





Article

Use of Corn-Steep Water Effluent as a Promising Substrate for Lactic Acid Production by *Enterococcus faecium* Strain WH51-1

Mohamed T. Selim ¹, Salem S. Salem ¹, Amr Fouda ¹, Mamdouh S. El-Gamal ¹
and Mohamed Ali Abdel-Rahman ^{1,2,*}

¹ Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Cairo 11884, Egypt; mohamed.talaat@azhar.edu.eg (M.T.S.); salemsalahsalem@azhar.edu.eg (S.S.S.); amr_fh83@azhar.edu.eg (A.F.); mamdouh.elgamal@azhar.edu.eg (M.S.E.-G.)

² Al-Azhar Center for Fermentation Biotechnology and Applied Microbiology, Al-Azhar University, Cairo 11884, Egypt

* Correspondence: mohamedali@kyudai.jp or mohamedali@azhar.edu.eg

Abstract: Various challenges facing the industrial production of bio-based lactic acid (LA) such as cost of raw materials and nitrogen sources, as well as contamination risk by mesophilic and neutrophilic producers, should be overcome for the commercial production. This study aimed to investigate the feasibility of corn steep water (CSW) as a raw material for LA production using a newly thermo-alkali-tolerant lactic acid bacterium. The physicochemical characteristics of CSW were investigated. The high carbohydrates, proteins, amino acids, vitamins, essential elements, minerals, and non-protein nitrogenous compounds content confirmed that the CSW is a promising substrate for LA production. Out of 67 bacterial isolates, *Enterococcus faecium* WH51-1 was selected based on its tolerance to high temperatures and inhibitory compounds (sodium metabisulfate, sodium chloride, sodium acetate, and formic acid). Fermentation factors including sugar concentration, temperature, inoculum size, and neutralizing agents were optimized for LA production. Lactic acid concentration of about 44.6 g/L with a high yield (0.89 ± 0.02 g/g) was obtained using 60 g/L of CSW sugar, inoculum size 10% (v/v), 45 °C, and sodium hydroxide or calcium carbonate as a neutralizing agent. These results demonstrated the potential of strain WH51-1 for LA production using CSW effluent as raw material.

Keywords: corn steep water; lactic acid production; LAB; fermentation; *Enterococcus faecium*



Citation: Selim, M.T.; Salem, S.S.; Fouda, A.; El-Gamal, M.S.; Abdel-Rahman, M.A. Use of Corn-Steep Water Effluent as a Promising Substrate for Lactic Acid Production by *Enterococcus faecium* Strain WH51-1. *Fermentation* **2021**, *7*, 111. <https://doi.org/10.3390/fermentation7030111>

Academic Editors: Mohamed Koubaa and Diomi Mamma

Received: 31 May 2021

Accepted: 6 July 2021

Published: 8 July 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Lactic acid (LA) is one of the most extensively used chemicals because it is integrated into various applications such as food, textile, pharmaceutical, and chemical industries [1]. In addition, it is used as feedstock in the production of polylactic acid (PLA) [2], as an attractive biodegradable candidate, instead of petrochemical polymers [3]. LA can be synthesized either through chemical methods or biotechnological fermentation processes. The latter approach is preferred due to its ability to produce pure LA forms with the utilization of renewable substrates, whereas a racemic mixture of DL-LA is produced by chemical methods [4]. Almost all LA produced in the world is currently obtained by microbial fermentation processes [5,6].

The global market size for LA was valued at USD 2.7 billion in the year 2020 and is expected to expand at a compound annual growth rate (CAGR) of 8.0% from 2021 to 2028 (<https://www.grandviewresearch.com/> (Accessed on 31 May 2021)). Therefore, the development of cost-effective LA fermentation processes is worth being undertaken to meet the huge rising demand. The cost of raw material represents approximately 40–70% of the total production costs; therefore, using cheap raw materials, including agricultural and industrial residues instead of expensive pure sugars, is one of the most important key factors that determine the success of the fermentation process [2,7].

Corn steep water (CSW) is a major by-product obtained from the wet-milling industry [8,9]. The major objectives of wet milling are to remove soluble protein, soften the kernels, and loosen the starch in the endosperm by disrupting the endosperm protein matrix and the endosperm cells using sulfur dioxide and endogenous proteases [10,11]. Several factors affect the starch quality and yields, such as broilers, heat damage, shrunken kernels, and foreign materials [12,13]. Taking these factors into consideration increases the amount of starch and free sugars in the steep water effluent [10]. Therefore, the effluents of the corn steep industry contain high sugar and nutrients content (nitrogen, amino acids, trace elements, and vitamins) that stimulate bacterial growth in a variety of fermentation processes [14–17]. However, the addition of SO₂ and elevated steeping temperatures inhibit the growth of microorganisms during the steeping process [11]. Additionally, the cost of the extraction and purification of sugars from steeping water limits its effective utilization. Moreover, the CSW can be combined with gluten, fibrous materials, and solids to use as animal feed [18,19], or to be used as a cost-effective nitrogenous source for microbiological purposes instead of a high-cost one [20,21]. In previous studies, CSW has been used as nitrogen source supplementation for LA [6,22], enzymes [23], and ethanol production [24] at low concentrations. However, until this time, there has been no study demonstrating the use of CSW as a carbon and nitrogen source due to the existence of the high content of inhibitory compounds for microbial growth in the concentrated raw material.

Another challenge for the effective LA fermentation process is the contamination risk by mesophilic and neutral lactic acid bacterial producers, because the fermentative media contains high nutritional contents and, hence, is considered an encouraging environment for microbial growth [25,26]. Consequently, the use of alkali- and thermo-tolerant strains would be greatly useful, not only to minimize the contamination risk but also to facilitate the non-sterilized fermentation which saves more energy during the production process [27].

Therefore, this study aimed to investigate the feasibility of CSW as a sole raw material (carbon and nitrogen source) for LA production, utilizing stress-tolerant LA producers. To achieve this goal, a complete analysis of CSW effluent was investigated. In addition, isolation of stress-tolerant bacterial isolates was achieved and screened to select and identify the most potent LA producing bacterial strain. Finally, the optimal fermentation conditions for increase the LA production using CSW as raw material were studied.

2. Materials and Methods

2.1. Substrate Collection

The CSW effluent was collected from the outlet of a steeping tank in a commercial wet milling plant for maize products, on the 10th of Ramadan, in the El-Sharqia Governorate, Egypt. The samples were stored at -20 °C for further study.

2.2. Seed and Fermentative Media

Modified yeast extract dextrose (mYD) agar medium (containing, g/L: yeast extract, 5; CSW, 20 as a sole carbon source; Agar, 15; pH was adjusted at 9.0 using 5N NaOH solution) was used for bacterial isolation and screening. Seed culture for the fermentation experiment was prepared by adding 1.0 mL of glycerol stock of bacterial culture to 9.0 mL of mYD and incubated at 50 °C for 24 h for refreshment. After this preculture was prepared, 1.0 mL of the refreshment culture was inoculated into the test tube containing 9.0 mL of the same fermentative media and incubated at 50 °C for 24 h before being used as inoculum for the main cultures at 10% (*v/v*). The CSW sugar was added to fermentative media at a different concentration as described in each experiment. All chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.3. Isolation and Screening of Lactic Acid Producers

Twenty-one dairy products (16 powder and 5 liquid products) were collected from different governorates in Egypt. One gram, or one milliliter, from each collected sample was inoculated in a 125-mL Erlenmeyer flask containing 40 mL of mYD media containing

20 g/L CSW sugars and adjusted pH at 9.0 before incubation at 50 °C for 48 h. At the end of the incubation period, 100 µL of each flask was streaked on mYD agar medium and incubated at 50 °C for 24 h. The appeared bacterial colonies were re-streaked on the same media for purification. The purified bacterial isolates were grown on mYD agar medium supplemented with 5 g/L CaCO₃. Positive acid-producing isolates were tested for catalase activity using 3% H₂O₂ for the primary selection of lactic acid bacteria. All bacterial isolates were kept in glycerol (30%) at −80 °C.

Further screening was conducted on broth media supplemented with 20 g/L of CSW sugar and incubated at 50, 55, and 60 °C for 48 h. To investigate the effect of inhibitors on sugar consumption, LA concentration, and LA production of the selected isolates, different concentrations of sodium metabisulfate (1.0–8.0 g/L), sodium chloride (2.5–10%), sodium acetate (5–20 g/L), and formic acid (2.5–10 g/L) were separately supplemented to mYD medium containing CSW as a sole carbon source.

2.4. Characterization and Identification of Isolate WH51-1

The most potent LA bacterial producer, coded WH51-1, was subjected to molecular identification through the extraction of genomic DNA using a modified method according to Miller et al. [28]. The 16S rRNA gene fragment was amplified using genomic DNA as a template and the bacterial universal primers of 27f (5-GAGTTTGATCACTGGCTCAG-3) and 1492r (5-TACGGCTACCTTGTTACGACTT-3) in a polymerase chain reaction (PCR) [29]. The PCR mixture contained 1 × PCR buffer, 0.5 mM MgCl₂, 0.25 mM dNTP, 2.5 U Taq DNA polymerase (QIAGEN), 0.5 µM of each primer, and 1 µg of genomic DNA. The PCR was conducted in a DNA Engine Thermal Cycler (PTC-200, Bio-Rad, Hercules, CA, USA) at the following conditions: hot starting performed at 94 °C for 3 min, followed by 30 cycles of 94 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1 min, with an extension performed for 10 min at 72 °C. The PCR product was commercially sequenced using both primers at Sigma Company (Cairo, Egypt) by using an ABI 3730xl DNA sequencer. The sequence was then compared with those in the GenBank database through BLASTN. Multiple sequence alignment was then performed on 1290 bp of 16S rRNA gene fragments by the ClustalX 1.8 software package, and the phylogenetic tree was established with a neighbor-joining method of the Kimura 2-parameter model to calculate genetic distance as the transitional and transversional substitution rates using MEGA (version 6.1) software. The level of confidence for each branch at 1000 repeats was tested by bootstrap analysis. The chimeric formation was detected using the Uchime2_NCBI tool to find chimeras that are >3% diverged from the closest sequences. The obtained 16S rRNA gene sequence of the isolated strain was deposited in GenBank with the accession number MZ093371.

2.5. Optimization of Fermentation Conditions

All optimization experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL working volume. To determine the effect of CSW concentration on LA production by strain WH51-1, different concentrations (Viz., 20, 40, 60, 80, and 100 g/L) from the total sugar were tested in batch fermentations. The experiment was conducted at pH 9.0 and 50 °C for 48 h. The fermentation temperature was also investigated at 35, 40, 45, 50, and 55 °C using a medium containing 60 g/L CSW sugar, inoculum size 10% (v/v), at pH 9.0 for 48 h. To investigate the influence of inoculum size on LA production, different inocula sizes (2.5–12.5%, v/v) were inoculated into the fermentation medium after maintaining the previous factors. Additionally, different neutralizing agents (NaOH and CaCO₃) were investigated to control the pH during fermentation at pH 9.0. A 5N NaOH was added during the fermentation process while CaCO₃ was added into the media components at 0.5 g/g-carbon source concentration.

2.6. Analytical Methods

The corn steep water samples' conductivity and total dissolved salts were determined using a digital InoLab system (InoLab PH 720, WTW GmbH, Weilheim in Oberbayern, Ger-

many), while pH was estimated by InoLab pH 720, (WTW GmbH, Weilheim in Oberbayern, Germany). Dissolved oxygen was determined using a digital WTW GmbH analyzer (model oxi197i). Chemical oxygen demand (COD) was measured using a COD Reactor (Model HACH, Loveland, CO, USA), whereas biological oxygen demand (BOD) was measured by adding standard microbial and air saturation for a 5-day incubation at 25 °C using a BOD Incubator (Velp R 10300141, Usmate Velate, Italy), followed by the determination of remaining oxygen [30].

Seventeen essential elements were determined by an Inductive Coupled Plasma Mass Spectrometer (ICP-MS) (model, Triple Quad 8800 G3663A, Stevens Creek Blvd. Santa Clara, CA, USA). The sample extract was clarified by centrifugation at 13,000 rpm for 15 min, and the supernatant was filtered with a 0.22 µm filter then acidified by using Nitric acid 69% with ratio (1%). Stock standard solutions were prepared by a single element or multi-elemental traceable standard solutions in acid matrices prepared specifically for plasma mass spectrometric analysis.

Protein content was estimated by the Lowry method [31], while the total sugars are estimated using the phenol sulfuric acid method [32]. Lactic acid analysis was performed using the method as described by Barker and Summerson [33] and measured at 570 nm.

Amino acids were quantified by a Sykam Amino Acid Analyzer (Sykam GmbH, Eresing, Germany) equipped with a Solvent Delivery System S 2100 (Quaternary pump with flow range 0.01 to 10.00 mL/min and maximum pressure up to 400 bar), Autosampler S 5200, Amino Acid Reaction Module S4300 (with built-in dual filter photometer between 440 and 570 nm with constant signal output and signal summary option), and Refrigerated Reagent Organizer S 4130 (Laird Thermal Systems, Durham, NC, USA). For standard preparation, a stock solution containing aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, ammonia, and arginine was used. All amino acid concentrations were 2.5 µMol/mL (except cystine which was 1.25 µMol/mL), diluted with 60 µL in a 1.5 mL vial with a sample dilution buffer, then filtered using a 0.22 µm syringe filter, then 100 µL was injected. For the sample preparation, 1.0 g of each sample was mixed with 5.0 mL hexane. The mixture was allowed to macerate for 24 h. Then, the mixture was filtered on Whatman no. 1 filter paper and the residue was transferred into a test tube where it was incubated in an oven with 10 mL 6N HCl for 24 h at 110 °C. After the incubation, the sample was filtered on Whatman no. 1 filter paper, evaporated on a rotary evaporator, and dissolved completely in a 3.0 mL dilution buffer. From this solution, the first dilution was prepared by diluting 100 µL to 1.0 mL dilution buffer, from which 100 µL was further diluted to a 1.0 mL dilution buffer, and filtered using a 0.22 µm syringe filter, then 100 µL was injected.

On the other hand, total vitamins were estimated by an HPLC system (Waters 2690 Separation Module) equipped with a Waters 996 photodiode array detector (Waters, Milford, MA, USA). For a standard preparation of water-soluble vitamins, 10 mg of 7 water-soluble vitamin reference standards (ascorbic acid, thiamine HCl, riboflavin, nicotinic acid, nicotinamide, pyridoxine HCl, and folic acid) was dissolved in 10 mL 0.05 M NaOH, diluted to a concentration of 100 µg/mL and filtered with a 0.22 µm syringe filter, then 10 µL was injected. A preparation of a stock solution of fat-soluble vitamins (vitamin E, D, and A) in 5.0 mL methanol was then diluted to obtain concentrations of 806.25 IU/mL vitamin A, 114 µg/mL vitamin E, and 400 IU vitamin D3, and filtrated using a 0.22 µm syringe filter before injection of 10 µL. The sample extract was filtered using a 0.22 µm syringe filter, then 10 µL was injected.

For estimation of the non-protein nitrogenous compound in CSW, a sample extract was clarified by centrifugation at 13,000 rpm for 15 min, and the supernatant was filtered with a 0.22 µm filter, then 10 µL was injected directly into a HPLC analyzer (Waters, Milford, MA, USA) equipped with a C18 column (3.9 × 150 mm) and a fluorescent detector, with 250 and 395 nm wavelengths of excitation and emission.

For fermentation experiments, bacterial growth was measured using the total viable count method. One mL of the fermentation medium was serially diluted and inoculated

on dishes containing plate count agar media. Consumed sugar was analyzed by a phenol-sulphuric acid method using glucose as the standard [32]. Intermittent samples were taken and centrifuged at 6000 rpm for 10 min to estimate LA concentration using the Barker and Summerson method [33]. The LA yield (g/g) based on consumed sugars was calculated as the ratio of LA (g/L) to CSW sugar (g/L). LA productivity (P_{LA} , g/L/h) is the ratio of LA produced to the time of the fermentation process. Maximum LA productivity (g/L/h) was determined by the difference between LA concentrations of two respective samples divided by the time difference.

3. Results and Discussion

3.1. Characterization of CSW as a Promising Substrate

3.1.1. Physicochemical Characteristics of CSW

The physicochemical characteristics of the CSW effluent are represented in Table 1. The effluent is viscous, with a deep brown color. The pH of CSW was 4.5 ± 0.2 . The TDS, COD, and BOD were 6452 mg/L, 78 mg/L, and 23 g/L, respectively. Besides this, CSW contains a high sugar content (250 g/L), high amino acid content (10.7 g/L), various water-soluble vitamins (14.5 g/L), and mineral ions that make it a promising substrate for LA fermentation.

Table 1. Physicochemical characterization and inorganic ions content of CSW used in the current study.

Parameters	Results
Form	Liquid
Appearance	Viscous
Color	Deep brown
Odor	Rottenness
pH	4.5 ± 0.2
Temperature (°C)	39.0 ± 1.1
Conductivity ($\mu\text{S}/\text{cm}$)	$11,400 \pm 0.42$
TDS (mg/L)	6452 ± 1.2
Dissolved oxygen (mg/L)	0.890 ± 0.04
COD (mg/L)	78.0 ± 3.4
BOD ₅ (g/L)	23.0 ± 1.4
Total Carbohydrates (g/L)	250.4 ± 1.3
Lactic acid (g/L)	3.41 ± 0.87
Total Protein (g/L)	11.3 ± 1.4
Total water-soluble vitamins (mg in 100 mL)	145.5
Total fat-soluble vitamins (mg in 100 mL)	0.0
Total amino acid (g/L)	10.7
Total non-protein nitrogenous components ($\mu\text{g}/\text{mL}$)	34.8
Inorganic Ions content (mg/kg of CSW)	
Aluminum	3.48
Boron	3.85
Barium	0.374
Calcium	132.6
Cadmium	<0.0006
Cobalt	<0.001
Chromium	0.235
Copper	0.221
Iron	2.84
Magnesium	392.65
Manganese	3.734
Molybdenum	0.2205
Nickel	0.4315
Lead	0.6125
Vanadium	<0.01
Zinc	29.21
Phosphorus	4515.5

The CSW not only contains low molecular weight substances which are leached out of the corn grain, but it also contains a large number of substances derived from the degradation and fermentative conversion of proteins, carbohydrates, and nucleic acids during steeping. Steep water is primarily handled by evaporation to a concentrated thick liquor that is a complex mixture of carbohydrates, amino acids, peptides, organic compounds, heavy metals, inorganic ions, and myo-inositol phosphates [34]. Watson [35] reported that the bacterial activities and any unconverted starch in the steep water increase the color formation in the evaporators due to non-enzymatic browning.

Hull et al. [34] reported that the steeping under acidic conditions is due in part to the initial presence of SO₂ and, later, lactic acid, a major fermentation product. Typical corn wet milling effluent contains various nutrients, including 250–300 mg/L of total soluble solids (TSS) [36]. Hence, if TSS is recovered from steep water, it can be used in animal feed after drying and mixing with other fibrous milling byproducts [36]. Unfortunately, since the steep water effluent is rather high in TSS, its COD and 5-day biological oxygen demand (BOD₅) is high [36]. Data represented in Table 1 show that the total carbohydrate of CSW is 250.44 ± 1.3 g/L. These high levels of sugars with other nutrients in CSW can stimulate the growth of microorganisms and, hence, lead to high levels of lactic acid production [37]. However, the addition of SO₂ and elevated temperatures can inhibit microbial growth during the steeping process [11]. Thus, the existence of high sugars with other nutrients would make these waste materials promising substrates for LA fermentation by suitable, potent microorganisms.

3.1.2. Inorganic Ions Content of CSW

Data represented in Table 1 reveal that phosphorus was the most prevalent ion present in CSW, with a value of 4515.5 mg/kg. Data also show that magnesium, calcium, and zinc existed in CSW at a concentration of 392.65, 132.65, and 29.2 mg/kg, respectively. Boron, manganese, aluminum, and iron were found at a slight concentration that ranges from (3.8–2.8 mg/kg). However, cadmium, cobalt, and vanadium were not detectable in CSW under study.

The CSW is not only rich in organic nutrients but also contains trace nutrients such as vitamins and metal ions [38,39]. Hull et al. [34] reported that the presence of inorganic phosphate in CSW is usually due to the de-phosphorylation of inositol phosphate isomers (InsPs) by phosphatase enzymes. Liggett et al. [38] reported that the presence of calcium, iron, magnesium, phosphorus, and potassium are at high concentrations in CSW compared to other elements. Hofer et al. [14] reported that the trace elements that exist in CSW are mostly bound to proteins or other molecules.

3.1.3. Amino Acid Analysis of CSW

Several earlier published studies reported the effectiveness of LAB to metabolize all amino acids, but this efficacy differs greatly between species [40–43]. Therefore, the analysis of amino acids in CSW would help to study their role in the growth of LAB. Data shown in Table 2 reveal that the CSW was rich in different amino acids (aspartic acid, threonine, serine, glutamic acid, proline, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine) and ammonium as had previously been reported [44,45]. Proline and glutamic acid were the most prevalent amino acids in CSW that reach 1.071 and 1.656 g/L respectively.

The results in this study confirmed that CSW is a naturally complex medium that is suitable for the growth of most LAB isolates. Wright [46] reported that the majority of the amino acid content that exists in CSW is originated from corn rather than from fermentation products. Because of the nutritional fastidiousness of LAB, they are unable to grow unless certain amino acids are supplied in the medium [5]. The high proline content is beneficial for microbial fermentation processes. Proline can stimulate the growth of some species of LAB and may be involved in osmoregulation, as previously proven [47]. Xu et al. [48] reported that proline, at a concentration of 1.0 g/L in culture media, can be

absorbed and accumulated in the cell, and hence the cell growth and pyruvate concentration improve by 59.0 and 22.1%, respectively. Tian et al. [49] reported that proline addition could protect *Lactocaseibacillus paracasei* under hyperosmotic stress, because it contributes to membrane stabilization, co-metabolizes as a carbon or nitrogen source for cell growth, or imports and accumulates in the cells as a compatible solute to counteract hyperosmotic stress. Tian et al. [49] reported that *Lactocaseibacillus paracasei* could import proline from the extracellular environment for improved sugar consumption and L-LA production.

Table 2. Total amino acid composition of CSW used in the current study.

NO.	Amino Acid	Retention Time (min)	Concentration (g/L)
1	Aspartic acid	8.40	0.766
2	Threonine	10.6	0.430
3	Serine	11.4	0.520
4	Glutamic acid	13.2	1.656
5	Proline	15.4	1.071
6	Glycine	19.4	0.580
7	Alanine	20.9	0.878
8	Cystine	22.0	0.180
9	Valine	22.9	0.556
10	Methionine	25.0	0.203
11	Isoleucine	27.2	0.303
12	Leucine	28.6	0.910
13	Tyrosine	31.0	0.210
14	Phenylalanine	32.3	0.331
15	Histidine	35.0	0.431
16	Lysine	39.3	0.527
17	Arginine	42.7	0.726

Glutathione amino acids, such as glutamic, glycine, and cystine, protect LAB against oxidative stress, osmotic stress, and acid stress [50]. Glutamate, glutamine, and arginine improve the acid resistance of LAB [45]. Additionally, arginine is an essential amino acid and a nitrogenous precursor of nitric oxide (NO) that is generated by NO synthase [51]. Lysine is an essential component of the peptidoglycan cell wall of most Gram-positive bacteria [52]. Qiao et al. [44] reported that aspartate was involved in the synthesis of lysine, indicating that lysine was found at an insufficient level, especially from the end-exponential growth phase to the stationary phase. Moreover, alanine metabolism plays an important role in the synthesis and defense of the cell wall [53]. Hofer et al. [14] found that the main amino acids in CSW are arginine, alanine, and glutamic acid with values of 44.3, 35.7 and 42.0 mg/g respectively. Liggett et al. [38] reported that over 95% of the total nitrogen in steep liquor, after hydrolysis, is accounted for by ammonia and amino acids.

3.1.4. Analysis of Fat-Soluble and Water-Soluble Vitamins in CSW

Vitamins are important growth factors that need to be added to the medium. They play an important role in the growth of microbial strains [54], so it was important to analyze the fat-soluble and water-soluble vitamins that exist in the CSW used in the current study. Data show the absence of fat-soluble vitamins (vitamins E, D, A) in CSW, whereas water-soluble vitamins including riboflavin, nicotinic acid, nicotinamide, and pyridoxine HCL at concentrations of 0.143, 106.31, 4.59, and 34.47 mg/100 mL, respectively, were detected in CSW effluent (Figure 1), while others including ascorbic acid, folic acid, and thiamine HCL were not detected.

The presence of nicotinic acid and riboflavin in CSW are important as reducing agents in the form of NADH/NAD and FADH/FAD, respectively, which are essential for energy generation in microbial cells [55]. Pyridoxine is a precursor of pyridoxal-5'-phosphate (PLP), which is involved in many reactions of amino acid metabolism [56]. The absence of folic acid may be due to the bad solubility of some B-vitamins in water; in addition, they compose

of just a small fraction of CSW [56]. Moreover, the absence of biotin was favorable for LA fermentation, as it acts as an inhibitor of some LAB [57]. The absence of thiamine in CSW used in the current study was compatible with Wright [46], who found that CSW contains considerable amounts of B-complex vitamins, except thiamine, which is usually low or absent. This phenomenon can be attributed to the hydrolysis of thiamine by SO₂ during the steeping process. Hofer et al. [14] also found that CSW contains water-soluble vitamins at different concentrations. Tanner et al. [58] found that CSW contains riboflavin, niacin, pantothenic acid, pyridoxine, and biotin at a concentration of 5, 819, 23.8, 19.1, and 0.125 µg/g, respectively. The differences between the values among published studies maybe due to actual differences in compositions and viscosity/dilution of effluent [59].

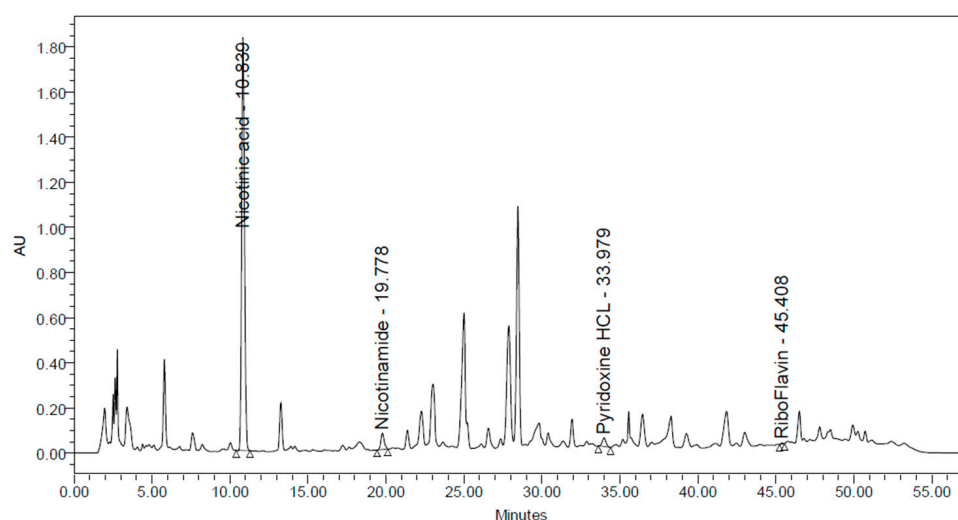


Figure 1. HPLC analysis of water-soluble vitamins in corn steep water obtained in the present study.

3.1.5. Analysis of Non-Protein Nitrogenous Components of CSW

The quantities of non-protein nitrogenous substances in CSW and their identification are critical steps because these substances can be added to fermentation media as high nutritional supplements. To achieve this goal, HPLC analysis of CSW confirmed the presence of several non-protein nitrogenous components as ethanolamine, ornithine, citrulline, taurine, γ -aminobutyric acid with a concentration of 5.12, 6.33, 7.41, 13.63, and 2.36 µg/mL, respectively (Table 3). From our data, taurine was the most prevalent non-protein nitrogenous component in CSW. Daniels [60] reported that taurine acts as a bio-stimulant in CSW. Moreover, taurine is known as a sole source of sulfur for the growth of aerobic microorganisms [61], as well as also being considered the main source of energy for many aerobic bacteria [62]. Denger et al. [63] reported that taurine nitrogen has been confirmed as the sole source of nitrogen for the growth of two *Rhodococcus* spp. in the presence of excess carbon sources. Clifford et al. [64] showed that taurine degradation can also be a significant source of other metabolically important S and N species (sulfate, sulfide, bisulfide, thiosulfate, ammonium, alanine, and other organo-sulfonates). Hull et al. [34] also found non-protein nitrogenous compounds such as γ -aminobutyric acid in CSW. Various non-protein nitrogenous components, such as ammonia, purine and pyrimidine derivatives, choline, and trigonelline were also detected in CSW [59]. Metabolic transformation of low molecular weight compounds occurs during steeping. For example, γ -aminobutyric acid is present in corn grain in low levels, whereas it is found free in steep water in high amounts. In contrast, the concentration of free glutamic acid is negligible in steep water, although this amino acid is in high concentrations in endosperm proteins and steep water proteins. Free glutamic acid is enzymatically de-carboxylated to γ -aminobutyric acid during steeping [65].

Table 3. HPLC analysis of non-protein nitrogenous compounds in CSW used in the current study.

Compound	Retention Time (min)	Concentration (µg/mL)
Ethanolamine	4.8	5.12
Ornithine	6.0	6.33
Citrulline	8.0	7.41
Taurine	9.0	13.6
γ-Aminobutyric acid	10.0	2.36

Citrulline is a non-protein amino acid and is represented as the main precursor for arginine [66]. Hwang and Lee [67] found that LAB has an arginine deaminase pathway that is involved in producing citrulline and ornithine from arginine, so we thought that presence of citrulline and ornithine in the CSW under study may have been due to the arginine deaminase pathway, and that this enhanced the bacterial growth [67]. Citrulline also plays an important metabolic intermediate in the urea cycle [68]. Citrulline could protect *Streptococcus pyogenes* from acid stress via the arginine deiminase pathway [69]. It also can act as a compatible solute against osmotic stress [70]. According to the previous analysis, it can be concluded that CSW is an ideal low-cost raw material for LA production by a suitable microbial producer.

3.2. Isolation and Screening of the Most Potent Lactic Acid Producers

To overcome the associated problems with LA fermentation, including substrate cost and contamination risk [25], isolation of LAB from 21 dairy products was achieved at thermo-alkaline conditions (pH 9 and 50 °C) using CSW-containing media. Out of 67 bacterial isolates, only 15 isolates had the efficacy to produce LA with a high yield (greater than 0.88 g/g). A second screening test was performed to determine the ability of these bacterial isolates to produce LA under higher temperatures at 55 °C and 60 °C. Out of these 15 bacterial isolates, only six isolates were able to tolerate 60 °C and could produce more than 10 g/L of lactic acid. Out of the previous six bacterial isolates, two isolates (coded as SSD16-1 and WH51-1) were selected as the highest producers and used to investigate the inhibitory compounds that existed in the effluent substrates on them. These isolates were catalase-negative and therefore preliminarily identified as lactic bacteria.

3.3. Effect of Inhibitors on LA Production by the Most Potent Isolates

To investigate the effect of inhibitors on sugar consumption and LA fermentation with the most potent selected bacterial isolates, different concentrations of sodium metabisulfate (1–8 g/L), sodium chloride (2.5–10, % w/v), sodium acetate (5–20 g/L), and formic acid (2.5–10 g/L) were separately supplemented to fermentation medium that contained CSW as the sole carbon source (Table 4).

Both isolates showed high LA yields ranging 0.77–0.87 g/g and 0.78–0.90 g/g in the presence of 1.0–4.0 g/L of sodium metabisulfate for SSD16-1 and WH51-1, respectively, while the productivity of LA was decreased to 0.25 and 0.31 g/g at 8.0 g/L of sodium metabisulfate for isolate SSD16-1 and WH51-1, respectively.

Similarly, a high LA yield (ranged 0.73–0.86 g/g) was achieved in the presence of 2.5–7.5% of sodium chloride for both isolates, while a lower yield (0.27 and 0.31 g/g) was achieved with 10% of NaCl for both isolates (SSD16-1 and WH51-1, respectively).

On the other hand, isolates SSD16-1 and WH51-1 showed a LA yield with values ranging 0.60–0.82 g/g and 0.57–0.87 g/g in the presence of 5.0–15.0 g/L of sodium acetate, respectively, while the productivity decreased to 0.26 and 0.36 g/g at 20.0 g/L of sodium acetate, respectively.

Lactic acid yield at 0.53–0.78 g/g and 0.62–0.72 g/g were achieved in the presence of 2.5–7.5 g/L of formic acid for isolates SSD16-1 and WH51-1, respectively, while lower LA yields at 0.25 g/g were achieved with 10 g/L formic acids for both isolates.

Table 4. Effect of different inhibitory compounds on sugar consumption, LA concentration, and LA yield by the two most potent selected isolates.

Bacterial Isolate	Sodium Metabisulfate				Sodium Chloride				Sodium Acetate				Formic Acid			
	Inhibitor Conc. (g/L)	Consumed Sugar (g/L)	LA Conc. (g/L) ^a	Y _{LA} (g/g) ^b	Inhibitor Conc. (%)	Consumed Sugar (g/L)	LA Conc. (g/L) ^a	Y _{LA} (g/g) ^b	Inhibitor Conc. (g/L)	Consumed Sugar (g/L)	LA Conc. (g/L) ^a	Y _{LA} (g/g) ^b	Inhibitor Conc. (g/L)	Consumed Sugar (g/L)	LA Conc. (g/L) ^a	Y _{LA} (g/g) ^b
SSD 16-1	1	15.09	13.12	0.87	2.5	14.09	12.12	0.86	5	14.84	12.14	0.82	2.5	14.41	11.21	0.78
	2	15.98	9.78	0.61	5	13.98	8.15	0.58	10	13.89	8.45	0.61	5	12.79	7.98	0.62
	4	6.01	4.65	0.77	7.5	5.81	4.22	0.73	15	8.33	5.02	0.60	7.5	9.22	4.92	0.53
	8	3.55	0.89	0.25	10	4.95	1.34	0.27	20	2.09	0.55	0.26	10	1.29	0.32	0.25
WH 51-1	1	17.69	15.89	0.90	2.5	16.59	14.29	0.86	5	10.99	9.56	0.87	2.5	15.99	11.56	0.72
	2	16.2	12.45	0.77	5	15.2	11.25	0.74	10	11.23	6.85	0.61	5	11.03	7.45	0.68
	4	6.25	4.85	0.78	7.5	5.25	3.85	0.73	15	6.81	3.85	0.57	7.5	6.51	4.05	0.62
	8	3.89	1.21	0.31	10	3.89	1.21	0.31	20	3.97	1.41	0.36	10	3.89	0.99	0.25

^a Maximum lactic acid concentration after 48 h, ^b Lactic acid yield.

According to the obtained results, isolate WH51-1 was considered the most potent isolate exhibiting greater stability under high-stress/inhibitory compounds that might exist in the waste materials under study, and therefore was selected for characterization and further studies. This isolate was identified as *Enterococcus faecium* WH51-1 using a 16S rRNA sequence (Figure 2).

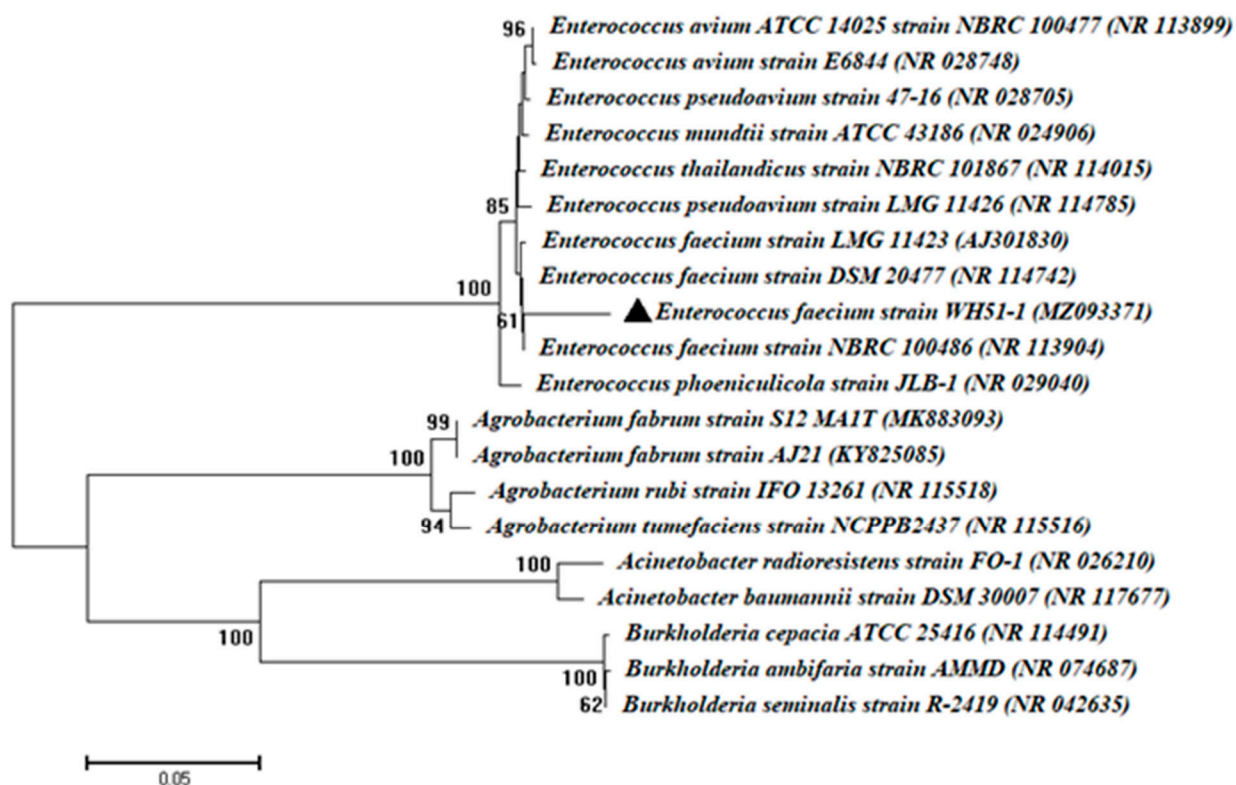


Figure 2. Phylogenetic analysis of 16S rRNA sequences of the bacterial isolate with the sequences from NCBI. The symbol ▲ refers to 16S rRNA gene fragments retrieved from this study. The analysis was conducted with MEGA 6 using the neighbor-joining method.

In the present study, the selected strain WH51-1 exhibited greater stability under high NaCl concentration. Generally, salt stress adversely affects the growth performance, survival rate, and activities of central carbon metabolism, which affect the efficiency of LA production [68]. Previous research demonstrated that salt stress led to changes in cell membrane fatty acid composition in many microorganisms including *Rhodococcus erythropolis*, *Lactocaseibacillus paracasei*, and *Desulfovibrio vulgaris* [71–73].

Moreover, corn is steeped in water containing 0.2% sulfur dioxide generated from sodium metabisulfate that attacks disulfide bonds of the protein matrix encapsulating starch granules. Once the endosperm protein matrix is dispersed, the starch granules become free, and the overall starch recovery is increased [74]. The steeping tanks on large scale contain SO₂ and elevated steeping temperatures to inhibit the growth of microorganisms during the steeping process [11], so we speculate that the selected strain, WH51-1, will be more applicable for LA fermentation because it exhibited high stability under different concentrations of sodium metabisulfate.

3.4. Optimization of the Fermentation Conditions for LA Production

3.4.1. Effect of Sugar Concentration on LA Production by *Enterococcus faecium* WH51-1

To investigate the influence of initial sugar concentrations on LA production by *E. faecium* WH51-1, fermentation processes were conducted using different concentrations of the initial sugars (20–100 g/L) in mYD media at 50 °C for 48 h. Data obtained in Table 5 and represented graphically in Figure 3 summarize the fermentation parameters and profiles

for LA production at different sugar concentrations. The maximum total viable cell was increased from $68.3 \pm 2.08 \times 10^{10}$ CFU/mL at 20 g/L, reaching the maximum value of $116.6 \pm 2.08 \times 10^{10}$ CFU/mL at 60 g/L of the total sugars. The cell growth was decreased to $31.3 \pm 1.52 \times 10^{10}$ CFU/mL at 80 g/L, giving the lowest value of $25.0 \pm 3.0 \times 10^{10}$ CFU/mL at total sugars of 100 g/L.

Table 5. Effect of different concentrations of CSW on the growth, sugar consumption, LA concentration, LA yield, LA productivity, and maximum LA productivity by *E. faecium* WH51-1.

CSW Conc. (g/L)	Total Viable Cell ($\times 10^{10}$)	Consumed Sugar (g/L)	LA Conc. (g/L) ^a	Y_{LA} (g/g) ^b	P_{LA} (g/L/h) ^c	Max P_{LA} (g/L/h) ^d at the Indicated Time
20	68.3 ± 2.08	18.3 ± 0.37	16.1 ± 0.55	0.88 ± 0.02	0.33 ± 0.01	0.68 ± 0.06 (36 h)
40	89.3 ± 7.02	28.0 ± 0.41	24.5 ± 0.11	0.87 ± 0.01	0.51 ± 0.01	0.65 ± 0.01 (12 h)
60	116.6 ± 2.08	36.9 ± 5.22	29.1 ± 0.87	0.80 ± 0.13	0.60 ± 0.01	0.96 ± 0.04 (12 h)
80	31.3 ± 1.52	23.2 ± 0.37	20.1 ± 0.2	0.86 ± 0.01	0.41 ± 0.01	0.62 ± 0.02 (36 h)
100	25.0 ± 3.0	13.2 ± 0.28	11.0 ± 0.1	0.82 ± 0.01	0.22 ± 0.01	0.33 ± 0.02 (12 h)

^a Maximum lactic acid concentration after 48 h, ^b Lactic acid yield, ^c Lactic acid productivity at the end of fermentation time, ^d Maximum lactic acid productivity at the indicated time. Data represented by Mean \pm SD ($n = 3$).

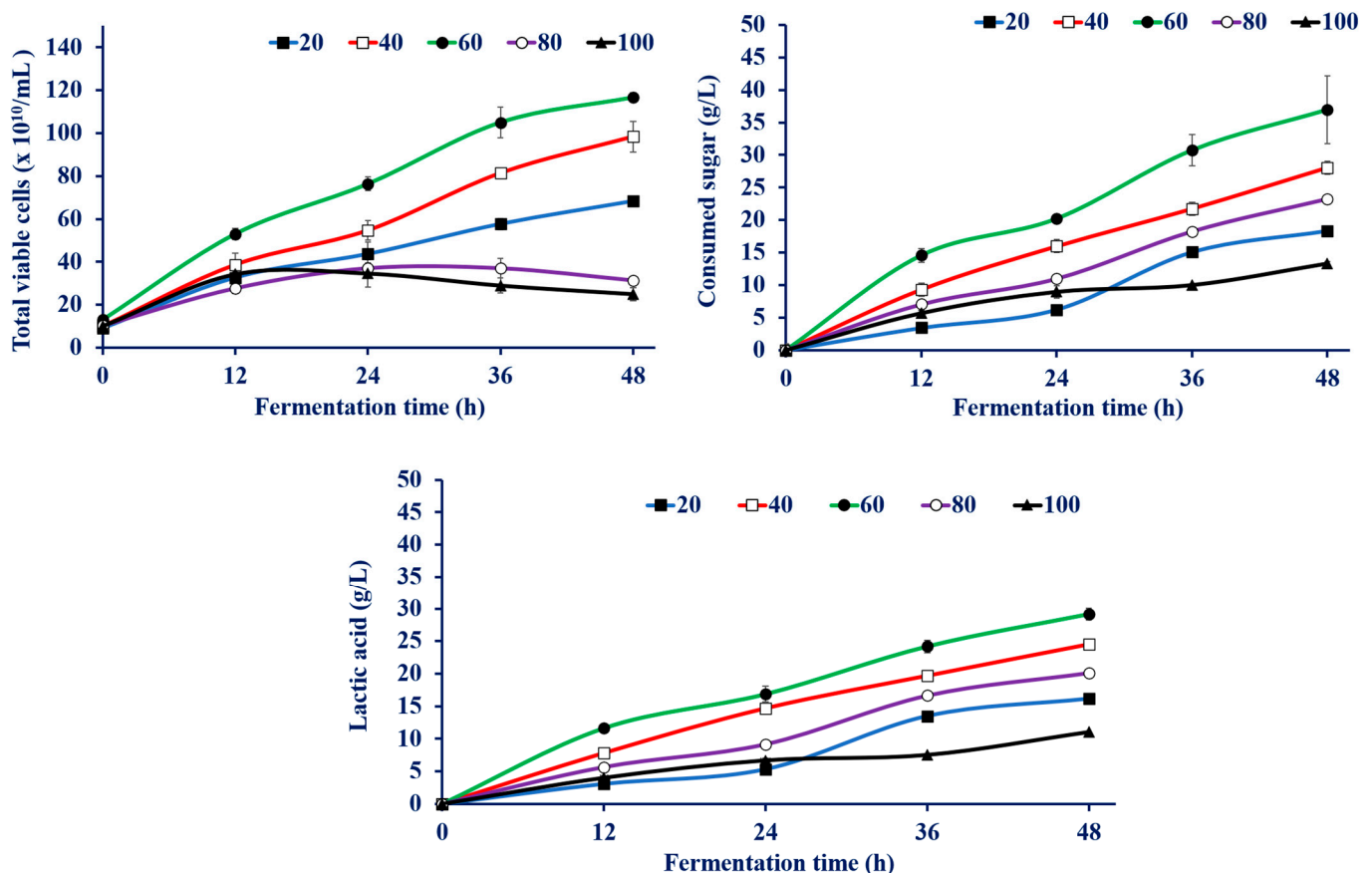


Figure 3. Effect of different concentrations of CSW on the fermentation parameters for lactic acid production by *E. faecium* WH51-1. The standard deviation is less than the size of symbols if no error bars are seen.

In contrast, sugar consumption by the WH 51-1 strain was increased from its minimal value of 18.3 ± 0.37 g/L at 20 g/L to give the highest value at 60 g/L of 36.9 ± 5.22 g/L, while it decreased after that to reach 13.2 g/L at 100 g/L of the total sugars. A similar pattern was given for LA production. The final LA concentration was increased from 1.16 ± 0.55 g/L at 20 g/L, achieving the highest value of 29.1 ± 0.87 g/L at 60 g/L of the total sugars and decreased after that, reaching its minimal value of 11.0 ± 0.1 g/L at 100 g/L of

the total sugars. On the other hand, LA yield showed a comparable range of 0.86–0.88 g/g of sugars consumed at 20–80 g/L of the total sugars while giving 0.82 ± 0.01 g/g of sugars consumed at a high concentration of sugars (100 g/L). LA productivity was also increased from 0.33 ± 0.01 g/L/h at 20 g/L to 0.60 ± 0.01 g/L/h at 60 g/L of the total sugars while it decreased after that, obtaining the minimal value of 0.22 g/L/h at 100 g/L. The maximum LA productivity ranged from (0.33 ± 0.02) to (0.96 ± 0.04) g/L/h giving the maximum value at 60 g/L of the total sugars.

From the previous results, a comparable LA yield and productivity were obtained at 40–60 g/L of the total sugars, while the maximum LA concentration and the maximum productivity (29.13 ± 0.87 g/L and 0.96 ± 0.04 g/L/h, respectively) were obtained when 60 g/L of the total sugars was used as the initial carbon source concentration.

Several authors also reported an increased LA concentration with the increase of initial sugar concentrations up to certain limits [2,75,76]. Interestingly, the very high concentrations of sugar resulted in increasing osmotic pressure and, hence, decreasing LA productivity [77]. Only a few LAB, such as *Enterococcus faecalis* CBRD01 [78], *Enterococcus mundtii* QU 25 [5], *Lactocaseibacillus paracasei* subsp. *paracasei* CHB2121 [79], and *Lb. mutant* G-03 [80], have been reported to be able to tolerate sugar concentrations greater than 60 g/L.

3.4.2. Effect of Inoculum Size on LA Production by *Enterococcus faecium* WH51-1

Inoculum size plays an important role in efficient fermentation and reduction of the lag phase duration [6]. To determine the effect of different inocula sizes on the production of LA by strain WH51-1, fermentation processes were achieved by using different values of inocula sizes ranging 2.5–12.5% in mYD media at 45 °C for 48 h. Data obtained in Table 6 show that the maximum total viable cell was increased from $78.0 \pm 3.60 \times 10^{10}$ CFU/mL at an inoculum size of 2.5% (v/v) reaching the maximum value of $121.33 \pm 2.88 \times 10^{10}$ CFU/mL at 10% (v/v). Cell growth was decreased to $86 \pm 6.24 \times 10^{10}$ CFU/mL at 12.5%. In contrast, the sugar consumption by WH 51-1 strain was increased from 25.43 ± 1.19 at 2.5% to give the highest value of 37.43 ± 1.01 g/L at 10% (v/v) while it decreased after that to reach 26.4 ± 0.62 g/L at 12.5% (v/v).

Table 6. Effect of inocula sizes of CSW on the growth, sugar consumption, LA concentration, LA yield, LA productivity, and maximum LA productivity by *E. faecium* WH51-1.

Inocula Sizes (v/v, %)	Total Viable Cell ($\times 10^{10}$)	Consumed Sugar (g/L)	LA Conc. (g/L) ^a	Y_{LA} (g/g) ^b	P_{LA} (g/L/h) ^c	Max P_{LA} (g/L/h) ^d at the Indicated Time
2.5	78.0 ± 3.60	25.4 ± 1.19	23.0 ± 0.15	0.90 ± 0.03	0.47 ± 0.01	0.83 ± 0.16 (36 h)
5	98.0 ± 7.0	29.5 ± 0.56	26.8 ± 0.9	0.90 ± 0.01	0.55 ± 0.01	0.82 ± 0.12 (36 h)
7.5	117.0 ± 2.64	36.6 ± 0.40	29.1 ± 0.87	0.79 ± 0.02	0.60 ± 0.01	0.96 ± 0.04 (12 h)
10	121.3 ± 2.88	37.4 ± 1.01	32.8 ± 1.01	0.87 ± 0.04	0.68 ± 0.02	1.11 ± 0.07 (12 h)
12.5	86.0 ± 6.24	26.4 ± 0.62	23.4 ± 1.34	0.88 ± 0.03	0.48 ± 0.02	0.59 ± 0.01 (12 h)

^a Maximum lactic acid concentration after 48 h, ^b Lactic acid yield, ^c Lactic acid productivity at the end of fermentation time, ^d Maximum lactic acid productivity at the indicated time. Data represented by Mean \pm SD ($n = 3$).

The final LA concentration was increased from 23.03 ± 0.15 g/L at an inoculum size of 2.5% (v/v) achieving the highest value of 32.86 ± 1.01 g/L at 10% (v/v) and decreased after that, reaching 23.4 ± 1.34 g/L at 12.5% (v/v). Lactic acid productivities were also increased from 0.47 ± 0.01 g/L/h at 2.5% (v/v) to 0.68 ± 0.022 g/L/h at 10% (v/v), while decreased to 0.48 ± 0.02 g/L/h at 12.5% (v/v). The maximum LA productivity ranged from (0.83 ± 0.16) to (1.11 ± 0.07) g/L/h, giving the maximum value of 10%. As a result, 10% (v/v) was selected as the optimal inoculum size for LA production by *E. faecium* WH51-1. Vishnu et al. [81] reported that the optimal inoculum size for LA production by *Lactobacillus amylophilus* GV6 was 10% (v/v). Panesar et al. [82] found that the maximum LA production was 33.72 g/L by *Lactocaseibacillus casei* NBIMCC 1013, which could be observed with an inoculum size of 2–4% (v/v).

3.4.3. Effect of Neutralizing Agents on LA Production by *Enterococcus faecium* WH51-1

Neutralizing agents are used during fermentation for overcoming product inhibition. Neutralizing the fermentation media prevents the progressive acidification of the medium and improves organic acid production [83]. For evaluating the effect of pH control on LA fermentation parameters by *E. faecium* WH51-1, CaCO_3 and NaOH solutions were used as neutralizing agents (Figure 4). The results showed that there were almost comparable total viable cells and sugar consumption in the case of neutralizing the fermentation media by NaOH ($179.33 \pm 3.51 \times 10^{10}$ CFU/mL and 50.06 ± 1.81 g/L, respectively) and CaCO_3 at $178.66 \pm 2.51 \times 10^{10}$ CFU/mL and 49.7 ± 1.25 g/L, respectively. Moreover, there was a comparable final concentration of LA (~ 44.5 g/L) using NaOH or CaCO_3 as a neutralizing agent that was highly increased as compared to the fermentation without neutralizing agents at 32.8 g/L. A high LA yield at 0.89 g/g of sugar consumed was obtained. However, a slight increase in LA productivity was obtained when NaOH was used for neutralizing the fermentation media achieving 0.46 ± 0.02 g/L/h, compared to neutralization by CaCO_3 , which obtained 0.41 ± 0.01 g/L/h. Furthermore, the highest value of maximum LA productivity (1.58 ± 0.07 g/L/h) was obtained using the NaOH solution.

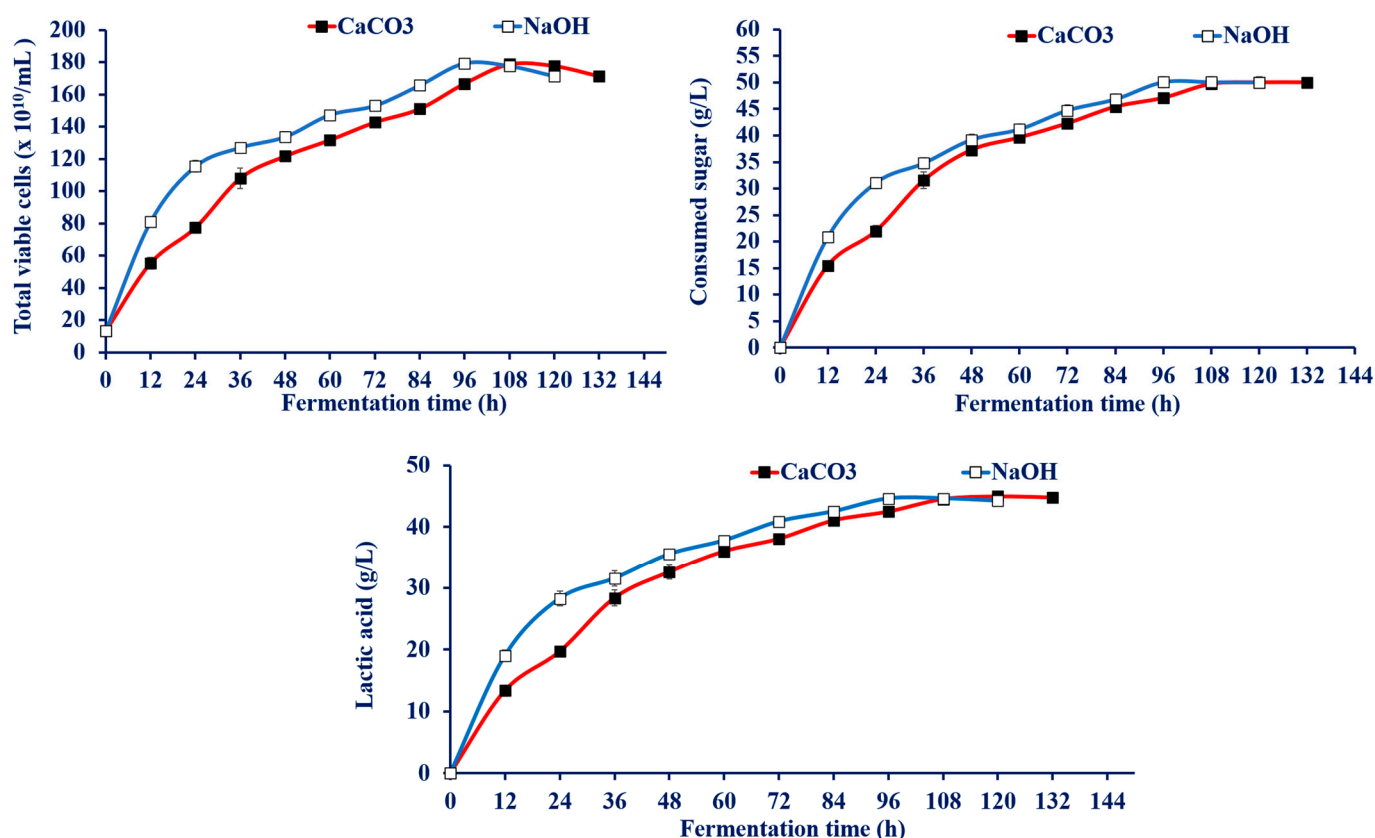


Figure 4. Effect of different neutralizing agents on LA production by *E. faecium* WH51-1. The standard deviation is less than the size of symbols if no error bars are seen.

As a result, NaOH seemed to be better than CaCO_3 as a neutralizing agent during LA production from CSW, not only because of higher fermentation parameters but also due to the avoidance of formation of gypsum waste materials as a result of CaCO_3 supplementation [26]. Several reports have been recorded on LA fermentation processes using an NaOH solution for controlling the pH of the fermentation medium [75,84,85].

4. Conclusions

In this work, a cost-effective system for increased LA production from CSW was achieved under thermo-alkaline conditions for decreasing the contamination risk. A com-

plete analysis of CSW indicated its suitability to be used as a raw material for LA production due to its high content of sugars, nitrogen, amino acids, trace elements, and vitamins. *Enterococcus faecium* WH51-1 has been proven to have a good stability toward various inhibitory compounds, and is a promising candidate for LA production from CSW effluent with minimal nutrient supplementation (5 g/L yeast extract). Optimizing the fermentation conditions increased LA production, achieving a maximum concentration of 44.6 g/L at a yield of 0.89 g/g-consumed sugar and productivity of 1.58 g/L/h using 60 g/L CSW sugar, inoculum size (10%, v/v), at 45 °C and pH 9.0 using NaOH as a neutralizing agent. Further optimization studies, including response surface statistical designs, will be investigated for improved sugar consumption and LA production from CSW effluent.

Author Contributions: Conceptualization, M.T.S., S.S.S., A.F., M.S.E.-G. and M.A.A.-R.; Methodology, M.T.S., S.S.S., A.F., M.S.E.-G. and M.A.A.-R.; Software, M.T.S., S.S.S., A.F., M.S.E.-G. and M.A.A.-R.; Validation, M.T.S., S.S.S., A.F., M.S.E.-G. and M.A.A.-R.; Formal Analysis, M.T.S., S.S.S., A.F., M.S.E.-G. and M.A.A.-R.; Investigation, M.T.S., S.S.S., A.F., M.S.E.-G. and M.A.A.-R.; Resources, M.T.S., S.S.S., A.F., M.S.E.-G. and M.A.A.-R.; Data Curation, M.T.S., S.S.S., A.F., M.S.E.-G. and M.A.A.-R.; Writing—Original Draft Preparation, M.T.S. and M.A.A.-R.; Writing—Review and Editing, M.T.S., S.S.S., A.F., and M.A.A.-R.; Visualization, M.T.S., S.S.S., A.F., M.S.E.-G. and M.A.A.-R.; Supervision, S.S.S., A.F., M.S.E.-G. and M.A.A.-R.; Project Administration, S.S.S., A.F., M.S.E.-G. and M.A.A.-R.; Funding Acquisition, M.A.A.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Din, N.A.S.; Lim, S.J.; Maskat, M.Y.; Mutalib, S.A.; Zaini, N.A.M. Lactic acid separation and recovery from fermentation broth by ion-exchange resin: A review. *Bioresour. Bioprocess.* **2021**, *8*, 31. [\[CrossRef\]](#)
- Gaber, M.A.; Abdel-Rahman, M.A.; Hassan, S.E.-D.; Azab, M.S. Efficient biorefinery process for lactic acid production from date wastes with alleviating substrate inhibition effect using thermo-alkaline repeated batch fermentation. *Biomass Convers. Biorefinery* **2021**, *11*, 1053–1066. [\[CrossRef\]](#)
- Vorawongsagul, S.; Pratumpong, P.; Pechyen, C. Preparation and foaming behavior of poly (lactic acid)/poly (butylene succinate)/cellulose fiber composite for hot cups packaging application. *Food Packag. Shelf Life* **2021**, *27*, 100608. [\[CrossRef\]](#)
- Cubas Cano, E. Valorisation of Lignocellulosic Residues for Lactic Acid and Bioethanol Production in a Biorefinery Context. Ph.D. Thesis, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, Madrid, Spain, 2021.
- Abdel-Rahman, M.A.; Tashiro, Y.; Sonomoto, K. Recent advances in lactic acid production by microbial fermentation processes. *Biotechnol. Adv.* **2013**, *31*, 877–902. [\[CrossRef\]](#)
- Wang, Y.; Tashiro, Y.; Sonomoto, K. Fermentative production of lactic acid from renewable materials: Recent achievements, prospects, and limits. *J. Biosci. Bioeng.* **2015**, *119*, 10–18. [\[CrossRef\]](#)
- Abdel-Rahman, M.A.; Hassan, S.E.D.; El-Din, M.N.; Azab, M.S.; El-Belely, E.F.; Alrefaey, H.M.A.; Elsakhawy, T. One-factor-at-a-time and response surface statistical designs for improved lactic acid production from beet molasses by *Enterococcus hirae* ds10. *SN Appl. Sci.* **2020**, *2*, 573. [\[CrossRef\]](#)
- Wang, F.; Hu, J.-H.; Guo, C.; Liu, C.-Z. Enhanced laccase production by *Trametes versicolor* using corn steep liquor as both nitrogen source and inducer. *Bioresour. Technol.* **2014**, *166*, 602–605. [\[CrossRef\]](#)
- Liu, X.; Wang, X.; Xu, J.; Xia, J.; Lv, J.; Zhang, T.; Wu, Z.; Deng, Y.; He, J. Citric acid production by *Yarrowia lipolytica* SWJ-1b using corn steep liquor as a source of organic nitrogen and vitamins. *Ind. Crop Prod.* **2015**, *78*, 154–160. [\[CrossRef\]](#)
- Wang, D.; Eckhoff, S.R. Effect of Broken Corn Levels on Water Absorption and Steepwater Characteristics. *Cereal Chem. J.* **2000**, *77*, 525–528. [\[CrossRef\]](#)
- Singh, N.; Shevkani, K.; Kaur, A.; Thakur, S.; Parmar, N.; Viridi, A.S. Characteristics of starch obtained at different stages of purification during commercial wet milling of maize. *Starch Stärke* **2014**, *66*, 668–677. [\[CrossRef\]](#)
- Eneje, L.; Ogu, E.; Aloh, C.; Odibo, F.; Agu, R.; Palmer, G. Effect of steeping and germination time on malting performance of Nigerian white and yellow maize varieties. *Process Biochem.* **2004**, *39*, 1013–1016. [\[CrossRef\]](#)

13. Brooker, D.B.; Bakker-Arkema, F.W.; Hall, C.W. *Drying and Storage of Grains and Oilseeds*; Springer Science & Business Media: Berlin, Germany, 1992.
14. Hofer, A.; Hauer, S.; Kroll, P.; Fricke, J.; Herwig, C. In-depth characterization of the raw material corn steep liquor and its bioavailability in bioprocesses of *Penicillium chrysogenum*. *Process Biochem.* **2018**, *70*, 20–28.
15. Li, X.; Xu, W.; Yang, J.; Zhao, H.; Pan, C.; Ding, X.; Zhang, Y. Effects of applying lactic acid bacteria to the fermentation on a mixture of corn steep liquor and air-dried rice straw. *Anim. Nutr.* **2016**, *2*, 229–233. [\[CrossRef\]](#)
16. Bolobova, A.V.; Kondrashchenko, V.I. Use of yeast fermentation waste as a biomodifier of concrete (Review). *Appl. Biochem. Microbiol.* **2000**, *36*, 205–214.
17. Pereira, F.B.; Guimarães, P.M.; Teixeira, J.; Domingues, L. Optimization of low-cost medium for very high gravity ethanol fermentations by *Saccharomyces cerevisiae* using statistical experimental designs. *Bioresour. Technol.* **2010**, *101*, 7856–7863. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Azizi-Shotorkhoft, A.; Sharifi, A.; Mirmohammadi, D.; Baluch-Gharaei, H.; Rezaei, J. Effects of feeding different levels of corn steep liquor on the performance of fattening lambs. *J. Anim. Physiol. Anim. Nutr.* **2015**, *100*, 109–117.
19. Tauqir, N.A. Impact of Corn Steep Liquor and Enzose Mixture on Growth Performance of Chicks. *Pak. J. Zool.* **2021**, 1–4.
20. Babakhani, S.; Fahmi, A.; Katebi, H.; Ouria, A.; Majnoui-Toutakhane, A.; Ganbarov, K.; Kafil, H. Non-sterile corn steep liquor a novel, cost effective and powerful culture media for *Sporosarcina pasteurii* cultivation for sand improvement. *J. Appl. Microbiol.* **2021**, *130*, 1232–1244. [\[PubMed\]](#)
21. Maddipati, P.; Atiyeh, H.K.; Bellmer, D.D.; Huhnke, R.L. Ethanol production from syngas by Clostridium strain P11 using corn steep liquor as a nutrient replacement to yeast extract. *Bioresour. Technol.* **2011**, *102*, 6494–6501. [\[CrossRef\]](#)
22. Liu, B.; Yang, M.; Qi, B.; Chen, X.; Su, Z.; Wan, Y. Optimizing l-(+)-lactic acid production by thermophile *Lactobacillus plantarum* As.1.3 using alternative nitrogen sources with response surface method. *Biochem. Eng. J.* **2010**, *52*, 212–219. [\[CrossRef\]](#)
23. Silveira, M.; Wisbeck, E.; Hoch, I.; Jonas, R. Production of glucose-fructose oxidoreductase and ethanol by *Zymomonas mobilis* ATCC 29191 in medium containing corn steep liquor as a source of vitamins. *Appl. Microbiol. Biotechnol.* **2001**, *55*, 442–445. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Saxena, J.; Tanner, R.S. Optimization of a corn steep medium for production of ethanol from synthesis gas fermentation by Clostridium ragsdalei. *World J. Microbiol. Biotechnol.* **2011**, *28*, 1553–1561. [\[CrossRef\]](#)
25. Abdel-Rahman, M.A.; Tashiro, Y.; Zendo, T.; Sonomoto, K. Improved lactic acid productivity by an open repeated batch fermentation system using *Enterococcus mundtii* QU 25. *RSC Adv.* **2013**, *3*, 8437–8445. [\[CrossRef\]](#)
26. Abdel-Rahman, M.A.; Sonomoto, K. Opportunities to overcome the current limitations and challenges for efficient microbial production of optically pure lactic acid. *J. Biotechnol.* **2016**, *236*, 176–192. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Abdel-Rahman, M.A.; Tashiro, Y.; Zendo, T.; Sakai, K.; Sonomoto, K. *Enterococcus faecium* QU 50: A novel thermophilic lactic acid bacterium for high-yield l-lactic acid production from xylose. *FEMS Microbiol. Lett.* **2014**, *362*, 1–7. [\[CrossRef\]](#)
28. Miller, D.; Bryant, J.E.; Madsen, E.L.; Ghiorse, W.C. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl. Environ. Microbiol.* **1999**, *65*, 4715–4724. [\[CrossRef\]](#)
29. Lane, D. 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*; Stackebrandt, E., Goodfellow, M., Eds.; John Wiley & Sons: New York, NY, USA, 1991; pp. 115–175.
30. Association, A.P.H. *Standard Methods for the Examination of Water and Wastewater*; Clesceri, L.S., Greenberg, A.E., Eaton, A.D., Eds.; American Public Health Association: Washington, DC, USA, 1912; Volume 2.
31. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [\[CrossRef\]](#)
32. Herbert, D. Chapter III Chemical analysis of microbial cells. VII-E Determination of nucleic acids in micro-organisms. In *Methods in Microbiology*; Academic Press: Cambridge, MA, USA, 1971; Part B; Volume 5, pp. 324–328.
33. Barker, S.; Summerson, W.H. The colorimetric determination of lactic acid in biological material. *J. Biol. Chem.* **1941**, *138*, 535–554. [\[CrossRef\]](#)
34. Hull, S.R.; Peters, E.; Cox, C.; Montgomery, R. Composition of corn steep water during experimental steeping. *J. Agric. Food Chem.* **1996**, *44*, 3521–3527. [\[CrossRef\]](#)
35. Watson, S.A. Measurement and maintenance of quality. In *Corn: Chemistry and Technology*; Watson, S.A., Ramstad, P.E., Eds.; American Association of Cereal Chemists: St. Paul, MN, USA, 1987; pp. 125–183.
36. Yasri, N.G.; Yaghmour, A.; Gunasekaran, S. Effective removal of organics from corn wet milling steepwater effluent by electrochemical oxidation and adsorption on 3-D granulated graphite electrode. *J. Environ. Chem. Eng.* **2015**, *3*, 930–937. [\[CrossRef\]](#)
37. Whistler, R.L.; BeMiller, J.N.; Paschall, E.F. *Starch: Chemistry and Technology*; Academic Press: Cambridge, MA, USA, 2012.
38. Winston Liggett, R.; Koffler, H. Corn steep liquor in microbiology. *Bacteriol. Rev.* **1948**, *12*, 297. [\[CrossRef\]](#)
39. Xiao, X.; Hou, Y.; Liu, Y.; Liu, Y.; Zhao, H.; Dong, L.; Du, J.; Wang, Y.; Bai, G.; Luo, G. Classification and analysis of corn steep liquor by UPLC/Q-TOF MS and HPLC. *Talanta* **2013**, *107*, 344–348. [\[CrossRef\]](#)
40. Tammam, J.; Williams, A.; Noble, J.; Lloyd, D. Amino acid fermentation in non-starter *Lactobacillus spp.* isolated from Cheddar cheese. *Lett. Appl. Microbiol.* **2000**, *30*, 370–374. [\[CrossRef\]](#) [\[PubMed\]](#)
41. Kieronczyk, A.; Skeie, S.; Langsrud, T.; Le Bars, D.; Yvon, M. The nature of aroma compounds produced in a cheese model by glutamate dehydrogenase positive *Lactobacillus* INF15D depends on its relative aminotransferase activities towards the different amino acids. *Int. Dairy J.* **2004**, *14*, 227–235. [\[CrossRef\]](#)

42. Williams, A.G.; Noble, J.; Banks, J.M. Catabolism of amino acids by lactic acid bacteria isolated from Cheddar cheese. *Int. Dairy J.* **2001**, *11*, 203–215. [\[CrossRef\]](#)
43. Liu, S.-Q.; Holland, R.; Crow, V.L. The potential of dairy lactic acid bacteria to metabolise amino acids via non-transaminating reactions and endogenous transamination. *Int. J. Food Microbiol.* **2003**, *86*, 257–269. [\[CrossRef\]](#)
44. Qiao, Y.; Liu, G.; Leng, C.; Zhang, Y.; Lv, X.; Chen, H.; Sun, J.; Feng, Z. Metabolic profiles of cysteine, methionine, glutamate, glutamine, arginine, aspartate, asparagine, alanine and glutathione in *Streptococcus thermophilus* during pH-controlled batch fermentations. *Sci. Rep.* **2018**, *8*, 12441. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Teixeira, J.; Seeras, A.; Sanchez-Maldonado, A.F.; Zhang, C.; Su, M.S.-W.; Gänzle, M.G. Glutamine, glutamate, and arginine-based acid resistance in *Lactobacillus reuteri*. *Food Microbiol.* **2014**, *42*, 172–180. [\[CrossRef\]](#)
46. Loy, D.D.; Wright, K. Nutritional properties and feeding value of corn and its by-products. In *Corn: Chemistry and Technology*; White, P.J., Johnson, L.A., Eds.; American Association of Cereal Chemists: St. Paul, MN, USA, 1987.
47. Limauro, D.; Falcioratore, A.; Basso, A.L.; Forlani, G.; De Felice, M. Proline biosynthesis in *Streptococcus thermophilus*: Characterization of the proBA operon and its products. *Microbiology* **1996**, *142*, 3275–3282. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Xu, S.; Zhou, J.; Liu, L.; Chen, J. Proline enhances *Torulopsis glabrata* growth during hyperosmotic stress. *Biotechnol. Bioprocess Eng.* **2010**, *15*, 285–292. [\[CrossRef\]](#)
49. Tian, X.; Wang, Y.; Chu, J.; Zhuang, Y.; Zhang, S. Enhanced L-lactic acid production in *Lactobacillus paracasei* by exogenous proline addition based on comparative metabolite profiling analysis. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 2301–2310. [\[CrossRef\]](#)
50. Zhang, Y.; Zhang, Y.; Zhu, Y.; Mao, S.; Li, Y. Proteomic analyses to reveal the protective role of glutathione in resistance of *Lactococcus lactis* to osmotic stress. *Appl. Environ. Microbiol.* **2010**, *76*, 3177–3186. [\[CrossRef\]](#) [\[PubMed\]](#)
51. Ho, S.W.; El-Nezami, H.; Shah, N.P. Effects of supplementation of citrulline and *Lactobacillus helveticus* ASCC 511 on intestinal epithelial cell integrity. *J. Funct. Foods* **2020**, *64*, 103571. [\[CrossRef\]](#)
52. Litchfield, J. Lactic Acid, Microbially Produced. In *Encyclopedia of Microbiology*, 3rd ed.; Schaechter, M., Ed.; Academic Press: Cambridge, MA, USA, 2009; pp. 362–372.
53. Reimundo, P.; Menendez, A.; Mendez, J.; Perez-Pascual, D.; Navais, R.; Gomez, E.; Brana, A.; Guijarro, J. dltA gene mutation in the teichoic acids alanylation system of *Lactococcus garvieae* results in diminished proliferation in its natural host. *Vet. Microbiol.* **2010**, *143*, 434–439. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Yao, C.; Chou, J.; Wang, T.; Zhao, H.; Zhang, B. Pantothenic Acid, Vitamin C, and Biotin Play Important Roles in the Growth of *Lactobacillus helveticus*. *Front. Microbiol.* **2018**, *9*, 1194. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Jørgensen, H.; Nielsen, J.; Villadsen, J.; Møllgaard, H. Metabolic flux distributions in *Penicillium chrysogenum* during fed-batch cultivations. *Biotechnol. Bioeng.* **1995**, *46*, 117–131. [\[CrossRef\]](#)
56. Hofer, A.; Herwig, C. Quantitative determination of nine water-soluble vitamins in the complex matrix of corn steep liquor for raw material quality assessment. *J. Chem. Technol. Biotechnol.* **2017**, *92*, 2106–2113. [\[CrossRef\]](#)
57. Carr, J.G. The vitamin requirements of lactic acid bacteria from ciders. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **1958**, *24*, 63–68. [\[CrossRef\]](#)
58. Tanner, F.W., Jr.; Pfeiffer, S.E.; Van Lanen, J.M. Vitamin and protein content of residues from the production of penicillin by sub-merged fermentation. *Arch. Biochem.* **1945**, *8*, 29–36.
59. Choi, J.-D.-R.; Jang, Y.-S.; Cho, J.-H.; Seung, D.; Lee, S.Y.; Papoutsakis, E.T.; Bennett, G.N.; Song, H. Characterization and evaluation of corn steep liquid in acetone-butanol-ethanol production by *Clostridium acetobutylicum*. *Biotechnol. Bioprocess Eng.* **2013**, *18*, 266–271. [\[CrossRef\]](#)
60. Daniels, R.S. Corn Steep Liquor as a Biostimulant Composition. Google Patents PCT/US2010/058315, 30 November 2010.
61. Cook, A.M.; Denger, K. Dissimilation of the C2 sulfonates. *Arch. Microbiol.* **2002**, *179*, 1–6. [\[CrossRef\]](#) [\[PubMed\]](#)
62. Cook, A.M.; Denger, K. Metabolism of taurine in microorganisms. *Taurine* **2006**, *6*, 3–13.
63. Denger, K.; Ruff, J.; Schleheck, D.; Cook, A.M. *Rhodococcus opacus* expresses the xsc gene to utilize taurine as a carbon source or as a nitrogen source but not as a sulfur source. *Microbiology* **2004**, *150*, 1859–1867. [\[CrossRef\]](#) [\[PubMed\]](#)
64. Clifford, E.L.; Varela, M.M.; De Corte, D.; Bode, A.; Ortiz, V.; Herndl, G.J.; Sintes, E. Taurine Is a Major Carbon and Energy Source for Marine Prokaryotes in the North Atlantic Ocean off the Iberian Peninsula. *Microb. Ecol.* **2019**, *78*, 299–312. [\[CrossRef\]](#) [\[PubMed\]](#)
65. Christianson, D.D.; Cavins, J.F.; Wall, J.S. Steep Liquor Constituents, Identification and Determination of Nonprotein Nitrogenous Substances in Corn Steep Liquor. *J. Agric. Food Chem.* **1965**, *13*, 277–280. [\[CrossRef\]](#)
66. Curis, E.; Nicolis, I.; Moinard, C.; Osowska, S.; Zerrouk, N.; Bénazeth, S.; Cynober, L. Almost all about citrulline in mammals. *Amino Acids* **2005**, *29*, 177–205. [\[CrossRef\]](#) [\[PubMed\]](#)
67. Hwang, H.; Lee, J.-H. Characterization of Arginine Catabolism by Lactic Acid Bacteria Isolated from Kimchi. *Molecules* **2018**, *23*, 3049. [\[CrossRef\]](#)
68. He, G.; Wu, C.; Huang, J.; Zhou, R. Metabolic response of *Tetragenococcus halophilus* under salt stress. *Biotechnol. Bioprocess Eng.* **2017**, *22*, 366–375. [\[CrossRef\]](#)
69. Cusumano, Z.T.; Caparon, M.G. Citrulline Protects *Streptococcus pyogenes* from Acid Stress Using the Arginine Deiminase Pathway and the F1Fo-ATPase. *J. Bacteriol.* **2015**, *197*, 1288–1296. [\[CrossRef\]](#)
70. Held, C.; Sadowski, G. Compatible solutes: Thermodynamic properties relevant for effective protection against osmotic stress. *Fluid Phase Equilibria* **2016**, *407*, 224–235. [\[CrossRef\]](#)

71. Zhou, A.; Baidoo, E.; He, Z.; Mukhopadhyay, A.; Baumohl, J.K.; Benke, P.; Joachimiak, M.P.; Xie, M.; Song, R.; Arkin, A.P.; et al. Characterization of NaCl tolerance in *Desulfovibrio vulgaris* Hildenborough through experimental evolution. *ISME J.* **2013**, *7*, 1790–1802. [[CrossRef](#)]
72. de Carvalho, C.C.C.R.; Marques, M.P.C.; Hachicho, N.; Heipieper, H.J. Rapid adaptation of *Rhodococcus erythropolis* cells to salt stress by synthesizing polyunsaturated fatty acids. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 5599–5606. [[CrossRef](#)]
73. Guillot, A.; Obis, D.; Mistou, M.-Y. Fatty acid membrane composition and activation of glycine-betaine transport in *Lactococcus lactis* subjected to osmotic stress. *Int. J. Food Microbiol.* **2000**, *55*, 47–51. [[CrossRef](#)]
74. Li, Q.; Singh, V.; Gonzalez de Mejia, E.; Somavat, P. Effect of sulfur dioxide and lactic acid in steeping water on the extraction of anthocyanins and bioactives from purple corn pericarp. *Cereal Chem. J.* **2019**, *96*, 575–589. [[CrossRef](#)]
75. Abdel-Rahman, M.A.; Hassan, S.E.-D.; Azab, M.S.; Mahin, A.-A.; Gaber, M.A. High Improvement in Lactic Acid Productivity by New Alkaliphilic Bacterium Using Repeated Batch Fermentation Integrated with Increased Substrate Concentration. *BioMed Res. Int.* **2019**, *2019*, 7212870. [[CrossRef](#)]
76. Kadam, S.R.; Patil, S.S.; Bastawde, K.B.; Khire, J.M.; Gokhale, D.V. Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production. *Process Biochem.* **2006**, *41*, 120–126. [[CrossRef](#)]
77. Michelson, T.; Kask, K.; Jögi, E.; Talpsep, E.; Suitso, I.; Nurk, A. L (+)-Lactic acid producer *Bacillus coagulans* SIM-7 DSM 14043 and its comparison with *Lactobacillus delbrueckii* ssp. *lactis* DSM 20073. *Enzym. Microb. Technol.* **2006**, *39*, 861–867. [[CrossRef](#)]
78. Mohan, D.; Sarswat, A.; Ok, Y.S.; Pittman, C.U. Organic and inorganic contaminants removal from water with biochar, a renewable, low cost and sustainable adsorbent—A critical review. *Bioresour. Technol.* **2014**, *160*, 191–202. [[CrossRef](#)] [[PubMed](#)]
79. Moon, S.-K.; Wee, Y.-J.; Choi, G.-W. A novel lactic acid bacterium for the production of high purity l-lactic acid, *Lactobacillus paracasei* subsp. *paracasei* CHB2121. *J. Biosci. Bioeng.* **2012**, *114*, 155–159. [[CrossRef](#)]
80. Ge, X.-Y.; Yuan, J.; Qin, H.; Zhang, W.-G. Improvement of l-lactic acid production by osmotic-tolerant mutant of *Lactobacillus casei* at high temperature. *Appl. Microbiol. Biotechnol.* **2010**, *89*, 73–78. [[CrossRef](#)] [[PubMed](#)]
81. Vishnu, C.; Seenayya, G.; Reddy, G. Direct fermentation of various pure and crude starchy substrates to L(+) lactic acid using *Lactobacillus amylophilus* GV6. *World J. Microbiol. Biotechnol.* **2002**, *18*, 429–433. [[CrossRef](#)]
82. Panesar, P.S.; Kennedy, J.F.; Knill, C.J.; Kosseva, M. Production of L(+) lactic acid using *Lactobacillus casei* from whey. *Braz. Arch. Biol. Technol.* **2010**, *53*, 219–226. [[CrossRef](#)]
83. Rivas, B.; Torrado, A.; Torre, P.; Converti, A.; Domínguez, J.M. Submerged Citric Acid Fermentation on Orange Peel Autohydrolysate. *J. Agric. Food Chem.* **2008**, *56*, 2380–2387. [[CrossRef](#)]
84. Bernardo, M.P.; Coelho, L.F.; Sass, D.C.; Contiero, J. L-(+)-Lactic acid production by *Lactobacillus rhamnosus* B103 from dairy industry waste. *Braz. J. Microbiol.* **2016**, *47*, 640–646. [[CrossRef](#)] [[PubMed](#)]
85. Hu, J.; Lin, Y.; Zhang, Z.; Xiang, T.; Mei, Y.; Zhao, S.; Liang, Y.; Peng, N. High-titer lactic acid production by *Lactobacillus pentosus* FL0421 from corn stover using fed-batch simultaneous saccharification and fermentation. *Bioresour. Technol.* **2016**, *214*, 74–80. [[CrossRef](#)] [[PubMed](#)]