Journal of the Taiwan Institute of Chemical Engineers 59 (2016) 262–266

Contents lists available at ScienceDirect

Journal of the Taiwan Institute of Chemical Engineers

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

Mg-modified zeolite as a carrier for *Lactobacillus rhamnosus* in L(+) lactic acid production on distillery wastewater

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ARTICLE INFO

Article history:
Received 8 May 2015
Revised 23 July 2015
Accepted 28 July 2015
Available online 17 August 2015

Keywords:
Lactic acid
Stillage
Zeolite
Probiotics feed additive
Lactobacillus rhamnosus ATCC 7469

ABSTRACT

Biorefinery concept in lactic acid (LA) fermentation based on parallel production of LA and biomass enriched feed on a waste substrate was investigated. In this study, nitrogen opulent liquid stillage was used as a media for repeated batch L(+) LA fermentation by probiotic strain *Lactobacillus rhamnosus* ATCC 7469 immobilized onto non-modified and Mg-modified zeolite. The highest LA concentration of 47.60 g/L, overall productivity of 1.41 g/L/h, a yield of 0.86 g/g and yield coefficient of 0.96 g/g were obtained in fermentation with utilization of immobilized biocatalyst onto Mg-modified zeolite and $Mg(OH)_2$ as a neutralizing agent. Very high number of 8×10^{10} CFU/g cells immobilized onto Mg-modified zeolite, proven beneficial effect of zeolite in animal health and probiotic characteristics of *L. rhamnosus* qualify solid remains after the fermentation as a high quality probiotic feed additive. High productivity of LA in the fermentation and valorization of the remains in animal nutrition could recommend this process which employs a simple modification of zeolite as a promising one for bio-based renewable LA production.

1. Introduction

Recent trends in lactic acid (LA) production obtrude aggressive cut down in production cost by utilization of cheap waste substrates and improved process performances for effective conversion of nutrients to LA. Demand for LA which is expected to reach 367,300 metric tons until 2017 [1] is mainly driven by wide application range of polydilactides (PLA). The cheaper production of LA could increase competitiveness of biodegradable polydilactides as alternative to petroleum-based plastics with negative environmental impact [2]. LA was mostly produced by batch fermentation on starch substrates with unavoidable supplementation of media with nitrogen sources [3]. However, high price of synthetic medium, competitiveness of starch substrates with food and high cost of nitrogen source supplementation reduce the sustainability of current LA production. Therefore, waste substrates and agro-food industry by-products are considered better substrates for bio-based LA production.

Bagasse-derived cellulose [2], cheese whey [4], broken rice, biowaste [5], corn stover [6] and other lignocellulosic hydrolysates have been recently studied as substrates for LA production. Also, immobilization of the production microorganism on membranes [7] and beads [8] was studied. Immobilization of cells offers significant advantages like higher cell densities, better stability of cells, enables recirculation of biomass and easier separation of bacterial biomass with higher overall productivity of the process [8].

With increased consumption of bioethanol as a fuel, amounts of distillery stillage, by-product of bioethanol production on various substrates, are rapidly growing. Depending on its origin, the stillage is a complex effluent with BOD_5 values in the range of 15–340 g/L [9], so it should be treated before its disposal into flows. The cost of treatment of the stillage significantly affects the sustainability and economy of bioethanol production. Stillage was previously used for the production of dried distillers' grains (DDG) or as a substrate for the production of methane, acetic acid, single cell protein, *etc.* [10]. In our previous studies, wasted bread stillage which is investigated in this paper has shown an adequate composition for the growth of lactic acid bacteria (LAB) and LA productivity attained a value of 1.41 g/L/h on the whole stillage in the process for parallel LA and high quality feed production [11,12]. Different carriers were studied for immobilization of LAB by adsorption: wood chips [13], ceramics [14], cotton fibers [15], plastic composite supports [16] and zeolite [17]. Zeolites are highly porous aluminosilicates with a wide range of applications and high adsorption capacity for different biopolymers [18]. Also, natural zeolite has been studied as a carrier for *Acinetobacter* sp. in wastewater treatment [19] and has shown high stability during the process. However, these previous studies were performed with natural zeolites, whilst commercial X type zeolite with well

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http://dx.doi.org/10.1016/j.jtice.2015.07.035
1876-1070/

defined basic structure and exchangeable sodium ions has been utilized in this study. It has been revealed that liquid stillage is short in content of Mg^{2+} ions which in particular have an important role in cell metabolism and could stimulate LA production [20,21,22].

In this work, we evaluated possibilities for zeolite modification with Mg^{2+} ions and its application as a carrier for *Lactobacillus rhamnosus* ATCC 7469 immobilization for LA production on liquid distillery stillage. Utilization of liquid fraction of the stillage for LA fermentation by immobilized biocatalyst enables separation and recirculation of biomass with easier recovery of LA, whilst solid fractions of the stillage could be valorized through feed production. Also, $Mg(OH)_2$ was evaluated as a neutralizing agent which served as additional source of Mg^{2+} ions.

2. Experimental

2.1. Liquid stillage preparation

The stillage remained after bioethanol production on wasted bread was obtained from Reahem Ethanol Plant (Reahem d.o.o., Srbobran, Serbia). After centrifugation (4500 rpm, 20 min, centrifuge: Sigma® model 2-16, Shropshire, UK) solid stillage fraction was separated and pH of the supernatant (liquid stillage) was adjusted to 6.5 with 30% NaOH (Sigma-Aldrich, USA). After adjustment, the liquid stillage was sterilized at 121 °C for 20 min. The concentration of reducing sugars in the sterile liquid stillage was set at approximately 50 g/L with addition of a sterile 70% glucose solution and used as a fermentation medium. The liquid stillage consisted of proteins (43.75% of dry matter), reducing sugar (24.30% dm), lipids (11.42% dm) and ash (14.49% dm) [23]. Also, it contained 155 mg/L of Mg, 1.34 mg/L of Mn, 210.55 mg/L of Ca, 3.02 mg/L of Fe, 3.78 mg/L of Zn, 398.02 mg/L of Na, 0.22 mg/L of Cu, and 0.06 mg/L of Co [23].

2.2. Preparation of non-modified and modified zeolite as a carrier for immobilization

Zeolite molecular sieves (type 13X, with exchangeable Na^+ ions, beads, 8–12 mesh) were purchased from Sigma Aldrich, Darmstadt, Germany. Before utilization it was powdered and washed twice with demineralized water. Average particle size was 4–7 μm (90%) with normal particle size distribution. Slightly modified procedure of Hrenović et al. [24] was used for exchange of Na^+ with Mg^{2+} ions. In brief, 10 g of powdered zeolite was washed with deionized water and transferred into the flask with 250 ml of 1 M $MgCl_2$ in order to allow exchange of Na^+ ions from zeolite with Mg^{2+} ions from the solution. This way prepared suspension was incubated for 48 h at 30 °C in an orbital shaker (200 rpm). After the modification, solid remains of modified zeolite were washed with deionized water until a negative chloride ion test with 1% silver nitrate solution was obtained. Then, prepared modified zeolite was dried and used as a carrier for immobilization of *L. rhamnosus* ATCC 7469.

2.3. Immobilization of L. rhamnosus ATCC 7469 onto non-modified zeolite and Mg-modified zeolite

L. rhamnosus ATCC 7469, a homofermentative L(+) LA producing strain, was obtained from American Type Culture Collection (ATCC, Rockville, USA). The culture was propagated at 37 °C in 200 ml of Man Rogosa Sharpe broth (MRS) with inoculum concentration of 10% (v/v) under anaerobic static conditions using Anaerocult® C bags (Merck KGaA, Darmstadt, Germany). After 16 h, the culture was centrifuged (10,000 rpm, 5 min, centrifuge: Sigma® model 2-16, Shropshire, UK), twice washed with sterile 0.8% (w/v) NaCl solution and used for immobilization onto powdered Na-zeolite or Mg-modified zeolite as described in study by Djukić-Vuković et al. [17]. This way prepared immobilized biocatalyst was used as an inoculum for fermentation.

2.4. Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) of samples

Washed samples of immobilized *L. rhamnosus* ATCC 7469 onto zeolite carrier were dried in vacuum dryer at 25 °C for 3 h. Dried samples were coated with Au–Pd alloy using a spatter coater. The morphology of the samples was studied by field emission scanning electron microscopy (FESEM) TESCAN Mira3 XMU at 20 kV. The chemical composition of samples was analyzed using an Energy Dispersive Spectrometer (EDS) Isis 3.2, with a SiLi X-ray detector (Oxford Instruments, UK) connected to a scanning electron microscope JEOL JSM-5800 and a computer multi-channel analyzer.

2.5. LA fermentation

All LA fermentations were performed as repeated batch cultures with recirculation of immobilized biomass, with shaking (90 rpm, KS 4000i control, IKA®, Werke GmbH & Co. KG, Staufen, Germany), at 41 °C. Inoculum size of 2% (w/v) of immobilized biomass was used in fermentations. The fermentations were performed in 500 ml flasks with 200 ml of the liquid stillage under microaerophilic conditions in a gas pack system. Maintaining of pH value at around 6.5 was performed by addition of NaOH or $Mg(OH)_2$, depending on the experiment, in 4 h intervals. After depletion of sugar from media below the concentration of 10 g/L, the fermentation media was centrifuged (1000 rpm, 5 min residual immobilized biomass was transferred into the fresh fermentation media after washing with sterile physiological solution. Four subsequent cycles were performed in both systems until the overall volumetric productivity decreased below 1.0 g/L/h.

2.6. Methods of analysis

The concentration of reducing sugars, calculated as glucose, was estimated by 3,5-dinitrosalicylic acid method [25]. Calibration curve was set at 505 nm using standard glucose solutions. LA concentration was determined by enzymatic method (L(+)/D(−) LA assay, Megazyme®, Wicklow, Ireland) after deproteinization of the sample. The number of viable *L. rhamnosus* ATCC 7469 cells was estimated using pour plate technique on MRS agar after detachment of cells from zeolite carrier by methodology reported by Djukić-Vuković et al. [17].

2.7. Statistical analysis

The experiments were done in triplicates. All values are expressed as means ± standard deviation. Mean values of treatments were compared by the analysis of variance (one-way ANOVA) followed by Tukey test for mean differences testing. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Immobilization of L. rhamnosus ATCC 7469 onto Na- and Mg-zeolite

In order to satisfy nutritional requirements of *L. rhamnosus* and increase the stability of *L. rhamnosus* biofilm, exchange of Na^+ ions in commercial zeolite with Mg^{2+} ions was performed and this way modified zeolite was studied as a carrier for *L. rhamnosus* ATCC 7469. In order to observe the stability of Mg-zeolite system, the surface of Mg-zeolite at the beginning and after three cycles of fermentation was examined by EDS and the results are presented in Table 1. During the fermentation, Mg^{2+} ions were gradually released from Mg-modified zeolite into the media and supported the growth of *L. rhamnosus*. At the beginning of the fermentation the content of Na^+ and Mg^{2+} ions was almost equal in Mg-modified zeolite. The values of the content of Mg^{2+} on the surface of Mg-modified zeolite (Table 1) in the first cycle are maximal achieved by the method used in this study and previously reported by Hrenović et al. [24]. After three cycles of repeated batch fermentation with using NaOH as neutralizing agent the difference between modified and non-modified zeolite