Biotechnol Lett (2014) 36:1303–1307
DOI 10.1007/s10529-014-1482-9

ORIGINAL RESEARCH PAPER

Homo-fermentative production of D-lactic acid by *Lactobacillus* sp. employing casein whey permeate as a raw feed-stock

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Received: 10 October 2013 / Accepted: 24 January 2014 / Published online: 22 February 2014

Abstract Casein whey permeate (CWP), a lactose-enriched dairy waste effluent, is a viable feed stock for the production of value-added products. Two lactic acid bacteria were cultivated in a synthetic casein whey permeate medium with or without pH control. *Lactobacillus lactis* ATCC 4797 produced D-lactic acid (DLA) at 12.5 g l^{-1} in a bioreactor. The values of Leudking–Piret model parameters suggested that lactate was a growth-associated product. Batch fermentation was also performed employing CWP (35 g lactose l^{-1}) with casein hydrolysate as a nitrogen supplement in a bioreactor. After 40 h, *L. lactis* produced 24.3 g lactic acid l^{-1} with an optical purity >98 %. Thus CWP may be regarded as a potential feed-stock for DLA production.

Keywords Casein whey permeate · D-Lactic acid · Homo-fermentative · *Lactobacillus lactis* · Optical purity

List of symbols

q_{DLA}	Specific DLA productivity (g DLA g^{-1} dry cell weight l^{-1})
α	Growth-associated product constant
β	Non-growth-associated product constant
μ	Specific growth rate (h^{-1})
$Y_{X/S}$	Biomass yield coefficient (g DCW g^{-1} lactose)
$Y_{P/S}$	Product yield coefficient (g DLA g^{-1} lactose)

Introduction

Casein whey permeate (CWP) is a byproduct generated by subjecting cheese whey to ultra-filtration to concentrate whey protein. Whey proteins are retained by the membrane, and smaller molecules, including lactose and mineral salts, pass through the membrane constituting the CWP. Liquid CWP is primarily composed of lactose (5 % w/v), water (93 % v/v) and minerals (0.53 % w/v), with minimal fat (0.36 % w/v) and protein (0.85 % w/v) (USDA 2004). Liquid CWP is generally concentrated to 12 % w/v dry matter using reverse osmosis. It is used in the food industry (ice cream, cake and milk derivatives), baby foods, and

Electronic supplementary material The online version of this article (doi:10.1007/s10529-014-1482-9) contains supplementary material, which is available to authorized users.

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dietetic products. However, its use is limited due to the "salty" taste and lactose intolerance by a minority of humans (Ozmihci and Kargi 2007). CWP is gaining attention as a potential feed-stock for production of several value-added bioactive compounds due to its high content of lactose (Barile et al. 2009). Fermentative conversion of lactose in whey permeate by native and genetically modified microbial strains yielded a variety of value-added products including poly hydroxy butyrate (Ahn et al. 2000), bacteriocins (Flores and Alegre 2001), sophorolipids (Otto et al. 1999).

Poly lactic acid (PLA) finds extensive application in food packaging (Yoshito et al. 1987) and the biomedical industry due to its mechanical and structural properties, which include biodegradability, high-strength, high-modulus, low toxicity and thermo plasticity (Södergård and Stolt 2002). Physical properties of PLA are determined by the isomeric composition of the lactic acid, e.g., blending poly D-lactic acid (PDLA) and poly L-lactic acid. Therefore, the biopolymer industry is showing a growing interest in fermentative production of optically pure isomers of lactic acid (Tashiro et al. 2011). Only a few reported studies are available on fermentative production of optically pure L-lactic acid from the whey permeate (Martin et al. 2010). No significant research study has focused on the fermentative production of DLA employing whey permeate as a feed stock.

This present study is the first of its kind to enumerate the technical feasibility of fermentative conversion of CWP to DLA. Batch cultivation of lactic acid bacteria (LAB) in synthetic casein whey permeate (SCWP) and CWP was performed under defined process conditions, and the DLA titer and its optical purity values were reported.

Materials and methods

Lactic acid bacteria (LAB) and media

Lactobacillus delbrueckii subsp. *bulgaricus* LB-12 (a commercial yoghurt strain donated by Chr Hansen's Laboratories) and *L. delbrueckii* subsp. *lactis* (ATCC 4797) were grown on the MRS medium containing 20 g glucose l^{-1}, 10 g peptone l^{-1}, 10 g beef extract l^{-1}, 5 g yeast extract l^{-1}, 5 g $CH_3COONa{\cdot}3H_2O$ l^{-1}, 2 g K_2HPO_4 l^{-1}, 2 g tri-ammonium citrate l^{-1}, 0.2 g $MgSO_4{\cdot}7H_2O$ l^{-1}, 0.05 g $MnSO_4{\cdot}4H_2O$ l^{-1} and 1 g Tween 80 l^{-1}. Synthetic casein whey permeate (SCWP) medium used for DLA production was containing 50 g lactose l^{-1}, 5 g casein hydrolysate l^{-1}, 3 g tri-ammonium citrate l^{-1}, 2 g Tween 80 l^{-1}, 0.5 g $CaCO_3$ l^{-1}, 0.508 g NaCl l^{-1}, 0.304 g $MgCl_2$ l^{-1}, 0.774 g KCl l^{-1}, 2 g K_2HPO_4 l^{-1}, and 0.05 g $MnSO_4$ l^{-1}. In all the shake-flask experiments, the initial pH of the medium was adjusted to 6.5 using 1 M HCl or 1 M NaOH, and the medium was sterilized at 121 °C at for 20 min. LAB used as inocula were grown in MRS medium at 37 °C in shaker cultures (200 rpm) until the culture reached its exponential growth phase ($\sim 10^6$ CFU ml^{-1}).

Casein whey permeate (CWP)

Casein whey was obtained from a cottage cheese factory located in Guwahati (India). It was ultra-filtered using a spiral wound membrane composed of hydrophilic polyamide, with a molecular mass cut off of 30,000 Da to obtain the whey protein concentrate and CWP.

D-lactic acid production

Shake-flask experiments for DLA production were performed with 100 ml medium in 250-ml baffled Erlenmeyer flasks with 5 % (v/v) inoculum in a shaking incubator at 37 °C and 150 rpm. DLA production was also performed in a 3 l bench-scale bioreactor (Biosole ADI 1025, Applikon Biotechnology, The Netherlands). The bioreactor operating parameters (pH, temperature, dissolved oxygen, stirring speed and air flow rate) were adopted from the values reported in the literature (Tango and Ghaly 1999; Ghasemi et al. 2009) and were maintained at 6.5, 37 °C, 20 %, 200 rpm and 0.2 vvm respectively. All the experiments were replicated and the mean values of the experimental data were obtained.

Analytical methods

Lactose concentration was determined by 3,5-dinitrosalicylic acid method. Casein concentration was estimated by Lowry's method. Casein hydrolysate was used as standard for obtaining the calibration curve. Cell growth was measured using a UV spectrophotometer and the absorbance was measured at

Table 1 Yield coefficient and net specific growth rate estimated for shake flask and bioreactor experiments

Lactobacillus sp.	Yield coefficients		Net specific growth rate (μ) (h^{-1})	Specific productivity (q_p) (g DLA g^{-1} DCW h^{-1})
	$Y_{X/S}$ (g DCW g^{-1} lactose)	$Y_{P/S}$ (g DLA g^{-1} lactose)		
L. bulgaricus LB-12 (Flask—SCWP)	0.05	0.04	0.08	0.10
L. bulgaricus LB-12 (Bioreactor—SCWP)	0.05	0.04	0.11	0.19
L. lactis ATCC 4797 (Flask—SCWP)	0.06	0.08	0.17	0.33
L. lactis ATCC 4797 (Bioreactor—SCWP)	0.15	0.16	0.38	0.43
L. lactis ATCC 4797 (Bioreactor—CWP)	0.22	0.49	0.31	0.38

600 nm. Dry cell weight (DCW) was determined by a calibration curve between DCW and absorbance measured at 600 nm. The presence of D-lactic acid was analyzed by a D-lactate dehydrogenase enzyme kit (K-DATE, Megazyme, Ireland) and L-lactic acid was analyzed using an L-lactate oxidase kit (K-LATE, Megazyme, Ireland). The optical purity of D-lactic acid (% w/v) was calculated as follows:

$$\text{Optical purity of D-lactic acid (\%)} = \frac{(\text{D-lacticacid} - \text{L-lacticacid})}{(\text{Total lactic acid})} \times 100 \quad (1)$$

Results and discussion

In this present study, the potential of lactose enriched CWP (35 g lactose l^{-1}) for DLA production has been assessed by two DLA producer strains *L. bulgaricus* and *L. lactis*. Both these bacterial strains are known to utilize lactose as a substrate for their growth in contrast to other reported DLA producer strains and therefore, have been employed in this study.

Initially, shake-flask experiments were performed for batch cultivation of *L. bulgaricus* and *L. lactis* ATCC 4797 in the SCWP medium. *L. lactis* exhibited a maximum DLA titer of 2.5 g lactic acid l^{-1} at the end of the exponential phase (35 h) and the biomass conc. was observed to be 0.8 g DCW l^{-1}. The biomass and product yield values for *L. lactis* were significantly higher than *L. bulgaricus* (Table 1). DLA production by *L. bulgaricus* and *L. lactis* resulted in gradual fall in pH >4, which suppressed the biomass growth in both the cases. Hence, automated pH control (bioreactor) during batch fermentation could enhance both DLA production and biomass growth.

Automated pH control during batch fermentation of SCWP by *L. bulgaricus* in the bioreactor resulted in 50 % increase of DLA titer compared with shake-flask cultivation (Fig. 1a). But, Table 1 indicates that the biomass and product yield values for *L. bulgaricus* cultivation in the shake flask and bioreactor doesn't exhibit a significant difference except a minor increase of the specific growth rate (0.11 h^{-1}). The presence of the initial high conc. of lactose and the lack of other essential nutrients indicates that *L. bulgaricus* may not be suitable for DLA production from SCWP.

Figure 1b substantiates *L. lactis* as a potential strain for DLA production due to the enhanced DLA production (12.5 g lactic acid l^{-1}) and was selected for further studies employing CWP. Moreover, the yield coefficients ($Y_{X/S} = 0.15$ g DCW g^{-1} lactose and $Y_{P/S} = 0.16$ g lactic acid g^{-1} lactose) and a net specific growth rate of 0.38 h^{-1} (Table 1) were observed to be significantly high compared with *L. bulgaricus*.

Luedeking–Piret model kinetics indicated that DLA produced by *L. lactis* is a growth associated product (further details: see Supplementary Material) and also suggests that high cell density cultivation would be an optimal fermentation strategy.

The composition of CWP was found to be similar with SCWP medium except that CWP had a 30 % lower concentration of lactose. In order to enable the comparative analysis of SCWP and CWP, the lactose conc. was maintained constant at 50 g lactose l^{-1} in both the media by supplementing the required

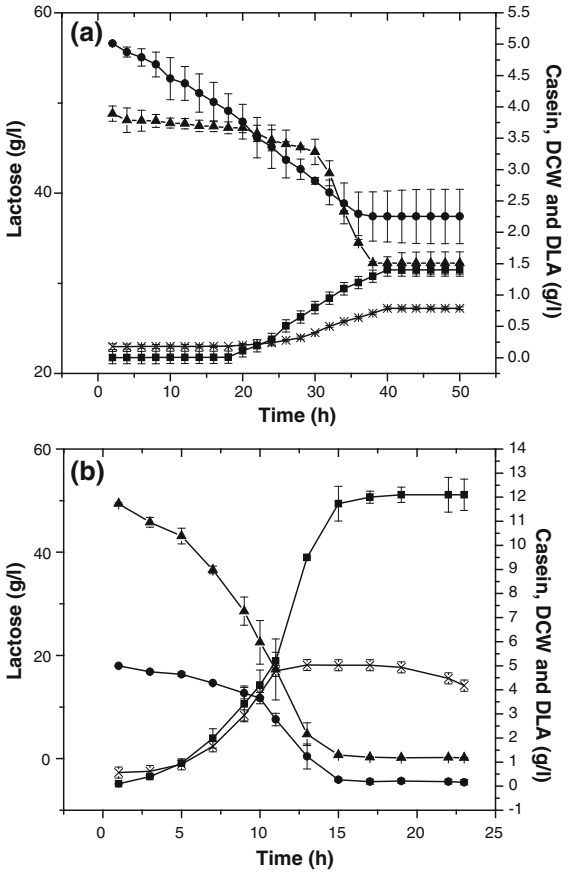

Fig. 1 Dynamic profile of DCW, lactose consumption, casein consumption and DLA production in batch fermentation of SCWP in a bioreactor (pH 6.5, 37 °C, 200 rpm). **a** *L. bulgaricus*, **b** *L. lactis* (*filled triangle* lactose, *filled circle* casein, *filled square* DLA, X-DCW)

nutrients. When *L. lactis* was cultured in bioreactor employing CWP as the fermentation medium the culture reached stationary growth phase by 35 h. Throughout the growth process, lactose consumption was observed to be very low (>10 %) and there was no significant casein consumption, yielding 1.2 g DLA l^{-1}. These results substantiates that casein present in CWP could not be utilized by *L. lactis* and was hypothesized that addition of readily available N-sources viz., casein hydro lysate (CH) or ammonium citrate (AC) may influence the growth of *L. lactis*.

To test this hypothesis, three independent shake-flask cultivation of *L. lactis* (5 % v/v of inoculum) were performed i.e. CWP, CWP supplemented with casein hydrolysate (CWP+CH) and CWP supplemented with ammonium citrate (CWP+AC) at 37 °C

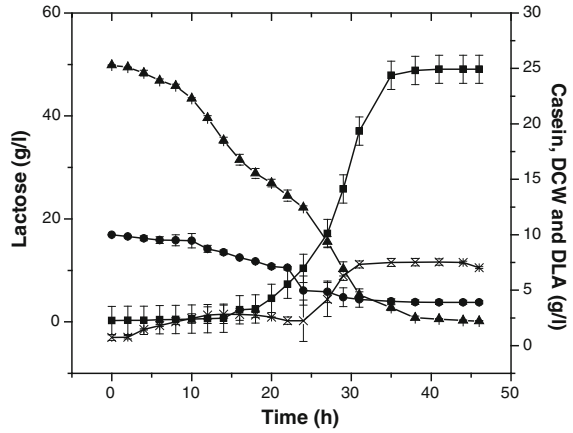

Fig. 2 Growth profile of *L. lactis* in bioreactor using CWP+CH as fermentation medium: *filled triangle* lactose, *filled circle* casein, *filled square* DLA, X-dry cell weight (DCW). By 47 h duration, 100 % consumption of lactose and casein hydrolysate was observed with the production of 24.3 g l^{-1} of DLA

and 200 rpm. From the experimental results, it was clearly evident that higher biomass conc., (3.08 g DCW l^{-1}) and lactose consumption was achieved in the CWP+CH medium (further details: see Supplementary Material). LAB exhibits limited biosynthetic capabilities and required free amino acids (or) protein hydrolysate to achieve high cell densities as well ferment lactose rapidly into lactic acid (Fitzpatrick and O'Keeffe 2001). Hence, CWP+CH was identified as the elite medium for DLA production in bioreactor. The dynamic profiles of biomass, DLA production, lactose and casein consumption observed during cultivation of *L. lactis* in CWP+CH medium were shown in Fig. 2. Interestingly, CWP+CH supported high cell density cultivation of *L. lactis* (11 g DCW l^{-1}) and a maximum DLA concentration of 24.3 g lactic acid l^{-1} was achieved in 40 h. Nearly, 100 % consumption of lactose and casein hydrolysate was observed by the end of the exponential growth phase. The yield coefficients ($Y_{X/S} = 0.22$ g DCW g^{-1} lactose and $Y_{P/S} = 0.49$ g lactic acid g^{-1} lactose) were also found to significantly high (Table 1).

The optical purity of final DLA extract was found to be 98.22 %, which ensures the suitability of DLA produced for PDLA synthesis. We envisage that further optimization of lactose, casein hydrolysate conc. in CWP+CH and fermentation conditions would further enhance DLA production. The results discussed in this present study enumerate the

feasibility of fermentative conversion of CWP to DLA and highlights its economical viability.

Conclusion

Technical feasibility of homo-lactic fermentative conversion of CWP by *L. lactis* to high value added DLA is successfully demonstrated in this study. *L. lactis* cultivation under controlled pH environment using CWP supplemented with casein hydrolysate resulted in a maximum DLA titer of 24.3 g lactic acid $l^{-1}$and the optical purity of DLA obtained was found to be >98 %, which is comparable with values reported in literature. Optimization of C/N ratio in CWP and achieving high cell density cultivation may enhance DLA yield, also reduce the fermentation time.

Acknowledgments Authors thank Dr. Chandan Das (Chemical Engineering Department, IIT Guwahati) for rendering valuable support to perform ultra-filtration of whey at his lab facility. Authors express their special thanks to Dr. Vimal Katiyar (Chemical Engineering Department, IIT Guwahati) for his valuable suggestions. The Authors thank Nitin More for critical evaluation of the text and grammatical corrections in the manuscript. This work was supported by Biotechnology Department, IIT Guwahati funded by Ministry of Human Resources and Development (MHRD), New Delhi, India.

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