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ORIGINAL PAPER

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Batch and repeated batch production of L(+)-lactic acid by *Enterococcus faecalis* RKY1 using wood hydrolyzate and corn steep liquor

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Abstract Lactic acid production was investigated for batch and repeated batch cultures of *Enterococcus faecalis* RKY1, using wood hydrolyzate and corn steep liquor. When wood hydrolyzate (equivalent to 50 g l^{-1} glucose) supplemented with 15–60 g l^{-1} corn steep liquor was used as a raw material for fermentation, up to 48.6 g l^{-1} of lactic acid was produced with, volumetric productivities ranging between 0.8 and 1.4 g l^{-1} h^{-1}. When a medium containing wood hydrolyzate and 15 g l^{-1} corn steep liquor was supplemented with 1.5 g l^{-1} yeast extract, we observed 1.9-fold and 1.6-fold increases in lactic acid productivity and cell growth, respectively. In this case, the nitrogen source cost for producing 1 kg lactic acid can be reduced to 23% of that for fermentation from wood hydrolyzate using 15 g l^{-1} yeast extract as a single nitrogen source. In addition, lactic acid productivity could be maximized by conducting a cell-recycle repeated batch culture of *E. faecalis* RKY1. The maximum productivity for this process was determined to be 4.0 g l^{-1} h^{-1}.

Keywords Corn steep liquor · *Enterococcus faecalis* · Lactic acid · Repeated batch · Wood hydrolyzate

Introduction

Lactic acid is widely used in the food, cosmetic, pharmaceutical, and chemical industries [1, 2]. As lactic acid exhibits excellent reactivity, attributable to its possession of both carboxyl (–COOH) and hydroxyl (–OH) groups, it can be readily converted into a host of potentially useful chemicals, including propylene oxide, propylene glycol, acrylic acid, 2,3-pentanedione, dilactide, and lactate ester [1, 3]. Recently, the scale of lactic acid production is considerably increasing, due to the increasing demand for biodegradable polylactic acid (PLA), whose principal producer is the Cargill-Dow LLC [4]. Although lactic acid can be manufactured by either petrochemical synthesis or biotechnological fermentation, the latter is widely preferred for current applications in the polymer industries. Whereas petrochemical synthesis generates racemic DL-lactic acid, optically active L(+)- or D(−)-lactic acid forms are produced via biotechnological fermentation from renewable resources [1, 2, 5, 6]. The production of optically pure lactic acid is crucial to polymer synthesis, because the physical properties of PLA rely heavily on the isomeric composition of the lactic acid used in the production process [6–8]. Optically pure L(+)-lactic acid can typically be polymerized into a highly crystalline polymer, which is suitable for the production of fibers and oriented films [9].

The biotechnological production of lactic acid requires cheap and renewable raw materials, as polymer producers and other industrial users require large quantities of lactic acid at a relatively low cost. Wood is currently considered as a fairly attractive raw material for use in its production [10–12]. However, it has become necessary, in recent years, to develop an alternative nitrogen source which is cheaper than the yeast extract traditionally employed in the cultivation of lactic acid bacteria, because production costs are highly dependent on the nitrogen source cost, as well as the carbon source cost [13]. Although agricultural waste products, including corn steep liquor which is a by-product of the corn steeping process, constitute an effective nutrient source for microbial cultivation, only a few studies have been conducted regarding the production of lactic acid from wood hydrolyzate and corn steep liquor.

In this study, we evaluated the production of lactic acid using wood hydrolyzate and corn steep liquor, in an

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attempt to develop a more economical method of lactic acid fermentation. Here, we report our findings regarding the effects of corn steep liquor concentration on lactic acid fermentation by *Enterococcus faecalis* RKY1 using wood hydrolyzate. We also report the improvement of lactic acid fermentation by the addition of trace amounts of yeast extract to the corn steep liquor medium. In addition, we evaluated a cell-recycle repeated batch process for producing lactic acid from wood hydrolyzate, with the objective of increasing lactic acid productivity.

Materials and methods

Microorganism

Enterococcus faecalis RKY1 was used throughout this study. This strain is stocked in the Korea Collection for Type Cultures (KCTC, Daejeon, Korea) under the collection number, KCTC 8890P [14]. Stock cultures were maintained at −20°C in 5-ml vials containing 50% (v/v) glycerol, until use in these experiments.

Preparation of wood hydrolyzate

Oak wood chips (2×4 mm), composed of 49.3% (w/w) cellulose, 25.9% (w/w) hemicellulose, and 21.7% (w/w) Klasson lignin, were kindly supplied by the Korea Institute of Energy Research (KIER, Daejeon, Korea). These chips were soaked with 0.5% (w/v) H_2SO_4 for 24 h, and then we steam exploded the swollen chips at 215°C for 5 min, in an 8-l exploder. The solid residues (450 g dry weight) obtained after steam explosion were then enzymatically hydrolyzed with Celluclast 1.5L (20 IU g^{-1} residue; Novozymes A/S, Bagsvaerd, Denmark) and Novozym 188 (30 IU g^{-1} residue; Novozymes A/S), in a shaking incubator (KMC-8480S; Vision Scientific Co., Daejeon, Korea), using a 2-l Erlenmeyer flask containing a 1.5-l working volume, at 50°C and 200 rpm for 2 days. The resulting wood hydrolyzate was then centrifuged at 15,540*g*, and the supernatant, which contained approximately 88 g l^{-1} glucose, was diluted with distilled water to the desired glucose concentration.

Culture media

The medium for cell growth or inoculum preparation contained wood hydrolyzate (equivalent to 30 g l^{-1} glucose), 10 g l^{-1} yeast extract, and 5 g l^{-1} K_2HPO_4. The medium used in the batch fermentations contained wood hydrolyzate (equivalent to 50 g l^{-1} glucose) and 15–60 g l^{-1} corn steep liquor. In order to improve lactic acid fermentation, a medium containing wood hydrolyzate (equivalent to 50 g l^{-1} glucose) and 15 g l^{-1} corn steep liquor was supplemented with 1.5 g l^{-1} yeast extract. This medium was also used in the cell-recycle repeated batch fermentations.

Batch fermentations

Batch lactic acid fermentations were performed in a 2.5-l fermentor (KF-2.5L; Kobiotech Co., Incheon, Korea), containing 1 l of fermentation medium. The culture pH was automatically maintained at 7.0 via the addition of 10 M NaOH. The culture temperature was maintained at 38°C, and the agitation speed was set at 200 rpm, in order to ensure thorough mixing of the fermentation broth. Samples were removed aseptically at regular intervals for further analysis.

Repeated batch fermentations

Repeated batch fermentations were performed in the same fermentor as the batch fermentations, but using a hollow-fiber module (SKUF-103-0830; SK Chemical Co., Suwon, Korea) for cell recycling. This module contained 100 hollow fiber membranes, each of which was 25 mm in inner diameter and 300 mm in length. The nominal molecular weight cut-off of the membranes was 30 kDa, and the effective surface area of the membranes was 0.06 m^2. The hollow fiber module was sterilized with 0.1 M NaOH and 200 ppm NaOCl for 12 h, followed by washing with sterile water until the pH inside the module reached 7.0. After the glucose in each batch was depleted, 900 ml of culture broth was withdrawn through the hollow fiber module, and an equal volume of fresh medium was fed into the fermentor.

Analytical methods

Cell growth was turbidimetrically measured by a spectrophotometer at 660 nm. These measurements were then converted to dry cell weights by calculation with a calibration curve which was relating optical density to dry cell weight. One unit of optical density corresponded to 0.8 g dry cell weight l^{-1}. Lactic acid was analyzed with a Waters HPLC (Millipore Co., Bedford, MA, USA), equipped with a Waters 486 tunable absorbance detector, which was set to 210 nm. An Aminex HPX-87H ion-exclusion column (300×7.8 mm; Bio-Rad, Hercules, CA, USA) was used with 5 mM H_2SO_4 as a mobile phase, at a flow rate of 0.6 ml min^{-1}, while the column temperature was maintained at 35°C. The quantity of L(+)-lactic acid present was determined with an enzymatic test kit (Sigma Co., St. Louis, MO, USA). The same test kit was used to quantify D(−)-lactic acid, after replacing the L(+)-lactate dehydrogenase with D(−)-lactate dehydrogenase (from *Leuconostoc mesenteroides*; Sigma Co.). The corn steep liquor employed in these experiments contained approximately 10% (w/w) lactic acid, composed of a roughly racemic mixture with similar amounts of L(+)- and D(−)-lactic acids. Therefore, we subtracted the lactic acid present in the corn steep liquor from the total lactic acid concentrations to obtain the results presented here. Glucose concentrations

were enzymatically measured via the glucose oxidase–peroxidase method, using a Glucose-E kit (YD Diagnostics, Seoul, Korea). All the analyses were conducted in triplicate, and mean values were reported.

Results and discussion

Enterococcus faecalis RKY1 was previously reported to be capable of producing high yields of succinic acid from fumaric acid and glycerol [14–17]. During this bioconversion process, glycerol was employed as a carbon source, in order to supply the fumaric acid with hydrogen. *E. faecalis* RKY1 was also determined to be capable of producing high yields of lactic acid from various carbohydrates, such as glucose, maltose, and fructose, via a homofermentative pathway [6, 12, 18, 19]. Since the RKY1 strain previously proved able to produce lactic acid from wood hydrolyzate and yeast extract [12], we attempted to develop a more efficient and economical process for the production of lactic acid from wood hydrolyzate and corn steep liquor.

Effects of corn steep concentrations on lactic acid production using wood hydrolyzate

In typical lactic acid fermentation methods, the raw material cost constitutes between 40 and 70% of the total production cost [1, 20]. It has been reported that the production of high yields of lactic acid from wood hydrolyzate and yeast extract could be accomplished via batch fermentation of *E. faecalis* RKY1 [12]. However, the development of an alternative nitrogen source is a prerequisite for the economical production of lactic acid from wood hydrolyzate, because yeast extract is a relatively expensive nitrogen source for industrial use. Therefore, in order to develop a more economical method for lactic acid fermentation, we evaluated the efficacy of fermenting wood hydrolyzate using corn steep liquor as a cheaper nitrogen source. In order to determine the influence of corn steep liquor concentration on lactic acid fermentation, we added corn steep liquor to wood hydrolyzate at an initial concentration of 15 g l^{-1}. The corn steep liquor concentration was then increased in increments of 15 g l^{-1}, to a final concentration of 60 g l^{-1}. Table 1 shows several fermentation parameters for batch lactic acid fermentations using these varying concentrations of corn steep liquor. As shown in Table 1, the dry cell weight of *E. faecalis* RKY1 increased directly with increased corn steep liquor supplementations up to 45 g l^{-1}, although little increase in dry cell weight was observed at higher concentrations. The lactic acid production rate was also stimulated by increased corn steep liquor concentration. Dry cell weight was observed to increase from 2.1 to 3.5 g l^{-1}, and lactic acid productivity was observed to increase from 0.8 to 1.4 g l^{-1} h^{-1}. The lactic acid produced was almost wholly L(+)-lactic acid, with an L(+)-isomer content of more than 96%. This result suggests that wood hydrolyzate and corn steep liquor, which are both cheap and renewable resources, should prove to be feasible as raw materials for the industrial production of L(+)-lactic acid.

When wood is employed as a raw material for lactic acid fermentation, it is necessary to pretreat and hydrolyze it to form fermentable sugars via steam explosion and enzymatic treatment, which may result in some extra cost. It was previously reported by von Sivers and Zacchi [21] that pretreatment and enzymatic hydrolysis for ethanol production from pine wood contributed approximately 30% to the total production cost. However, pure glucose (US \$0.5 kg^{-1}) is far more expensive than wood (US \$0.025 kg^{-1}) [22, 23]. Therefore, although the production of lactic acid from wood requires additional steps, it should still prove to be more economical than lactic acid production from pure glucose.

Enhancement of lactic acid fermentation by supplementation of trace amounts of yeast extract

In order to reduce the necessity for corn steep liquor supplementation, some of the corn steep liquor was replaced with trace amounts of yeast extract. Table 1 shows the kinetic parameters of lactic acid fermentations with wood hydrolyzate and corn steep liquor, as well as with the same medium supplemented with 1.5 g l^{-1} yeast extract. As can be seen in Table 1, when a medium containing wood hydrolyzate (equivalent to 50 g l^{-1} glucose) and 15.0 g l^{-1} corn steep liquor was supplemented with 1.5 g l^{-1} yeast extract, maximum dry cell weight and lactic acid productivity were obtained, at 3.4 g l^{-1} and 1.5 g l^{-1} h^{-1}, respectively. The maximum dry cell weight and productivity observed in this experiment were, respectively, 1.6 and 1.9 times higher than those values measured using the same medium, but without the 1.5 g l^{-1} yeast extract. This result indicates that fermentation efficiency (as evidenced by cell growth and productivity values) can be considerably improved by adding trace amounts of yeast extract to the cheap wood hydrolyzate and corn steep liquor medium.

Based on these results, the production of 1 kg lactic acid should require approximately 312.5 g corn steep liquor and 31.3 g yeast extract. If the cost of corn steep liquor is US \$0.4 kg^{-1}, and the cost of yeast extract is US \$3.0 kg^{-1} [22, 24], the nitrogen source cost for the production of 1 kg lactic acid would be approximately US \$0.22. This value corresponds to approximately 23% of the nitrogen source cost for lactic acid fermentation from wood hydrolyzate using 15 g l^{-1} yeast extract as a single nitrogen source (US \$0.94 kg lactic $acid^{-1}$). Table 2 shows several reports regarding lactic acid production from wood via the batch culture of several strains of lactic acid bacteria [10–12]. As can be seen in Table 2, *Lactobacillus delbrueckii* NRRL-B445 exhibited the highest productivity (2.7 g l^{-1} h^{-1}), but was also associated with the highest nitrogen source cost (US

Table 1 Effect of corn steep liquor concentrations on lactic acid production, L(+)-lactic acid content, yield, dry cell weight, and productivity in lactic acid fermentation using wood hydrolyzate as a carbon source by batch culture of *Enterococcus faecalis* RKY1

Corn steep liquor (g l^{-1})	Fermentation time (h)	Lactic acid (g l^{-1})	L(+)-Lactic acid content[a] (%)	Yield[b] (g g^{-1})	Dry cell weight (g l^{-1})	Productivity (g l^{-1} h^{-1})
15	60	47.0 ± 0.5	97.0 ± 1.0	0.96 ± 0.01	2.1 ± 0.1	0.8
30	48	48.5 ± 0.2	97.1 ± 0.9	0.98 ± 0.00	2.4 ± 0.2	1.0
45	36	46.3 ± 0.7	96.7 ± 0.5	0.94 ± 0.01	3.3 ± 0.1	1.3
60	36	48.6 ± 0.9	96.7 ± 0.2	0.97 ± 0.02	3.5 ± 0.1	1.4
15 (+1.5 g l^{-1} YE)[c]	33	48.0 ± 0.3	97.0 ± 0.6	0.98 ± 0.01	3.4 ± 0.2	1.5

Data are presented as mean values of three independent analyses, and ± denotes standard deviation among the replicates
[a] g-L(+)-lactic acid g-total lactic acid^{-1} × 100
[b] g-lactic acid produced g-glucose consumed^{-1}
[c] The medium containing 15 g l^{-1} corn steep liquor was supplemented with 1.5 g l^{-1} YE, and YE represents yeast extract

Table 2 Data reported on lactic acid production from wood by batch fermentation using several types of lactic acid bacteria

Microorganisms	Lactic acid (g l^{-1})	Yield (g g^{-1})	Productivity (g l^{-1} h^{-1})	Nitrogen source (g l^{-1})	Nitrogen source cost (US$ kg-lactic acid^{-1})	References
Lactobacillus delbrueckii NRRL-B445	108	0.67	0.9	YE 5 + PEP 10	0.60	[11]
Lactobacillus delbrueckii NRRL-B445	36.0	0.97	2.7	YE 5 + PEP 10	1.81	[12]
Enterococcus faecalis RKY1	93.0	0.93	1.7	YE 15	0.48	[22]
Enterococcus faecalis RKY1	48.6	0.97	1.4	CSL 60	0.49	This work
Enterococcus faecalis RKY1	48.0	0.98	1.5	CSL 15 + YE 1.5	0.22	This work

YE, PEP, and CSL represent yeast extract, peptone, and corn steep liquor, respectively

$1.81 kg lactic acid^{-1}) [11]. Although only a few studies have been conducted regarding lactic acid production from wood using the genus *Enterococcus*, *E. faecalis* RKY1 appears to be a good choice for the cheap production of lactic acid with wood hydrolyzate and corn steep liquor. In addition, the nitrogen source cost for the production of lactic acid can be substantially reduced by replacing some of the corn steep liquor with amounts of yeast extract as small as 1.5 g l^{-1}.

Repeated batch production of lactic acid using wood hydrolyzate and corn steep liquor

The cell-recycle repeated batch culture of *E. faecalis* RKY1 was carried out, in order to achieve higher volumetric productivity of lactic acid. The medium for all the repeated batch culture experiments comprised 15 g l^{-1} corn steep liquor, 1.5 g l^{-1} yeast extract, and wood hydrolyzate containing 50 g l^{-1} glucose. If the glucose concentration of each batch fell below 2 g l^{-1} during fermentation, subsequent batch cultures were initiated. In total, 11 of these batch experiments were conducted. As shown in Fig. 1, although the first batch run was completed after 33 h of fermentation, fermentation time became shorter with subsequent batch runs. The second batch run was completed after 24 h, the third batch after 18 h, the fourth and fifth after 15 h, and all the remaining batch runs were completed after 12 h of fermentation. During all of the repeated batch runs, we obtained lactic acid yields above 0.92 g g^{-1}, and L(+)-lactic acid contents above 96%. Both lactic acid productivity and dry cell weight gradually increased with repeated batch runs. The maximum productivity was 4.0 g l^{-1} h^{-1}, which was measured on the eighth batch run, and the maximum dry cell weight was 19.3 g l^{-1}, which was measured on the final batch run, respectively. To the best of our knowledge, although several reports have been published regarding batch production of lactic acid from wood [10–12] using lactic acid bacteria, only a small amount of research has focused on cell-recycle repeated batch production using wood and corn steep liquor as primary raw materials. In addition, the lactic acid productivity observed in this study, 4.0 g l^{-1} h^{-1}, appears to be much higher than any values reported thus far from other experiments on the production of lactic acid from wood.

In conclusion, although most studies concerning the production of lactic acid from wood have focused specifically on the genus *Lactobacillus*, *E. faecalis* RKY1 proved able to efficiently produce L(+)-lactic acid from renewable resources, such as wood and corn steep liquor. It has been demonstrated that the lactic acid thus produced was almost completely L(+)-lactic acid, showing an L(+)-isomer content of more than 96%. The nitrogen source cost for producing 1 kg lactic acid was reduced to approximately US $0.22, which is approximately 23% of the nitrogen source cost for lactic acid fermentation using 15 g l^{-1} yeast extract as a single nitrogen source. Lactic acid productivity was enhanced

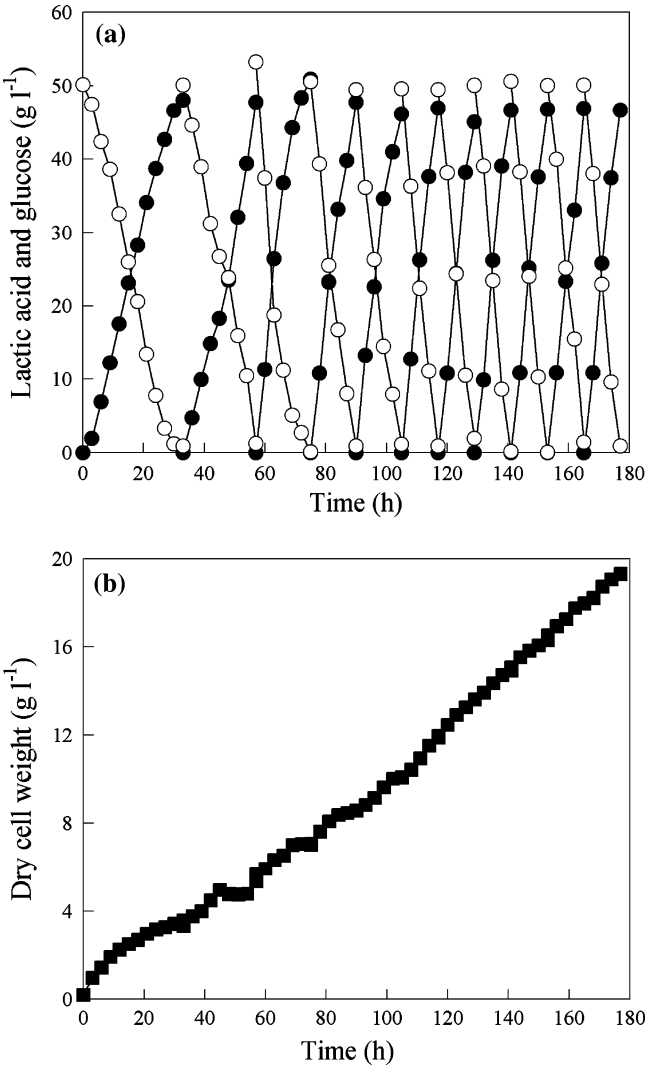

Fig. 1 Profiles of lactic acid production (**a**) and cell growth (**b**) during cell-recycle repeated batch lactic acid fermentation using wood hydrolyzate and corn steep liquor as the main raw materials by *Enterococcus faecalis* RKY1. *Filled circles* lactic acid, *open circles* glucose, and *filled squares* dry cell weight. All analyses were conducted in triplicate, and mean values are presented

substantially when cell-recycle repeated batch cultures of *E. faecalis* RKY1 were conducted, with a maximum productivity of 4.0 g l^{-1} h^{-1}.

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