

Evaluation of Lactic Acid Production by different *Bacillus Subtilis* Strains Isolated from *Theobroma Cacao* Crops in Ecuador

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Lactic acid (LA) is an organic compound with numerous applications in the chemical, pharmaceutical and food industries. In addition, the study of its production has garnered renewed interest due to its applicability in the generation of biopolymers, seeking to lower operating costs and cultivation conditions. Ecuador has a growing demand for LA, but it lacks local production, despite having a microbial diversity that can be used for this purpose. Among the microorganisms that stand out for their production potential is *Bacillus subtilis*. In the present project, 7 strains of *Bacillus subtilis* isolated from cocoa crops, a cultivar of great local importance, were studied. These were identified by sequencing the 16s ribosomal and API50 galleries. The strains which were tested for LA production from glucose. The 3 best strains for LA production were identified and their yields were evaluated under aerobic, anaerobic and microaerobic conditions. The highest production of LA was evidenced under aerobic conditions with efficiencies of approximately 0.25 grams of LA per gram of glucose in the three best strains, equivalent to 5 g / L LA. Through the present investigation, it was possible to establish methodologies and achieve reasonable LA production with *Bacillus subtilis* strains, in addition, 3 promising strains for the production of LA in the country were identified.

1. Introduction

Lactic acid (LA) can be produced in two main ways; the first one is through chemical hydrolysis of lacto nitrile with strong acids (Komesu et al., 2017), while the second one is through bioprocesses. Microorganism-mediated production is preferred as it can be directed towards one optical isomer, thereby having more potential for different applications (Nguyen et al., 2012). Additionally, raw materials in bioprocesses can be cheaper, and operation conditions can result in lower energy costs (Harrison et al., 2015). Due to these reasons, over 90% of LA production is focused on fermentative processes, equivalent to 72,000 annual tons (Romero-García et al, 2009). Despite these benefits, there are limitations and spaces for improvement in LA production. Most commonly used species, such as *Lactobacillus delbrueckii*, *Lactobacillus casei*, *Lactobacillus lactis* present low yield and high nutritional requirements (Orozco & Zuluaga 2014). Among new researched species due to their potential for LA production, *Bacillus subtilis* stands out for its higher growth rate and substrate versatility, along with its ability to grow in simple media (Ohara & Yahata 1996).

B. subtilis is a sporulating bacteria, catalase positive, classified as GRAS (generally recognized as Safe) by the United States Food and Drug Administration (FDS). Despite being considered a strictly aerobic microorganism,

B. subtilis can grow under anaerobic conditions with cellobiose, due to the presence of the RABCD operon that codifies EII permease, and phosphor-beta glycosidic proteins, as an anaerobic way through nitrate ammonification and several fermentative processes (Rhee et al., 2016). Under aerobic conditions, *B. subtilis* uses glucose as a carbon source to produce L. lactide and 2,3 butanediol as main products.

LA production through *B. subtilis* is a relevant alternative due to not only high yields, but also ease of culture (Gao et al., 2012). However, there are differences in productivity, depending on the strain, which, along with substrates and operation conditions, can produce between 1.5 and 150 g/L, with wild type strains resulting in the 2-4 g/L range (Romero-Garcia et al., 2009). Being a mega-diverse and mainly agricultural country, Ecuador offers a rich variety of climates and conditions that affect microbiota. Particularly, cacao, of which Ecuador is one of the main producers in the world, is cultivated in different climatic regions, that affect not only its composition but also the microorganisms associated to the crops. Therefore, in the present work, the viability of LA production from different *B. subtilis* strains, isolated from cacao crops from different locations in Ecuador, was tested to find new, cost-effective alternatives.

2. Materials and Methods

2.1 Isolation of microorganisms: Different Bacillus strains were isolated from healthy cocoa cultivars (*Theobroma cacao L.*). Fine aroma cacao (Nacional), and a clone (Naranjal, CCN-51) were sampled in two locations: Naranjal and Balao, Guayas Province-Ecuador. Bacterial strains were isolated using a protocol previously described by Silva et al. (2012).

2.2 Molecular Identification and metabolic profiling: Isolated strains were molecularly identified by analysis of the 16s rDNA gene. Genomic DNA was extracted using a modified protocol from Ausubel et al. (2011). For the amplification of the DNA fragment, the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (CGGTTACCTTGTTACGACTT) were used. The PCR products were purified and sequenced at Macrogen Inc. (Seoul, South Korea), and the sequences were compared to the NCBI nucleotide sequence database using BLASTN suite. Strains identified as *B. subtilis*, were then characterized through their metabolic profile using API50CH/B galleries. The percentages of similarity according to the metabolic profile, with those expected in a *B. subtilis* strain were obtained using the software, provided by the API50CH / B bioMérieux gallery provider, apiweb™.

2.3 Screening of LA Production: A preliminary screening of 7 strains was carried out in MT culture media, proposed by Gao et al. (2012). Fermentations were carried out in triplicate, in 100 mL flasks, closed with gauss-cotton stoppers, at 37 °C, for 72 h, with shaking at 180 rpm. For the inoculation into MT media, a strain colony previously cultured in nutritive agar for 15 h was used. After fermentation, three strains with the largest LA production rates were chosen for subsequent experimentation. These were cultured under the same conditions, by triplicate, and kinetics of cell growth, glucose consumption and LA production were generated by analyzing 1 mL samples taken at 0,3,6, 9, 24,27,30, 33 h, after inoculation.

2.4 Effect of oxygen conditions on LA production: Due to the fact that *B. subtilis* is a facultative anaerobic microorganism, and that some of the genes associated with anaerobic respiration are exacerbated when anoxic conditions occur, an analysis of the implication of different oxygen concentrations in the production of LA was carried out, in order to determine the most appropriate operating conditions. LA production was evaluated for the three chosen strains under anaerobic, aerobic and micro-aerobiosis conditions. As a positive control, a *Lactobacillus spp.* strain (V1.1), from USFQ microorganism bank, was used. In these experiments, culture was carried out up to 72 h to achieve equilibrium, after which samples were taken for LA quantification. For anaerobic and micro-aerobic conditions, shaking speed was 100 rpm (according to preliminary laboratory assays), and 180 rpm for aerobic conditions. To achieve anaerobiosis, a nitrogen purge was done for 30 s, at 2 L/min. For micro-aerobiosis, Gaspak jars were used.

2.5 Analytical methods: For LA quantification, the methodology proposed by Borshchevskaya et al. (2016), based on iron (III) chloride was used. Glucose determination was done through the phenol-sulphuric acid method, based on the protocol by Dubois et al. (1956). Biomass quantification through dry weight was used as a measurement of cell growth, based on the method proposed by Gao et al. (2012).

2.6 Statistical analyses: All fermentations were carried out in triplicates, and three replicates were measured for every parameter. The results are reported as average \pm standard deviation. For the statistical analysis, ANOVA was used, followed by Tuckey pair-wise comparison tests, with 95 % confidence. For the statistical analysis of LA and glucose quantification methods, a Gage R&R analysis was performed through MINITAB

3.2 Preliminary assessment of LA production potential

Strains were assessed for their capacity to produce LA from glucose and are presented in Figure 2.

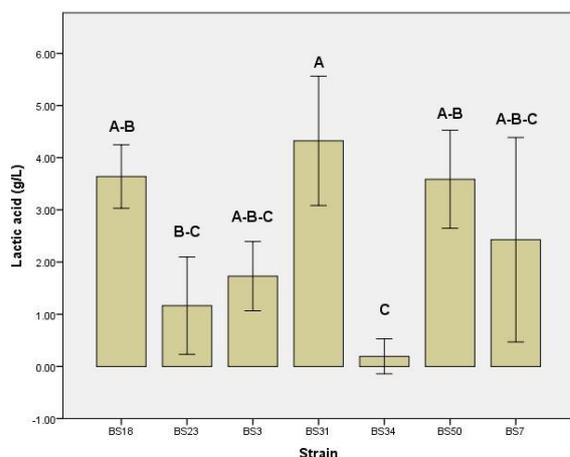


Figure 2. LA production of 7 *Bacillus subtilis* strains, under aerobic conditions.

The strains with the highest production rates were BS18 (3.64 g/L), BS31 (3.61 g/L) and BS50 (3.59 g/L). The mean of LA production of the BS31 strain is statistically different from those presented by the BS50 and BS18 strains, which do not present statistically significant differences between them. Strains BS03, BS34, BS07, BS23 produced lower LA concentrations. Therefore, due to their higher LA potential, and their ability to metabolize common sugars (i.e. glucose, fructose and sucrose, Fig. 3), strains BS18, BS31 and BS50 were chosen for subsequent experimentation.

3.3 Analysis of the effect of oxygen conditions on LA production

Once the strains with the highest productivity were identified, the effect of oxygen concentration on LA production was assessed. The results obtained from 72 h cultures under aerobic, anaerobic, and micro-aerobic conditions are shown in Figure 3. As a positive control, a *Lactobacillus spp.* (V1.1) was used. The aerobic culture presented a statistically higher LA production than anaerobic and micro-aerobic cultures. Strain 18 presented significant acid production under all culture conditions, unlike strains 31 and 50, which presented production only under aerobic and micro-aerobic cultures. There were no statistical differences between the strains at aerobic conditions, while under micro-aerobiosis, once again, strain 18 had the highest production level.

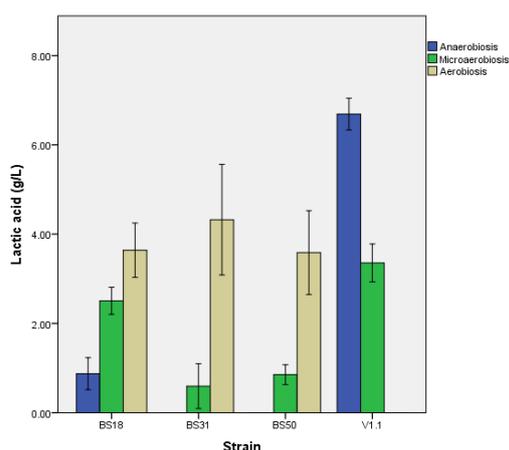


Figure 3. LA production from BS18, BS31 and BS50 strains under different oxygen concentrations.

The fermentation process begins with the formation of pyruvate through the Embden-Meyerhof pathway in which glucose is converted into 2 pyruvate molecules; After this step, the lactate dehydrogenase enzyme transforms pyruvate into LA generating energy in the form of NADH +. Although the absence of oxygen should stimulate

the metabolic routes associated with anaerobic respiration, along with fermentative processes, this was not evidenced in this work. *B. subtilis* generally has a reduced ability to grow anaerobically in media that does not contain pyruvate or added amino acids. Results similar to those obtained in terms of a reduction in the production of LA in anaerobiosis were evidenced by Ohara & Yahata in 1996 and Romero-Garcia et al. in 2009. The BS18 strain was robust against changes in oxygen availability, and, although the difference between the average production between aerobiosis and micro aerobiosis is statistically significant, the decrease in the average production is small, becoming an interesting option to reduce future operating costs.

3.4 Kinetics of biomass change, glucose consumption and LA production

In all three strains, a production of LA concordant with glucose consumption is observed, as observed in Figure 4, beginning at 9 h of fermentation, and stabilizing at 24 h. The production of LA by the 3 strains is homogeneous and does not present any difference according to the statistical analysis. On the other hand, with respect to biomass production, an exponential growth can be observed between 9 and 27 h of culture. After this, during the 27 and 30 h, the strains presented a stage after which a second phase of cell growth continues. Differences in strain growth are not statistically relevant, with the exception of strain 18, in which a stage of cell death prior to the second growth stage is evidenced. The mean LA production obtained in BS18, B531 and BS50 was 4.66 g, 5.86 g and 4.38 g under aerobic conditions, respectively, consistent with that evidenced in other Wild type strains of *B. subtilis*. (Ohara & Yahata, 1996; Romero-García et al., 2009) Maximum LA production prior to 24 h of culture in all the strains. However, as the time intervals studied were wide, more analysis is required to establish the fermentation time required to reach equilibrium. As the theoretical values of LA production correspond to 1 g glucose / 1 g LA, efficiencies were between 21.9 % to 29.33 %.

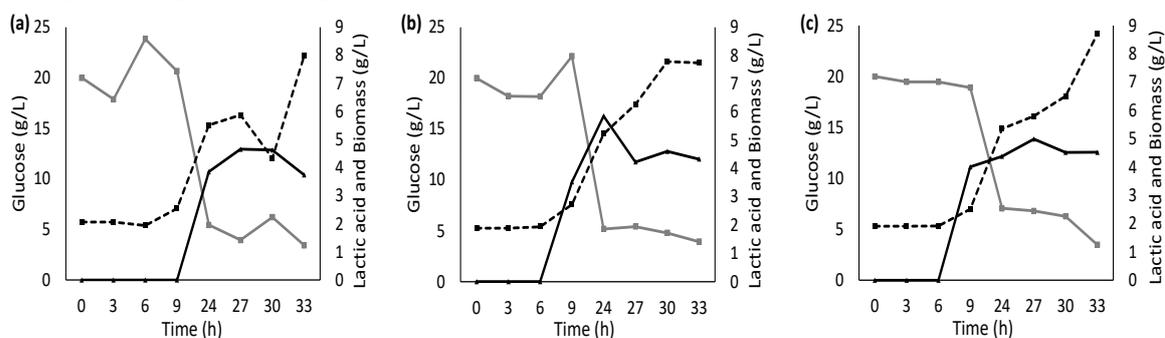


Figure 4. Kinetics of biomass growth (dashed line), glucose consumption (grey line) and LA production (black line) for different *B. subtilis* strains: (a) BS18, (b) BS31, and (c) BS50.

Even though fermentations from glucose maintained significant LA concentrations, these are low to be considered within the framework of industrial application. However, analysis regarding the optimization of LA production by adding elements known for their ability to improve the fermentation process through *B. subtilis*, such as pyruvate, and optimal concentrations of yeast extract, would be important to reach cost efficient conditions. Additionally, despite certain advantageous qualities identified in the strains, genetic improvement processes could be carried out to obtain yields close to those presented by lactobacillus species, while maintaining low costs in raw materials that work with *B. subtilis*. High LA productions have been obtained from modified strains such as *Bacillus subtilis* MUR1, with which 159 g / L were reached (Gao et al., 2012).

4. Conclusions

Through this study, the identification of 6 *Bacillus subtilis* strains was achieved through the analysis of the biochemical profile allowed by the API50CH / B galleries. Molecular analysis by sequencing the 16S region did not allow an effective identification of the strains analyzed, being necessary other markers for the determination of species in *Bacillus subtilis*. Of these strains, the highest LA production rate was achieved under aerobic conditions, with LA concentrations of approximately 5 g / L, starting with a glucose concentration of 20 g/L. Despite the potential for LA production from these strains, improvements can be made through optimization of medium composition and genetic improvement.

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