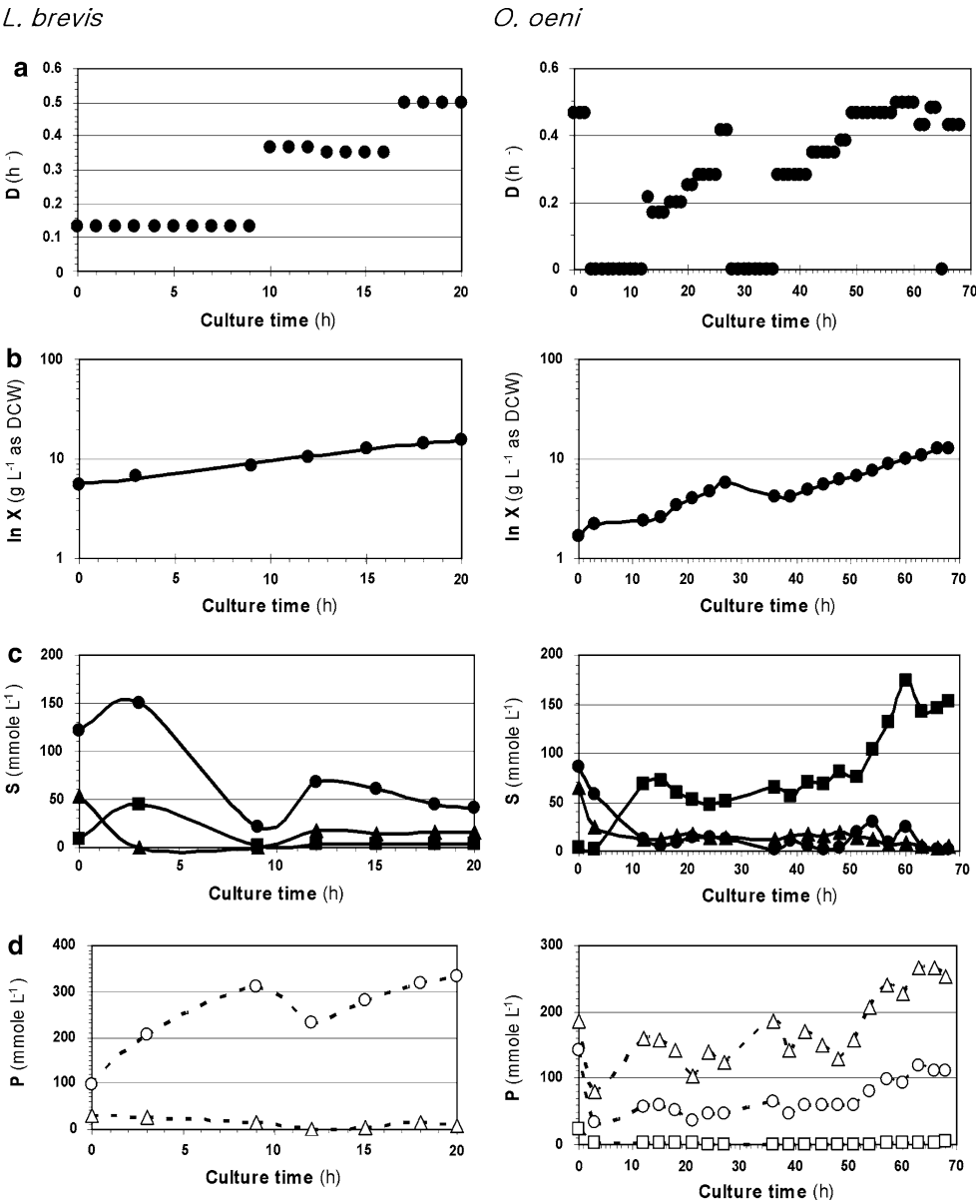

Fig. 2 The growth of *Lactobacillus brevis* (*left column*) and *Oenococcus oeni* (*right column*) in the MBR. **a** Dilution rate (D) of growth media, **b** cell mass of both organisms (ln X as DCW), **c** glucose (*filled circles*) and fructose (*filled triangles*) as carbon sources, and mannitol (*filled squares*) as product. **d** Lactic acid (*open circles*), acetic acid (*open triangle*) and ethanol (*open squares*) as products. All assays of organic acids, biomass, and pH were performed in triplicate and standard error rate was ± 3%

retardation of growth. The product membrane (Fig. 1, no. 6) showed a serious reduction of membrane flux during either the propagation of *L. brevis* or in the MLF by *L. brevis* and limited further increases in the biomass concentration. The dilution rate (*D*) therefore could not increase above 0.5 h^{-1}.

Growth of *O. oeni* in the MBR was restricted by membrane fouling in the feed membrane due to a low growth rate of *O. oeni*. The feed membrane was used for sterilization and particle removal of growth medium during the growth of MLF organism and then green cider during the MLF. The prolonged use of the feed membrane for the strain cultivation prior to the MLF could affect flux decline in the feed membrane during the MLF. To overcome this problem either feed pretreatment or intermittent membrane cleaning would be required.

Biomass concentration in MBR during MLF

Green cider was then used as substrate for the MLF-mediated maturation, using the typical pH 3.4–3.7 with 90–130 g ethanol l^{-1}. Microorganisms for the MLF should be tolerant to low pH, the organic acids, and the ethanol in green cider or grape must. In this investigation, the performance of the cell biocatalyst was assessed by its efficiency in carrying out the malolactic transformation with adverse hydraulic retention time and the ethanol concentration during the MLF.

In the MLF of green cider using *O. oeni* a steady growth of biomass was observed from 14.8 to 18 DCW g l^{-1} (Fig. 3c) while the hydraulic retention time of green cider was changed between 3 and 12 h (Fig. 3a). Similarly, when the ethanol concentration was increased from 90 to

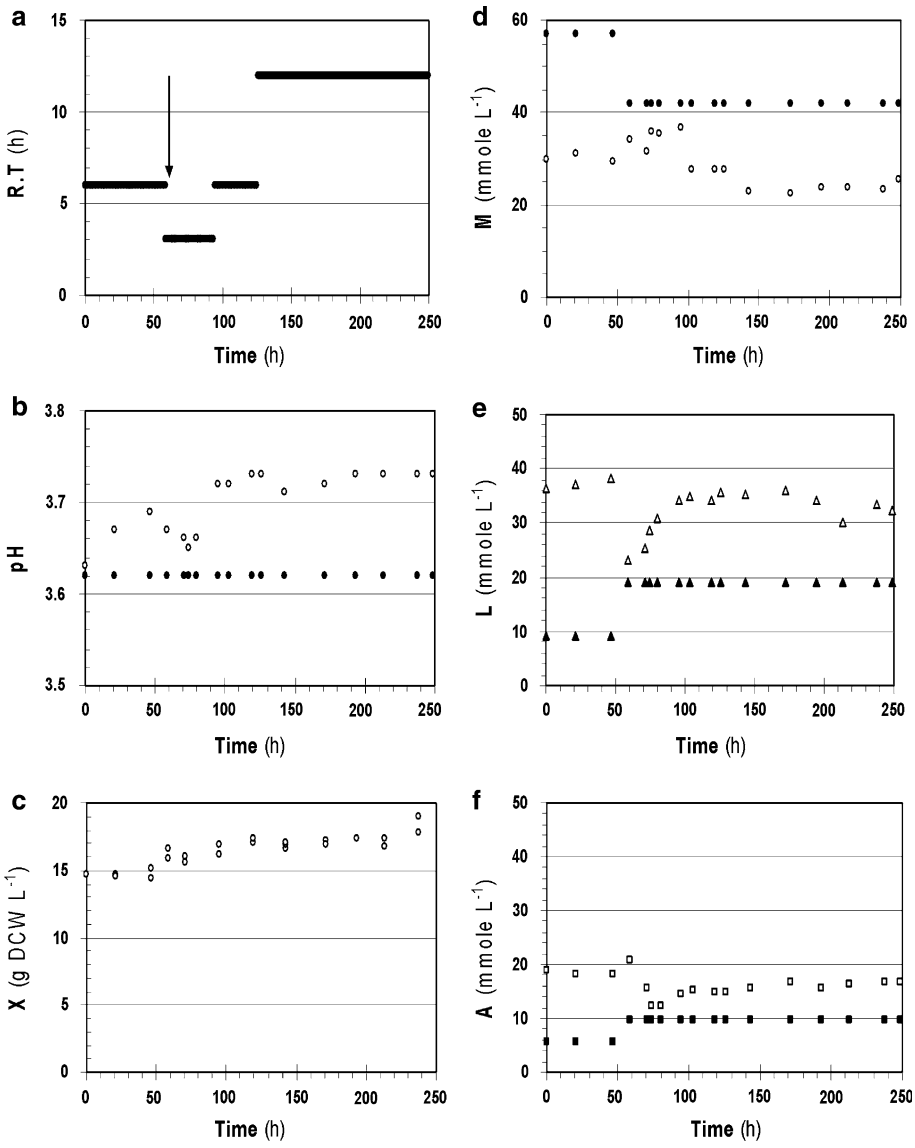

Fig. 3 The influence of the hydraulic retention time (**a**) on pH (**b**), biomass (**c**), malic acid (**d**), lactic acid (**e**), and acetic acid (**f**) in substrate (*closed symbols*) and product (*open symbols*) during the MLF of green cider by *O. oeni* in the MBR. The ethanol concentration of green cider was 90 g l^{-1}. All assays of organic acids, biomass, and pH were performed in triplicate and standard error rate was ± 3%

130 g l^{-1} (Fig. 5a) cell mass of *O. oeni* also increased from 15.5 to 25 DCW g l^{-1} (Fig. 5c).

In contrast, under the same conditions, cell mass of *L. brevis* slightly declined from 8.94 to 7.57 DCW g l^{-1} (Fig. 4c) in the hydraulic retention time below 5 h (Fig. 4a). The effect of increasing the alcohol concentration of green cider (Fig. 6a) reduced the biomass concentration although the biomass remained active. Biomass of *L. brevis* was 9.0 DCW g l^{-1} initially in 90.0 g ethanol l^{-1}, but it gradually reduced to 7.0 DCW g l^{-1} in 130.0 g ethanol l^{-1}(Fig. 6c) green cider.

The effect of retention time on organic acids during MBR

Having produced the biomass in a propagation phase above, the influence of hydraulic retention time on the MLF performance of green cider by two organisms using the MBR was investigated. The ethanol concentration of green cider was diluted to 90.0 g l^{-1} with tap water resulting in a malate concentration between 41.6 and 56 mmol l^{-1} (Figs. 3, 4).

The effect of retention for *O. oeni* (Fig. 3) and *L. brevis* (Fig. 4) shows that the malate concentration in green cider substrate was significantly reduced in product cider, whereas lactic acid and acetic acid concentrations simultaneously increased. Using *O. oeni* and increasing the hydraulic retention time from 3 to 12 h, the amounts of malate removed in green cider substrate increased from 7.01 to 18.4 mmol l^{-1}(Fig. 3d) with concomitant increases in the amounts of acetate from 4.2 to 6.8 mmol l^{-1} (Fig. 3f). Lactate concentrations also increased slightly (Fig. 3e).

Using *L. brevis* for the MLF of green cider the effect of increasing the hydraulic retention time from 3 to 12 h increased the amounts of malate removed from 21.5 to

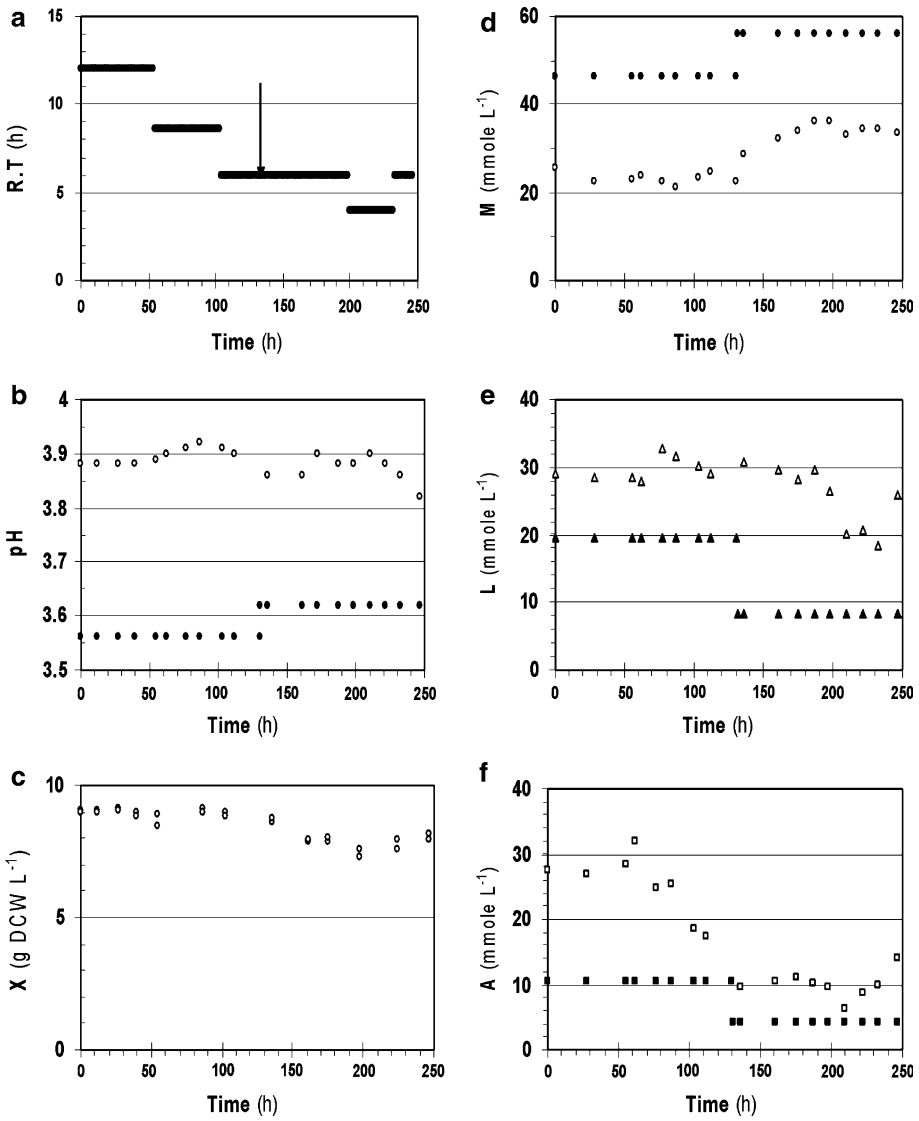

Fig. 4 The influence of the hydraulic retention time (**a**) on pH (**b**), biomass (**c**), malic acid (**d**), lactic acid (**e**), and acetic acid (**f**) in substrate (*closed symbols*) and product (*open symbols*) during the MLF of green cider by *L. brevis* in the MBR. The ethanol concentration of green cider was 90.0 g l^{-1}. All assays of organic acids, biomass, and pH were performed in triplicate and standard error rate was ± 3%

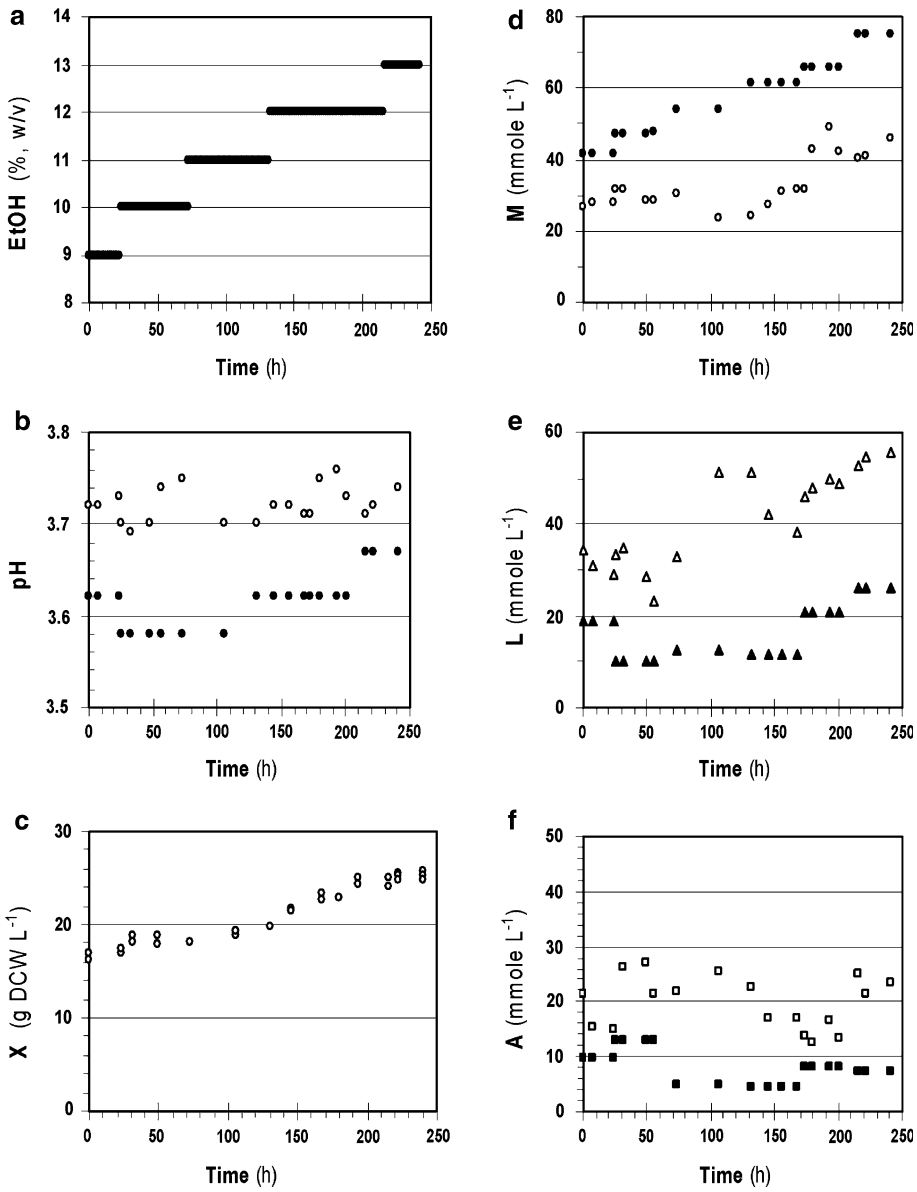

Fig. 5 The influence of ethanol concentration (**a**) on the pH (**b**), biomass (**c**), malic acid (**d**), lactic acid (**e**), and acetic acid (**f**) in substrate (*closed symbols*) during the intensive malolactic fermentation of green cider by *O. oeni* in the MBR. The dilution rate was 6 h. All assays of organic acids, biomass, and pH were performed in triplicate and standard error rate was ± 3%

23.7 mmol l^{-1} (Fig. 4d). At the same time the concentrations of acetic acid increased from 6.04 to 17.1 mmol l^{-1} on average (Fig. 4f) while lactic acid increased from 9.0 to 20 mmol l^{-1} on average (Fig. 4e).

The effect of ethanol concentration on organic acids in MBR

The effect of alcohol concentration on the MLF of green cider was also assessed. Figure 5d shows the variation on malic acids concentration during the MLF using *O. oeni*. The amounts of malic acid removed increased from 14.4 mmol l^{-1} in 90.0 g ethanol l^{-1} to 30.3 mmol l^{-1} in 110.0 g ethanol l^{-1}. Above 110 g ethanol l^{-1}, the amounts of removed malic acid did not increase further, but were maintained. At the same time the amounts of lactic acid increased from 12.4 mmol l^{-1} in the 90.0 g ethanol l^{-1} to 38.7 mmol l^{-1} in 110.0 g ethanol l^{-1} but then slightly decreased to 130.0 g ethanol l^{-1} (Fig. 5e).

When *L. brevis* was used in the MBR under the same conditions, the amounts of malic acid removed increased from 24.1 mmol l^{-1} in 90.0 g ethanol l^{-1} to 34.2 mmol l^{-1} in 110.0 g ethanol l^{-1}. However, when the alcohol concentration increased to 130.0 g ethanol l^{-1}, the amounts of malic acid removed were substantially decreased to 16.1 mmol l^{-1} (Fig. 6d). The lactic acid profile also changed in response to high concentrations of ethanol (Fig. 6e). The amounts of acetic acid during the MLF increased from 7.6 mmol l^{-1} in 90.0 g ethanol l^{-1} to 19.2 mmol l^{-1} in 110.0 g ethanol l^{-1}, but declined in 130.0 g ethanol l^{-1} (Fig. 6f). Clearly this organism is inhibited by the high ethanol concentrations.

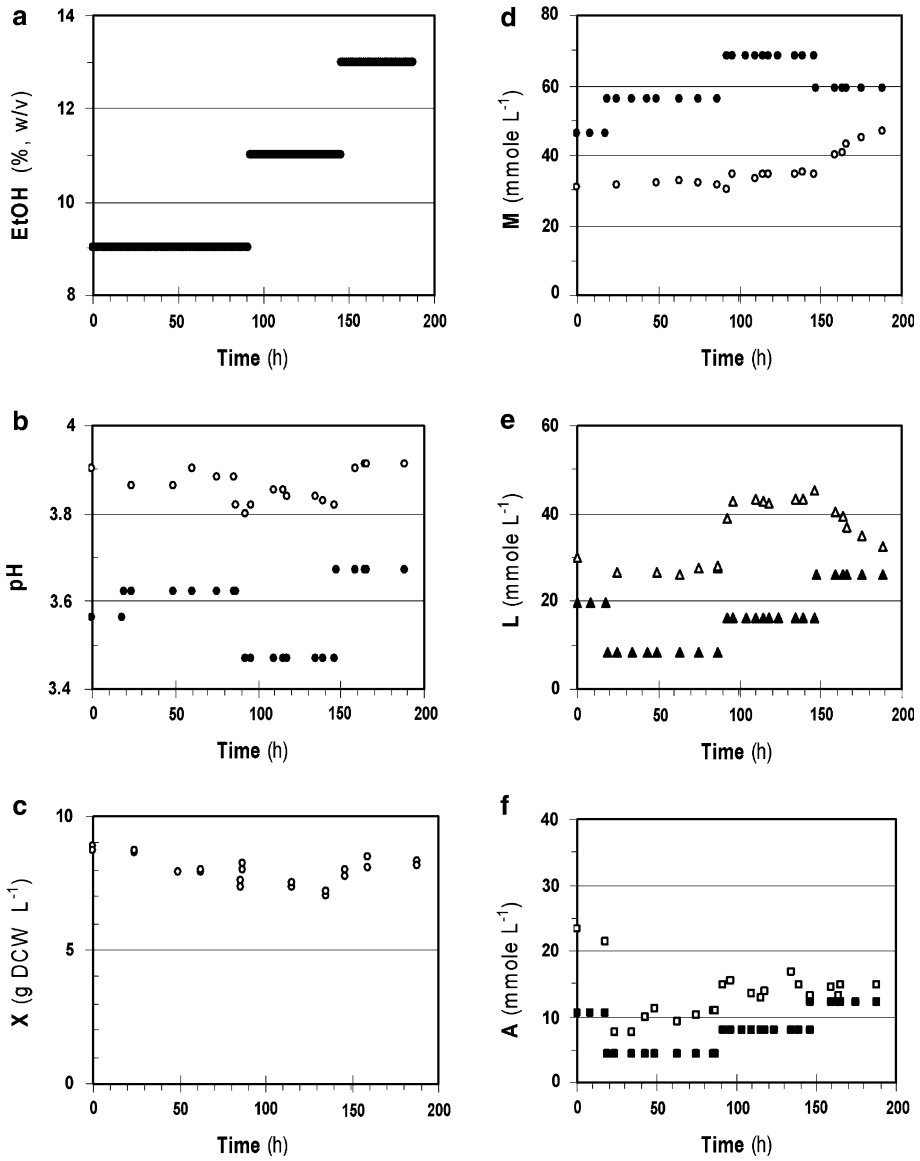

Fig. 6 The influence of ethanol concentration (**a**) on the pH (**b**), biomass (**c**), malic acid (**d**), lactic acid (**e**), and acetic acid (**f**) in substrate (*closed symbols*) during the intensive malolactic fermentation of green cider by *L. brevis* in the MBR. All assays of organic acids, biomass, and pH were performed in triplicate and standard error rate was ± 3%

The pH of cider before and after MBR processing

During the green cider treatment in the MBR, the pH of the cider was increased to pH 3.75 from pH 3.4–3.7 using *O. oeni* and to pH 3.9–4.0 using *L. brevis*. This change is thought to be mainly due to de-acidification by degradation of malic acid to lactic acid and CO_2 during the MLF.

Altering the hydraulic retention time or the ethanol concentrations has little effect on the final pH of the cider; however, the pH of the product cider did not increase over pH 4.0. The pH of green cider substrate varied considerably between batches, but the pH of the product cider became consistent when the MBR treatment was used. The pH of the product cider was typically maintained around pH 3.7 using *O. oeni* while using *L. brevis* it was maintained between pH 3.8 and pH 3.9. The reason why pH did not increase over pH 4.0 during the MLF was thought to be because of the buffering capacity of the product cider enhanced by organic acids and CO_2.

Malolactic activity in the MBR

The malolactic activity (MA) and specific malolactic activity (SA) of both organisms in the MBR were calculated from kinetics of the amounts of malic acid removed and the concentration of biomass within the reactor. The hydraulic retention time (Fig. 7) and the ethanol concentration (Fig. 8) during the MLF significantly influenced the MA and SA of both organisms.

The malolactic activity of both organisms showed a steady increase until the hydraulic retention time of about 9 h. Specific malolactic activity of *O. oeni* proportionally

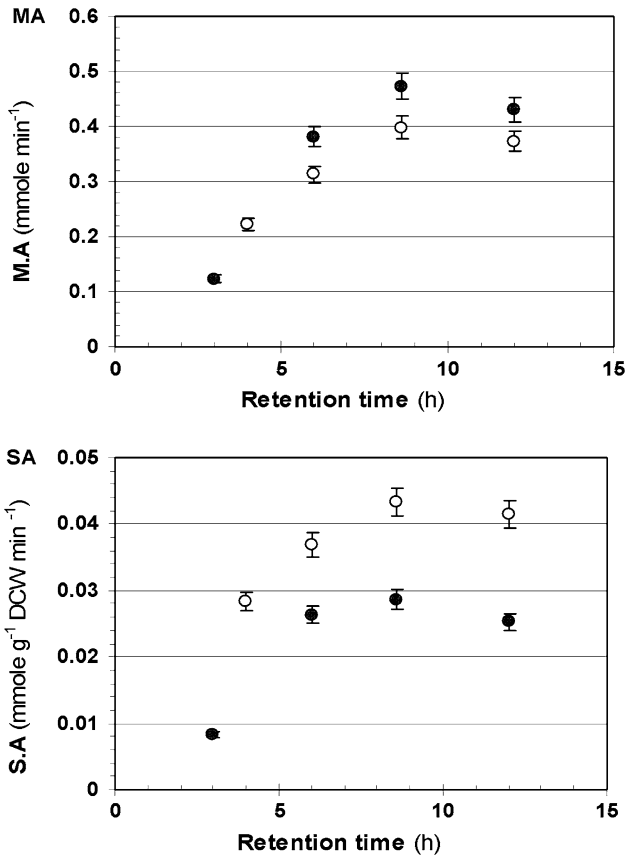

Fig. 7 The influence of the hydraulic retention time on the malolactic activity (*MA*; mmol min^{-1}) and specific malolactic activity (*SA*; mmol min^{-1} DCW g^{-1}) of *O. oeni* (*filled circles*) and *L. brevis* (*open circles*) during the MLF of green cider The ethanol content of green cider was maintained at 90.0 g l^{-1}. All assays of organic acids, biomass, and pH were performed in triplicate and standard error rate was ± 3%

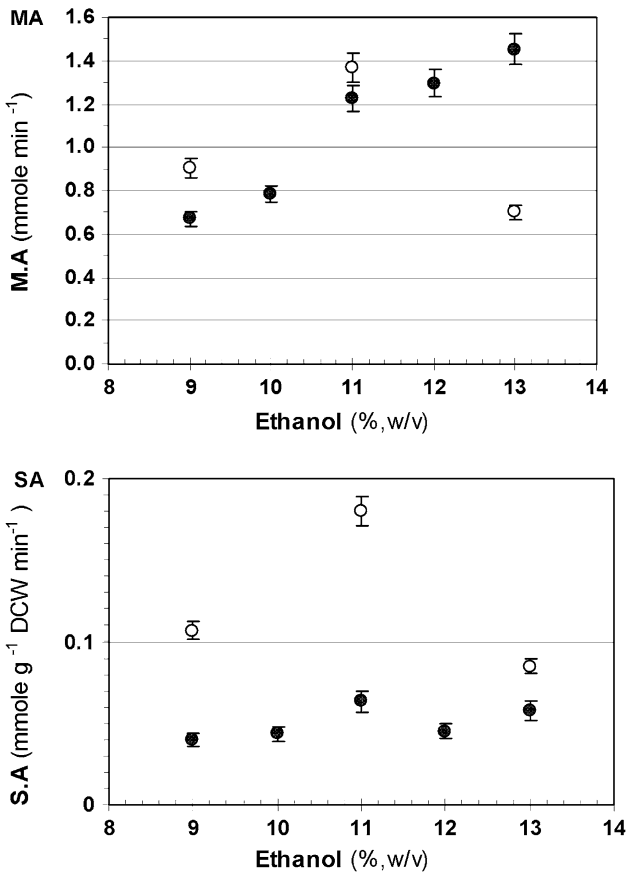

Fig. 8 The influence of the ethanol concentration on the malolactic activity (*MA*; mmol min^{-1}) and specific malolactic activity (*SA*; mmol min^{-1} DCW g^{-1}) of *O. oeni* (*filled circles*) and *L. brevis* (*open circles*) during the MLF of green cider. Dilution rate was 6 h. All assays of organic acids, biomass, and pH were performed in triplicate and standard error rate was ± 3%

increased with the hydraulic retention time increasing to 6 h and then showed a saturation in the higher hydraulic retention times. Specific malolactic activity of *L. brevis* also increased in proportion to the hydraulic retention time increasing from 3 to 9 h and thereafter became saturated. *O. oeni* showed higher malolactic activity than *L. brevis* over the whole range because cell density of *O. oeni* was higher than *L. brevis* under the same process conditions within the MBR (Fig. 7).

Figure 8 shows the inhibition by ethanol in the malolactic activity and specific malolactic activity during the MBR processing of green cider by the two organisms. Specific malolactic activity of *O. oeni* was not seriously affected, but maintained at 0.04–0.07 mmol min^{-1} DCW g^{-1}. The malolactic activity of *O. oeni* was in proportion to the ethanol concentration from 90.0 to 130.0 g l^{-1} and cell density increased from 15.5 to 25 DCW g l^{-1} (Fig. 5c). The improved MLF can therefore be attributed to the substantial tolerance of *O. oeni* to ethanol rather than an increase in specific malolatic activity.

The malolactic activity and specific malolactic activity of *L. brevis* were significantly inhibited above 110.0 g ethanol l^{-1}. Specific malolactic activity of *L. brevis* was relatively higher than that of *O. oeni* during the MBR processing, but *L. brevis* was more sensitive to ethanol than *O. oeni.*

Discussion

In wine and cider making, lactic acid bacteria have been used as a significant part of the maturation processes. A key process is the malolactic fermentation (MLF) which occurs after the primary alcoholic fermentation by yeast of grape or apple juice. Many strains of *O. oeni* which have been isolated in the wine-making process are distinctive in carrying out the MLF, their growth at low pH, and resistance

to high concentrations of organic acids and ethanol [7, 10, 31, 42]. *L. brevis* is a lactic acid bacterium found in cider maturation and in the spoilage of beer. They also are very common in post-alcoholic fermentations and can perform the MLF and have a good resistance to organic acids and ethanol [33, 34].

Malic acid, citric acid, alcohols, and aldehydes are the main substances that give grape must and green cider astringent, bitter, and sour flavor. In the secondary fermentation where sugar concentrations are low, MLF organisms are capable of catalyzing several conversions to remain viable and maintain their biomass. The most important of these is the decarboxylation of malate to lactate and CO_2 and this has the effect of reducing the acidity and astringency of the beverage. Small but significant amounts of flavor compounds are also produced by several reductive transformations during the MLF. The nutrient status and energy level of the organisms used for the MLF are also considered as a significant factor to determine efficiency of the MLF [11].

In this investigation, *L. brevis* and *O. oeni* were compared in terms of their efficiency as an MLF organism to ensure the MLF in green cider in the MBR. Both showed a good efficiency for rapid transformation of malate and at much faster rates in the MBR than in batch reactors [45]. This work also confirmed and extends the basic concept described by Goa and Fleet [5] in which a membrane reactor could be used as a contactor for rapid MLF of wine for prolonged periods. Our system was over 100 times larger and operated for 250 h. Our system also addresses a significant practical problem of providing high concentrations of an active MLF culture (Fig. 2), by allowing rapid propagation of the LAB prior to the use as an MLF contactor (Fig. 3). Using a simple growth medium containing yeast extract, glucose, and fructose, *L. brevis* grew to 15.5 g l^{-1} in 20 h and *O. oeni* was grown to 12.8 g l^{-1} in 68 h in the MBR (Table 1). Volumetric production rate in the MBR was improved to 28.8 times that of *L. brevis* in STR, but only 9.5 times in *O. oeni* because of low growth rate. Likewise, more energy, cost, and time are required to gain high density *O. oeni* than *L. brevis* in the MBR.

The MBR technology has been applied to a high alcohol green cider preparation for the first time. The system allowed both of the organisms to remain active even though they were only surviving or growing slowly in the extreme environment of green cider. The green cider has a relatively low nutrient status, and the MLF and small amounts of carbohydrate and organic acids (citrate) provide sufficient energy to maintain the activity of the LAB.

Once high density *O. oeni* has been obtained in the MBR, *O. oeni* showed a good tolerance against the acidity and 130 g ethanol l^{-1} of green cider (Fig. 8) and could grow, albeit slowly (Fig. 4). Although *L. brevis* was more efficient than *O. oeni* in affording a high density biocatalyst in the MBR, *L. brevis* was more problematic in the processing of green cider than *O. oeni*. The malolactic activity and biomass of *L. brevis* were inhibited when the ethanol concentration was increased over 110 g l^{-1}. *O. oeni* can grow through degradation from malic acid to lactic acid or acetic acid [40, 41], chemical reduction from acetaldehyde to ethanol [17, 27], and consumption of residual carbohydrates from the primary alcoholic fermentation. The malolactic activity and specific malolactic activity of *L. brevis* were drastically reduced at the alcohol concentration above 110 g l^{-1} (Fig. 8). In this investigation, specific malolactic activity varied from 0.04 to 0.07 mmol min^{-1} g^{-1} DCW in *O. oeni* and from 0.07 to 0.18 mmol min^{-1} g^{-1} DCW in *L. brevis*. Specific malolactic activity of *O. oeni* previously reported varied from 0.06 to 0.12 mmol min^{-1} g^{-1} as DCW [11]. This investigation has shown that *O. oeni* can perform MLF in more concentrated green cider than *L. brevis* because of better tolerance of *O. oeni* to the alcoholic stress. Better tolerance of *O. oeni* to the alcohol stress can help improve efficiency by saving production cost and time of the MLF.

A key factor in maintaining active LAB in the MBR is the nutrient status of the feed. Careful supplementation could improve the performance of the system considerably.

The robustly designed MBR allowed its long-term operation, the accumulation of biomass, and maturation of a large quantity of cider with continuous removal of the product materials through the externally fitted ceramic microfiltration membrane. This practical design could easily be scaled to commercial production.

However, there still several technical problems that would significantly improve the system. The flux decline in the feed membrane and the product membrane was a significant factor impeding the growth of lactic acid bacteria and efficient MLF as this membrane fouling ultimately limits the operation of the MBR. The retention time can influence the residual concentration of organic acids in product cider and the economic processing of MLF. The MLF with *O. oeni* suffered from flux decline in both of the feed membranes and the product membrane because of their long exposure time to feed medium and bacterial cultures caused by the slow growth rate. The MBR with *L. brevis* also had a serious flux decline in the product membrane even in the short time needed for its growth. *L. brevis* can produce extracellular polymers like S-layer proteins and polysaccharides [35, 43] and an exopolysaccharide (EPS) [22] and these are thought cause reduced membrane flux as the membrane surface is fouled by *L. brevis*.

This work shows that the choice of a suitable MLF organism is not a straightforward one and will be a function of the nature and composition of the fluid to be matured.

The strength of the alcohol and its acidity are key criteria. Another factor not considered here is the final flavor of the beverage; there are many small but important transformations that give a distinctive character to a beverage: what is desirable in cider may not be so in wine and vice versa.

There is also considerable scope to optimize the design and operation of the MBR to improve the MLF process and make them more controllable and easy to use.

Bubbles of CO_2 gas produced in MLF can be a problem of MLF with high density lactic acid bacteria in MBRs leading to abnormal operation of pumps. The MBR designed for this investigation has the ceramic membrane externally fitted. Typically the membrane part of the product membrane operates under high pressure (50–100 kPa) and the bioreactor part including the main vessel and heat exchanger operates at ambient pressure. CO_2 gas produced as the result of the MLF is dissolved in the high pressure of the membrane part of the product membrane and can be released at ambient pressure in the bioreactor part causing bubbling. Foaming in the main vessel can cause an abnormal operation of the level indicator, and bubbling in the pump can also cause a problem with liquid circulation. MBR with the internal membrane incorporated into the main vessel can help structurally form an equal pressure throughout whole MBR [37, 38] to reduce bubbling during the MLF. An alternative solution may be the active purging of the system with nitrogen to remove the CO_2 and maintain it at low levels in solution.

In this investigation, the use of MBR to obtain a high density biocatalyst for process intensification of the MLF of green cider was carried out. To produce high density biocatalysts, *L. brevis* showed faster kinetics and higher final cell concentrations than with *O. oeni*. However, the better tolerance of *O. oeni* to the alcoholic stress and the acidic condition means that it should be more efficient and economical in treating large volumes of concentrated green cider.

References

1. Bibal B, Vayssier Y, Goma G, Pareilleux A (1991) High-concentration cultivation of *Lactococcus cremoris* in a cell-recycle reactor. Biotechnol Bioeng 37:746–754
2. Bourdineaud J-P, Nehmé B, Tesse S, Lonvaud-Funel A (2003) The ftsH gene of the wine bacterium *Oenococcus oeni* is involved in protection against environmental stress. Appl Environ Microbiol 69(5):2512–2520
3. Cogan TM (1987) Co-metabolism of citrate and glucose by *Leuconostoc* spp. effect on growth, substrates and products. J Appl Bacteriol 63:551–558
4. Crespo JPSG, Xavier AMRB, Barreto MTO, Gonçalves LMD, Almeida JS, Carrondo MJT (1992) Tangential flow filtration for continuous cell recycle culture of acidogenic bacteria. Chem Eng Sci 47:205–214
5. Drews A, Kraume M (2005) Process improvement by application of membrane bioreactors. Chem Eng Res Des 83:276–284
6. Elferink SJWHO, Krooneman J, Gottschal JC, Spoelstra SF, Faber F, Driehuis F (2001) Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. Appl Environ Microbiol 67(1):125–132
7. Fortier L-C, Maréchal RT, Diviés C, Lee BH, Guzzo J (2003) Induction of *Oenococcus oeni* H^+-ATPase activity and mRNA transcription under acidic conditions. FEMS Microbiol Lett 222:165–169
8. Gao C, Fleet GH (1994) The degradation of malic acid by high cell density suspensions of *Lueconostoc oenos*. J Appl Bacteriol 76:632–637
9. Gao C, Fleet GH (1995) Cell cycle membrane bioreactor for conducting malolactic fermentation. Aust J Grape Wine Res 1:32–38
10. Guerrini S, Bastianini A, Granchi L, Vincenzini M (2002) Effect of oleic acid on *Oenococcus oeni* strains and malolactic fermentation in wine. Curr Microbiol 44:5–9
11. Herrero M, García LA, Díaz M (2003) Malolactic conversion using a *Oenococcus oeni* strain for cider production; effect of yeast extract supplementation. J Ind Microbiol Biotechnol 30:699–704
12. Holownia AT (2008) Wastewater treatment in a microbial membrane bioreactor—a model of the process. Desalination 221:552–558
13. Kamoshita Y, Ohashi T, Suzuki T (1998) Improvement of filtration performance of stirred ceramic membrane reactor and its application to rapid fermentation of lactic acid by dense cell culture of *Lactococcus lactis*. J Ferment Bioeng 85(4):422–427
14. Kulozik U, Wilde J (1999) Rapid lactic acid production at high cell concentrations in whey ultrafiltrate by *Lactobacillus helveticus*. Enz Microbial Technol 24:297–302
15. Laera G, Pollice A, Saturno D, Giordano C, Sandulli R (2009) Influence of sludge retention time on biomass characteristics and cleaning requirements in a membrane bioreactor for municipal wastewater treatment. Desalination 236:104–110
16. Liu S-Q (2002) A review. Malolactic fermentation in wine—beyond deacidification. J Appl Microbiol 92:589–601
17. Liu S-Q, Asmundson RV, Holland R, Crow VL (1997) Acetaldehyde metabolism by *Leuconostoc mesenteroides* subsp. *cremoris* under stress conditions. Int Dairy J 7:175–183
18. Lonvaud-Funel A (1999) Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie van Leeuwenhoek 76:317–331
19. Maicas S (2001) The use of alternative technologies to develop malolactic fermentation wine. Appl Microbiol Technol 56:35–39
20. Maicas S, Natividad A, Ferrer S, Pardo I (2000) Maloactic fermentation of wine with high densities of non proliferating cells *Oenococcus oeni*. World J Microbiol Biotechnol 16:805–810
21. Maréchal RT, Gaboriau D, Beney L, Diviés C (2000) Membrane fluidity of stress cells of *Oenococcus oeni*. Int Food Microbiol 55:269–273
22. Mårtensson O, Chasco M-D, Irastorza A, Öste R, Holst O (2003) Comparison of growth characteristics and exoplysaccharide formation of two lactic acid bacteria strains, *Pedicoccu damnosus* 2.6 and *Lactobacillu brevis* G-77, in an oat-based, nondairy medium. Lebensm Wiss Technol 36:353–357
23. Martinez G, Barker HA, Horecker BL (1963) A specific mannitol dehydrogenase from *Lactobacillus brevis*. JBC 238:1598–1603
24. Nedovic VA, Durieux A, Van Nedervelde L, Rosseels P, Vandegans J, Plaissant A-M, Simon J-P (2000) Continuous cider fermentation with co-immobilized yeast and *Leuconostoc oenos* cells. Enz Microbial Technol 26:834–839
25. Ng ANL, Kim AS (2007) A mini-review of modeling studies on membrane bioreactor (MBR) treatment for municipal wastewaters. Desalination 212:261–281

26. Nielsen JC, Richelieu M (1999) Control of flavor development in wine during and after malolactic fermentation by *Oenococcus oeni*. Appl Environ Microbiol 65:740–745
27. Osborne JP, Mira De Orduña R, Pilone GJ, Liu S-Q (2000) Acetaldehyde metabolism by wine lactic acid bacteria. FEMS Microbiol Lett 191:51–55
28. Pal P, Sikder J, Roy S, Giorno L (2009) Process intensification in lactic acid production: a review of membrane based processes. Chem Eng Process 48:1549–1559
29. Peterson WH, Fred EB (1920) Fermentation of fructose by *Lactobacillus pentoaceticus*, n. sp. JBC XLI:431–450
30. Pollice A, Laera G, Saturno D, Giordano C (2008) Effects of sludge retention time on the performance of a membrane bioreactor treating municipal sewage. J Mem Sci 317:65–70
31. Ramos A, Poolman B, Santos H, Lolkema JS, Konings WN (1994) Uniport of Anionic citrate and proton consumption in citrate metabolism generates a proton motive force in *Leuconostioc oenos*. J Bacteriol 176:4899–4905
32. Rodriguez SB, Amberg E, Thornton RJ, McLellan MR (1990) Malolactic fermentation in Chardonnay: growth and sensory effects of commercial strains of *Leuconostoc oenos*. J Appl Bacteriol 68:139–144
33. Sakamoto K, Margolles A, Van Veen HW, Konings WN (2001) Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette multidrug transporter HorA. J Bacteriol 183:5371–5375
34. Sakamoto K, Van Veen HW, Saito H, Kobayashi H, Konings WN (2002) Membrane-bound ATPase contributes to hop-resistance of *Lactobacillus brevis*. Appl Environ Microbiol 68:5374–5378
35. Savijoki K, Kahala M, Palva A (1997) High level heterologous protein production in *Lactococcus* and *Lactobacillus* using a new secretion system based on the *Lactobacillus brevis* S-layer signals. Gene 186:255–262
36. Soomro AH, Masud T, Anwaar K (2002) Role of lactic acid bacteria (LAB) in food preservation and human health—a review. Pak J Nutr 1:20–24
37. Suzuki T (1996) A dense cell culture system for microorganisms using a stirred ceramic membrane reactor incorporating asymmetric porous ceramic filters. J Ferment Bioeng 82:264–271
38. Suzuki T, Sato T, Kominami M (1994) A dense cell retention culture system using a stirred ceramic membrane reactor. Biotechnol Bioeng 44:1186–1192
39. Texeira H, Gonçalves MG, Rozés N, Ramos A, San Romão MV (2002) Lactobacillic acid accumulation in the plasma membrane of *Oenococcus oeni*: a response to ethanol stress. Microb Ecol 43:146–153
40. Tracey RP, Britz TJ (1989) Cellular fatty acid composition of *Leuconostoc oenos*. J Appl Bacteriol 66:445–456
41. Tracey RP, Britz TJ (1989) The effect of amino acids on malolactic fermentation by *Leuconostoc oenos*. J Appl Bacteriol 67:589–595
42. Van De Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E (2002) Stress responses in lactic acid bacteria. Antionie van Leeuwenhoek 82:187–216
43. Yasui T, Yoda K, Kamiya T (1995) Analysis of S-layer proteins of *Lactobacillus brevis*. FEMS Microbiol Let 133:181–186
44. Zhang D, Lovitt RW (2006) Performance assessment of malolactic fermenting bacteria *Oenococcus oeni* and *Lactobacillus brevis* in continuous culture. Appl Microbiol Biotechnol 69:658–664
45. Zhang D, Lovitt RW (2006) Review; strategies for enhanced malolactic fermentation in wine and cider maturation. J Chem Technol Biotechnol 81:1130–1140
46. Zhao Z, Chen L (2009) Dynamic analysis of lactic acid fermentation in membrane bioreactor. J Theor Biol 257:270–278