Vol. 18, No. 1 (2019) 131-141
Revista Mexicana de Ingeniería Química

A PLACKETT-BURMAN DESIGN FOR SUBSTITUTING MRS MEDIUM COMPONENTS WITH AVOCADO SEED HYDROLYSATE FOR GROWTH AND LACTIC ACID PRODUCTION BY *Lactobacillus* sp.

UN DISEÑO PLACKETT-BURMAN PARA SUSTITUIR LOS COMPONENTES DEL MEDIO MRS CON HIDROLIZADO DE SEMILLA DE AGUACATE PARA EL CRECIMIENTO Y LA PRODUCCIÓN DE ÁCIDO LÁCTICO POR *Lactobacillus* sp.

S. Espinel-Ríos, D.M. Palmerín-Carreño, A.L. Hernández-Orihuela, A. Martínez-Antonio*
Biological Engineering Laboratory, Genetic Engineering Department, Center for Research and Advanced Studies of the National Polytechnic Institute (Cinvestav), Campus Irapuato
Received: April 24, 2018; Accepted: May 1, 2018

Abstract
Lactic acid is a platform chemical with many commercial applications. Even though the biotechnological production is the leading approach, medium cost is a serious bottleneck. Starch from agroindustrial residues, such as avocado seeds, stands out as a promising feedstock. A Plackett-Burman design was used for screening and selection of MRS medium components that could be substituted with avocado seed hydrolysate (ASH) for growth and lactic acid production by *Lactobacillus* sp. The screening was performed using 20 g/L initial reducing sugar from the ASH; then, the effect of an increasing carbon-source concentration was evaluated. Five out of nine components could be substituted from the MRS medium for lactic acid production; this represented a medium cost reduction of at least 17%. Peptone from casein (10 g/L), meat extract (8 g/L), yeast extract (4 g/L), and sodium acetate (5 g/L) had to be maintained. The highest lactic acid concentration was estimated at 5.7 ± 0.7 g/L, achieved at 40.0 ± 2.0 g/L initial reducing sugar in experiments carried out in a stirred tank bioreactor, despite the presence of some inhibitory growth effects. Kinetic parameters were also obtained. This study may serve as a starting point for further research since this is the first study reported in the literature exploring the production of lactic acid from ASH as a feedstock.
Keywords: lactic acid, *Lactobacillus* sp., avocado seed hydrolysate, Plackett-Burman design.

Resumen
El ácido láctico es un compuesto químico con muchas aplicaciones comerciales. Se produce principalmente por vía biotecnológica, siendo el costo del medio de cultivo de gran importancia económica. El almidón de residuos agroindustriales como el de las semillas de aguacate, es una alternativa prometedora para emplearse en este proceso. En este estudio se utilizó un diseño Plackett-Burman para la selección de componentes del medio MRS que pudieran sustituirse con hidrolizado de semilla de aguacate (ASH) para el crecimiento y producción de ácido láctico por *Lactobacillus* sp. Se partió con una cantidad inicial de 20 g/L de azúcares reductores en el ASH; luego, se evaluó el efecto de una concentración creciente de la fuente de carbono. Cinco de nueve componentes del medio MRS pudieron ser sustituidos para la producción de ácido láctico, representando una reducción del costo del medio de cultivo en al menos 17%. Se mantuvo la concentración de peptona de caseína (10 g/L), extracto de carne (8 g/L), extracto de levadura (4 g/L) y acetato de sodio (5 g/L). La concentración máxima de ácido láctico fue de 5.7 ± 0.7 g/L, con 40.0 ± 2.0 g/L de azúcares reductores iniciales en los experimentos en biorreactor tipo tanque agitado, observándose también indicios de inhibición del crecimiento. Asimismo, se obtuvieron los parámetros cinéticos asociados al proceso. Este estudio sirve como punto de partida para futuras investigaciones, no encontrándose en la literatura un trabajo similar en donde se explore el uso de ASH como sustrato para la producción de ácido láctico.
Palabras clave: ácido láctico, *Lactobacillus* sp., hidrolizado de semilla de aguacate, diseño Placket-Burman.

1 Introduction

The world is facing a crucial situation where challenges like climate change, depletion of non-renewable resources, and a growing human population, to mention just a few, are seriously menacing present and future generations (Philippidis *et al*., 2016). The resource pressure is such that, if the population keeps growing at the same rates without changing the current production systems, two and three planets will be needed in terms of natural resources by 2030 and 2050, respectively. Thus, the conventional linear economic model of "take-make-dispose" is being reconsidered, driving the development of the so-called circular economy, characterized by a closed-loop system approach ("reduce-reuse-recycle") (Goyal *et al*., 2016).

* *Corresponding author. E-mail*: agustino.martinez@cinvestav.mx
doi: https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2019v18n1/Espinel ; *issn-e*: 2395-8472

In this sense, the pertinence of new working concepts such as biorefineries and bioeconomy become clearer and of urgent application. As formulated by the International Energy Agency (IEA): "Biorefinery is the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power, heat)" (Jong *et al*., 2012). The biorefinery concept is then a key aspect for the development of the bioeconomy, an economy where materials, chemicals and energy are obtained from renewable biological resources, in concordance with the circular economic model (McCormick and Kautto., 2013).

Avocados, mainly produced in North and Central America, are a major agroindustrial commodity. In 2016, global avocado production was estimated to be 5.57 tons of which Mexico contributed with 1.89 tons (FAO, 2016). The industrial processing of this fruit, mostly for guacamole and oil production, usually yields considerable amounts of by-products such as husks and seeds; the latter accounts up to 26% of the fruit mass. The chemical composition (dry weight) of lyophilized avocado peeled seeds (Hass variety) consists mainly of carbohydrates (79.5%), and other minor components, among them: lipids (5.5%), proteins (3.4%), crude fiber (4.0%), and ashes (4.2%) (Domínguez *et al*., 2014). It is worth noting that starch represents nearly 60% of the seed (dry matter basis) (Kahn, 1987), resulting in large amounts of potential fermentable sugars. Consequently, the avocado seed stands out as a very promising feedstock for industrial fermentations and biorefining.

On the other hand, lactic acid (2-hydroxypropanoic acid, CH_3-CHOHCOOH) has a wide range of applications within different industries (primarily in the food, chemical, cosmetic, and pharmaceutical sectors), to such extent that it is said to be the most widely occurring and important carboxylic acid (Gao *et al*., 2011; Pandey and Garg, 2013; Alsaheb *et al*., 2015). In a recent revision (Bozell and Petersen, 2010), lactic acid has been included in the list of the new "Top 10" chemical opportunities for biorefinery carbohydrates, this based on the following criteria: the extensive recent literature about this molecule, its multiple product applicability, the fact that it is an existing commercial product with large production volumes, and its platform potential.

Lactic acid market is greater than 300,000 t/year, with a price in the range of $1,300-1,500/t (Blanco *et al*., 2016). According to a recent market analysis (Grand View Research, 2017), the global lactic acid market size accounted for $2.08 billion in 2016 and it is expected to reach $9.8 billion by 2025. Regarding the demand for this compound, it is estimated to grow at an annual rate of 16.2% from 2017 to 2025. Lactic acid can be produced either biotechnologically (through sugar fermentation) or by chemical synthesis. Generally speaking, the latter consists in the hydrolysis of lactonitrile with strong acids, although it yields a racemic mixture of D(−) and L(+) isomers. In contrast, the fermentative process is considered a better option as it is more environmentally-friendly and has a high product specificity, generating pure L− or D−lactic acid depending on the biocatalyst used. In fact, the biotechnological approach accounts for around 90% of the world lactic acid production (Lasprilla *et al*., 2012). Lactic acid bacteria, mainly *Lactobacillus*, are the largest group of lactic acid-producing microorganisms (Gao *et al*., 2011; Pandey and Garg., 2013; Alsaheb *et al*., 2015; Bozell and Petersen, 2010; Blanco *et al*., 2016; Lasprilla *et al*., 2012). They are of industrial value due to their resistance against acidic environments and their high growth rates and productivities (John, 2009). Media composition is a crucial factor for the global efficiency and economics of lactic acid fermentation processes (John, 2009). Indeed, a very serious bottleneck of the biological production is the substrate cost, mainly the carbon-source cost. Then, finding cheap and renewable feedstocks such as agroindustrial residues represents a field of opportunity for this industry (Gao *et al*., 2011).

Therefore, the aim of this work was to evaluate the use of the avocado seed hydrolysate (ASH) as a feedstock for growth and lactic acid production by *Lactobacillus* sp., and statistically select supplementary nutrients that may enhance the product yield. Another goal was to explore the kinetic behavior of lactic acid fermentation from the ASH-based medium, at both flask and bioreactor levels, to facilitate further improvement and optimization research.

2 Materials and methods

2.1 *Microorganism*

A strain of lactic acid bacteria, isolated from a fermented dairy product, was identified as *Lactobacillus* sp. after 16S rDNA gene sequencing and phylogenetic analysis. It was maintained as a glycerol stock (15%), stored at −80 °C and grown in MRS broth (Sigma-Aldrich, 2017) at 37 °C under static conditions.

2.2 *Chemicals*

The main chemicals used in this study were as follow: lactic acid (85%) (Sigma-Aldrich), hydrochloric acid (Karal), sulfuric acid (Karal), sodium hydroxide (Karal), dextrose (Sigma-Aldrich), dinitro salicylic acid (Sigma-Aldrich), peptone from casein (Merck Millipore), meat extract (Becton Dickinson), yeast extract (Becton Dickinson), polysorbate (Tween 80) (Sigma-Aldrich), dipotassium phosphate (Sigma-Aldrich), sodium acetate (JT Baker), ammonium citrate (Sigma-Aldrich), magnesium sulfate (Sigma-Aldrich), and manganese sulfate (Sigma-Aldrich).

Table 1. Medium components used in the Plackett-Burman design at their low and high levels.

Variable	Medium component	Low level (−)	High level (+)
X_1	Peptone (g/L)	0	10
X_2	Meat extract (g/L)	0	8
X_3	Yeast extract (g/L)	0	4
X_4	Polysorbate (Tween 80) (mL/L)	0	1
X_5	Dipotassium phosphate (g/L)	0	2
X_6	Sodium acetate (g/L)	0	5
X_7	Ammonium citrate (g/L)	0	2
X_8	Magnesium sulfate (g/L)	0	0.2
X_9	Manganese sulfate (g/L)	0	0.05

2.3 Avocado seed hydrolysate (ASH)

The avocado (Persea americana Mill) seeds were finely ground and then hydrolyzed with hydrochloric acid (HCl) (1.0% v/v) under a thermal pressure treatment (120 °C, 0.1 MPa) for 15 min, following the methodology reported by Tzintzun-Camacho *et al*. (2016) and described in the patent application WO/2016/079568 (Martínez-Antonio *et al*., 2016). The amount of ground seed per liter of hydrolysate ranged from 10-20% w/v, depending on the desired reducing sugar concentration.

2.4 Analytical methods

Reducing sugars in the hydrolysate and in samples taken during experiments were measured by the dinitro salicylic acid (DNS) method (modified from Miller (1959)). DNS reagent was prepared as reported by Saqib and Whitney (2011). Samples mixed with the DNS reagent were boiled for 15 min, and then transferred to ice. Finally, distilled water was added prior to the measurement of absorbance at 575 nm. For the assay, the following dilution ratio was used (v/v): 1:1:8 (sample/DNS reagent/distilled water), with a final volume of 10 mL. A calibration curve with dextrose (0-1.0 g/L) was carried out as part of the method.

For lactic acid determination during the screening experiments, supernatants were recovered by centrifugation (DuPont Sorvall RC-5B, Wilmington, DE) at 3000 rpm for 15 min, and 5 mL samples were titrated with 0.1 N NaOH to a pH value of 8.3 (1 mL of 0.1 N NaOH = 9 mg of lactic acid) (Pandey and Garg, 2013). However, for the bioreactor experiment, concentrations of lactic acid were determined by high-pressure liquid chromatography (HPLC) (Agilent 1200, Santa Clara, CA) using an Aminex HPC-87H column (Bio-Rad, Richmond, CA) and a refractive index detector. Samples were run at 50 °C and eluted at 0.8 mL/min with 5 mM sulfuric acid. Reference lactic acid (Sigma) was chromatographed to determine its retention time.

Optical density (OD) was measured at 660 nm using a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, USA) to estimate bacterial growth (Chauhan *et al*., 2007). In all screening experiments, the size of the inoculum was adjusted to ~0.1 OD (absorbance) units. Inoculation and biomass quantification throughout the bioreactor cultivation are described in section 2.7.

2.5 Statistical screening of important nutrients

A Plackett-Burman design was selected to search for significant medium components affecting lactic acid and biomass production. Nine parameters, each taken from the composition of the MRS medium, were chosen for the screening. Only main effects were considered; that is, possible interaction effects between medium constituents were ignored. The statistical design was generated and the results for the 12 screening experiments were analyzed using Minitab® 17. The independent variables and their respective levels used in the study can be observed in Table 1. The positive control (PC) was the ASH supplemented with all medium components at their high levels (+). The high levels corresponded to the MRS formulation. All experiments were conducted in triplicate; they had an initial level of 20 g/L reducing sugar provided by the ASH (matching the glucose concentration present in the MRS culture medium). Additionally, a validation experiment of the model found with the Plackett-Burman design was performed under the same cultivating conditions, considering the components that resulted to be statistically significant for lactic acid production. The initial average pH was ~ 6.5, and cultures containing 10 mL of medium in 15 mL falcon tubes were incubated at 37 °C for 72 h under static conditions. Once the nutrients were statistically selected, the effect of an increasing carbon-source (ASH) concentration was analyzed in terms of growth and lactic acid production. Experiments were performed in duplicate, and carried out in 125 mL flasks containing 30 mL medium at different initial reducing sugar concentrations, ranging from 10.6 ± 1.8 to 67.6 ± 3.3 g/L. The latter concentration was the maximum achieved one through the hydrolysis treatment described above. Flasks were incubated under static conditions at 37 °C for 65 h; initial pH ~ 6.5.

Table 2. Results of the Plackett-Burman experiments. PC: positive control.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	Latic acid (g/L) (t=72h)	Biomass (OD_{660nm}) (t=72h)
1	+	+	-	+	-	-	-	+	+	2.412 ± 0.158	4.424 ± 0.158
2	+	-	+	-	-	-	+	+	+	1.482 ± 0.135	1.014 ± 0.031
3	+	+	-	+	+	-	+	-	-	2.973 ± 0.419	3.144 ± 0.053
4	+	-	+	+	-	+	-	-	-	3.540 ± 0.135	3.040 ± 0.122
5	-	-	-	-	-	-	-	-	-	0.246 ± 0.019	0.222 ± 0.043
6	-	+	-	-	-	+	+	+	-	2.310 ± 0.352	1.132 ± 0.085
7	-	+	+	+	-	+	+	-	+	3.259 ± 0.496	1.542 ± 0.098
8	+	+	+	-	+	+	-	+	-	3.846 ± 0.145	3.028 ± 0.523
9	-	+	+	-	+	-	-	-	+	2.150 ± 0.061	2.374 ± 0.078
10	-	-	-	+	+	+	-	+	+	1.461 ± 0.161	0.966 ± 0.151
11	-	-	+	+	+	-	+	+	-	1.906 ± 0.144	1.738 ± 0.097
12	+	-	-	-	+	+	+	-	+	3.342 ± 0.092	1.762 ± 0.149
PC	+	+	+	+	+	+	+	+	+	4.398 ± 0.120	2.554 ± 0.177

2.6 *Study of batch kinetics in flask*

After an adequate initial reducing sugar concentration was chosen, a kinetic experiment was set up using the statistically-selected medium. Four variables were followed and measured during the experiment: optical density, pH, and lactic acid and reducing sugar concentrations. Experiments were carried out in duplicate under the aforementioned cultivating conditions (37 °C, static conditions, initial pH ~ 6.5), using 250 mL flasks containing 150 mL culture medium.

2.7 *Bioreactor cultivation*

Lactobacillus sp. was cultivated in a 3 L stirred-tank bioreactor (Applikon, The Netherlands) (working volume 1.5 L) using the statistically-selected medium, and inoculated with a 10% v/v inoculum size. The culture was stirred at 50 rpm with two six-bladed Rushton-type impellers. pH was controlled at 6.0 by the addition of 4 M KOH/1 M HCl and temperature was maintained at 37 °C. No aeration was supplied. A silicone-based antifoaming agent (VRF-30) was added. Parameters were automatically controlled using a PID my-Control system, while kinetic data was collected with the BioXpert software, both supplied by the manufacturer.

Bacterial growth was monitored for 48 hours, and culture viability, expressed as colony forming units (CFU/mL), was determined by plate count. Additionally, biomass production was quantified as dry cell weight (DCW). For this, samples were centrifuged at 3000 rpm/20 min at 4 °C, and the cellular pellets were subsequently dried in an oven (PRONALAB, Mexico) at 80 °C for 24 h in order to be determined by gravimetry.

3 Results and discussion

3.1 *Effect of medium components on lactic acid production and biomass growth (Plackett-Burman experiment)*

Table 2 shows the results for lactic acid production and biomass growth for the Plackett-Burman experiment, and the regression analyses are presented in Tables 3 and 4, respectively. To begin with, the model for lactic acid production showed a good fit (R-sq = 95.20%). Using a significance level of α = 0.10, peptone and sodium acetate were found to be of relevant contribution for lactic acid production. The same would apply to meat and yeast extracts if α was widened to 0.15 and 0.20, respectively. On the other hand, the model for biomass growth was somewhat poor (R-sq = 88.44%).

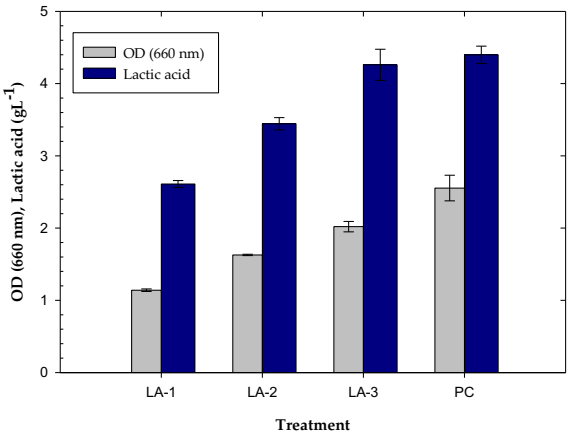

Fig. 1. Second round of experiments aimed at selecting the best nutrients for lactic acid production.

Table 3. Regression analysis of the Plackett-Burman design for lactic acid production.

	Medium component	Effect	Standard Error	T-value	P-value
X_1	Peptone (g/L)	1.044	0.155	3.37	0.078
X_2	Meat extract (g/L)	0.829	0.155	2.69	0.116
X_3	Yeast extract (g/L)	0.573	0.155	1.85	0.206
X_4	Polysorbate (Tween 80) (mL/L)	0.362	0.155	1.17	0.363
X_5	Dipotassium phosphate (g/L)	0.405	0.155	1.30	0.322
X_6	Sodium acetate (g/L)	1.098	0.155	3.54	0.071
X_7	Ammonium citrate (g/L)	0.269	0.155	0.87	0.477
X_8	Magnesium sulfate (g/L)	−0.349	0.155	−1.12	0.378
X_9	Manganese sulfate (g/L)	−0.119	0.155	−0.38	0.738

Model: Lactic acid production (g/L) = $0.354 + 0.1044X_1 + 0.1036X_2 + 0.1432X_3 + 0.362X_4 + 0.202X_5 + 0.2196X_6 + 0.135X_7 - 1.74X_8 - 2.38X_9$; R-sq = 95.20%.

Supposedly, no medium component alone was significant with an $\alpha = 0.10$. This is evidently false since a considerable drop in optical density was observed when no nutrients were supplemented to the medium (see run 5 in Table 2). However, peptone and meat extract were found to substantially influence cell growth considering an α-value of 0.15 and 0.20, respectively. Therefore, peptones and meat extract seemed to promote both lactic acid production and biomass growth, while sodium acetate would be essential to achieve high lactic acid concentrations.

Aiming at validating the model found with the Plackett-Burman experimental design and selecting with more certainty the significant medium components for lactic acid production, new experiments were performed under the same cultivating conditions, based on the previous significance analysis (see Table 5 and Fig. 1). Applying a Fisher's LSD (Least Significant Difference) test (95% confidence level), only treatments LA-3 and PC were not significantly different between each other regarding lactic acid production. With respect to biomass growth, all treatments were significantly different among each other. Nevertheless, ASH had the potential to replace five out of nine of the nutrients analyzed for lactic acid production, while peptone (10 g/L), meat extract (8 g/L), yeast extract (4 g/L), and sodium acetate (5 g/L) would need to be supplemented to the hydrolysate.

It is noticeable that the first three nutrients are all different nitrogen-sources. Indeed, lactic acid bacteria are regarded as "fastidious" microorganisms as they require various amino acids and peptides for growth. Due to their limited ability to synthesize their own amino acids from inorganic N-sources, exogenous amino acids from organic N-sources are fundamental (Nadra, 2007; Coelho *et al*., 2011; Hayek and Ibrahim, 2013). The fourth nutrient, sodium acetate, has been reported in the literature for positively influencing bacterial growth and lactic acid production in *Lactobacillus* spp. by retaining for longer times the enzyme activity of lactate dehydrogenase (Iino *et al*., 2003).

Previous studies have shown similar results. Samansoranakun and Adthalungrong (2012) evaluated 13 medium components for lactic acid production from tapioca starch hydrolysate by *Lactobacillus casei* TISTR 453. They found 10 nutrients that significantly influenced lactic acid production, among them were peptones, meat extract, winery yeast disposal, and sodium acetate. Chauhan *et al*. (2007) carried out an analogous study where they screened for significant medium components for lactic acid production from date (*Phoenix dactylifera*) juice by *Lactobacillus* sp. KCP01. They found that peptones, meat (beef) extract, yeast extract, and sodium acetate were also significant components.

3.2 *Effect of the initial reducing sugar concentration of ASH on lactic acid production and biomass growth*

Setting the medium composition of treatment LA-3 as constant based on the previous results, and varying the initial reducing sugar concentration from 10.6 ± 1.8 to 67.6 ± 3.3 g/L (see Table 6 and Fig. 2), resulted in a maximum lactic acid concentration of 4.3 ± 0.3 g/L at around 47.7 g/L initial reducing sugar. After this point, there was no significant difference on lactic acid production if more initial reducing sugars were added to the medium.

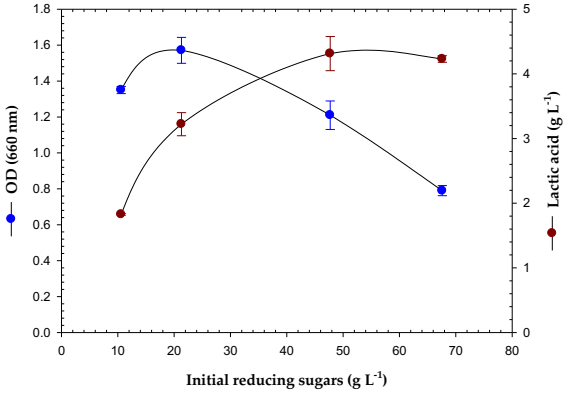

Fig. 2. Effect of increasing initial reducing sugar concentration on lactic acid production and biomass growth.

Table 4. Regression analysis of the Plackett-Burman design for biomass growth.

	Medium component	Effect	Standard Error	T-value	P-value
X_1	Peptone (g/L)	1.406	0.275	2.55	0.125
X_2	Meat extract (g/L)	1.150	0.275	2.09	0.172
X_3	Yeast extract (g/L)	0.181	0.275	0.33	0.774
X_4	Polysorbate (Tween 80) (mL/L)	0.887	0.275	1.61	0.249
X_5	Dipotassium phosphate (g/L)	0.273	0.275	0.50	0.669
X_6	Sodium acetate (g/L)	−0.241	0.275	−0.44	0.704
X_7	Ammonium citrate (g/L)	−0.620	0.275	−1.13	0.377
X_8	Magnesium sulfate (g/L)	0.036	0.275	0.07	0.953
X_9	Manganese sulfate (g/L)	−0.037	0.275	−0.07	0.953

Model: Biomass (OD_{660nm}) = $0.514 + 0.1406X_1 + 0.1438X_2 + 0.045X_3 + 0.887X_4 + 0.136X_5 - 0.048X_6 - 0.310X_7 + 0.18X_8 - 0.7X_9$; R-sq = 88.44%.

Regarding biomass growth, however, some inhibition was observed after 21.3 g/L initial reducing sugar. This inhibitory effect is widely reported in the literature and different toxicity mechanisms involving lactic acid production and accumulation have been postulated; for example, dissipation of the cell membrane potential, acidification of the cytosol, and intracellular anion accumulation. Although *Lactobacillus* sp. is more resistant against high lactic acid concentrations compared to other microorganisms, it is still inhibited by this organic acid after a certain threshold. Furthermore, the resistance capability varies among different lactic acid bacteria strains (Pieterse *et al*., 2005).

This inhibition could also be correlated with the residual reducing sugar concentration among the different treatments. At concentrations below 21.3 g/L, there was almost a 45% substrate consumption, but when the initial reducing sugar concentration was increased to 47.7 and 67.6 g/L, the overall substrate consumption dropped to 25.0 and 15.1%, respectively. Nevertheless, the average yield of product on substrate (lactic acid on reducing sugars) was considerably stable among the experiments, ranging only from 0.4 to 0.5 g/g. An approximately 47.7 g/L initial reducing sugar concentration seemed to be the more appropriate treatment, and thus was selected for the kinetic experiment.

3.3 Study of batch kinetics in flask

The flask kinetics using the statistically-selected medium, having an initial reducing sugar concentration of 49.0 ± 5.0 g/L, is presented in Fig. 3. Additionally, as can be seen in Table 7, similar results were obtained compared to treatment 3 in the previous experiment (Table 6). An important assumption made for the calculations was that no additional reducing sugars were produced during the fermentation process. This is based on previous laboratory experiments regarding the hydrolysis process that has been patented in our laboratory (Martínez-Antonio *et al*., 2016).

Several authors have reported lactic acid production from starchy biomass materials using *Lactobacillus* sp., and have obtained, in their best scenarios, yields (YP/S) up to 0.70 to 0.98 g/g and lactic acid concentrations up to 73-129 g/L (Gao *et al*., 2011; John *et al*., 2009; Samansoranakun and Adthalungrong, 2012). These yields are much more superior than those obtained in this study, indicating that for commercial purposes a different strain should be chosen. The low yields achieved in this study can be associated with the large amounts of reducing sugars that remained unconsumed at the end of the fermentations.

Thus, growing different *Lactobacillus* strains in the ASH-based medium may result in more competitive yields and product concentrations. In fact, strain improvement of industrial lactic acid bacteria is something commonly done in industry, including strategies such as random mutagenesis, directed evolution and dominant selection (Derkx *et al*., 2014). This is an interesting topic for future research. In terms of biomass growth, the specific growth rate and the doubling time was 0.50 h^{-1} and 1.39 h, respectively, close to those ones reported for *Lactobacillus* strains growing in MRS medium (0.49-0.55 h^{-1}, 1.2-1.4 h) (Brizuela *et al*., 2001).

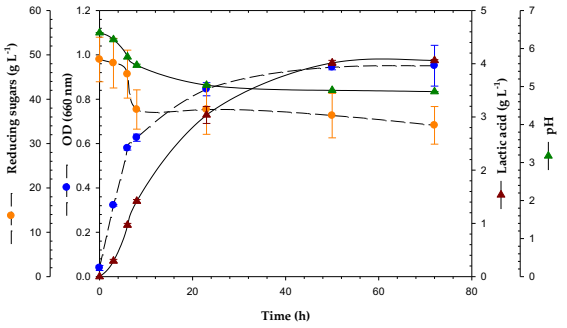

Fig. 3. Kinetics of *Lactobacillus* sp. in the statistically-selected medium at 47 g/L initial reducing sugars in Erlenmeyer flasks at 37 °C under static conditions. Error bars represent the standard deviation of triplicate experiments.

Table 5. Treatments aimed at validating the Plackett-Burman results.

Treatment	X_1	X_2	X_3	X_6	Lactic acid (g/L) (t = 72 h)	Biomass (OD_{660nm}) (t = 72 h)
LA-1	+	-	-	+	2.610 ± 0.048	1.140 ± 0.018
LA-2	+	+	-	+	3.444 ± 0.085	1.628 ± 0.009
LA-3	+	+	+	+	4.260 ± 0.216	2.020 ± 0.072

In the industrial batch production of lactic acid, the culture is usually generated in a series of inoculum vessels (size of inoculum 5-10% v/v) until it is finally transferred to the main fermenter (Ghaffar *et al*., 2014). In this and above-mentioned experiments, the criteria for the addition of the inoculum was the initial OD, resulting in an inoculum size less than 1% v/v. This, in turn, may have affected the overall fermentation results. That is why an inoculum size of 10% was used in the bioreactor experiment.

In the literature, lactic acid production has been tested using different fermentation regimes such as batch, fed-batch, and continuous. The highest lactic acid concentrations have been achieved through batch and fed-batch fermentations, while higher productivities have been obtained in continuous cultures; the latter fermentation process having the additional advantage of lasting for longer periods of time (Ghaffar *et al*., 2014).

Initial substrate inhibition and product inhibition have been reported in lactic acid fermentation processes. Similar to that observed in this study, significant inhibitory effects of sugar concentrations greater than 50 g/L have been found in batch cultures (Djuki *et al*., 2013). Therefore, it would be interesting to explore different fermentation regimes for lactic acid production such as fed-batch or continuous to minimize substrate inhibition. For instance, it was reported (Nancib *et al*., 2005) that maintaining a glucose concentration lower than 10 g/L during a date waste fermentation by *Lactobacillus casei* subsp. *rhamnosus* could significantly enhance lactic acid production.

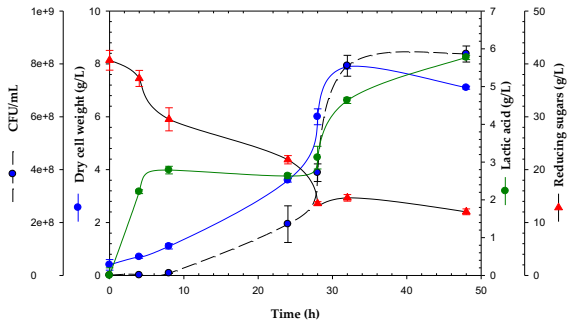

Fig. 4. Kinetics of *Lactobacillus* sp. growing on the statistically-selected medium in bioreactor at 47 g/L initial reducing sugar, 50 rpm, 37 °C, and pH 6.0. Data from two independent experiments are shown.

Regarding product inhibition, this phenomenon is clearly observed in Table 6 and Fig. 3, especially when the pH drops below 5. To avoid this problem, a widely-adopted strategy has been to maintain the pH between 5.0 to 6.5 by adding a base like ammonium hydroxide (Ghaffar *et al*., 2014). Therefore, it was decided to control the pH at 6.0 in the following bioreactor fermentation.

3.4 Growth of Lactobacillus sp. in bioreactor

The bioreactor kinetics of *Lactobacillus* sp. growing on the statistically-selected medium, with an initial reducing sugar concentration of 40.0 ± 2.0 g/L, is presented in Fig. 4. A photo of the bioreactor culture can be seen in Fig. 5. As can be seen in Table 7, the kinetic parameters in the bioreactor slightly improved when compared to the flask culture.

Glucose was not fully metabolized in 48 h; even after 24 h of incubation, approximately 50% of reducing sugars remained. The viable cell count in the ASH-based medium was 8.3 $\times 10^8$ CFU/mL after 48 h of fermentation. The maximum lactic acid concentration was 5.7 ± 0.7 g/L, achieved also after 48 h of fermentation.

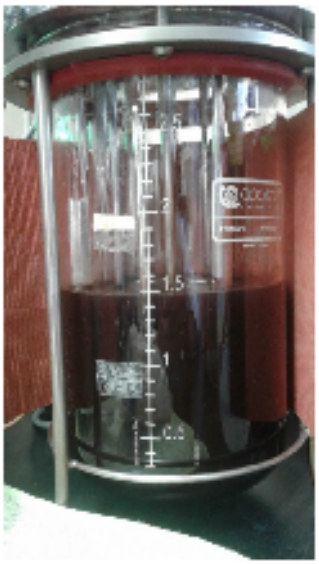

Fig. 5. *Lactobacillus* sp. growing on the ASH-based medium in the 3 L bioreactor.

Table 6. Results of different fermentation procedures, varying the initial reducing sugar concentration.

Treatment	Initial reducing sugars (g/L)	Residual reducing sugars (g/L)	Lactic acid production (g/L)	Final OD (660 nm)	$Y_{P/S}$ (g/g)	Final pH
1	10.6 ± 1.8	5.7 ± 0.2	1.8 ± 0.0	1.35 ± 0.02	0.4 ± 0.1	5.5 ± 0.0
2	21.3 ± 2.6	12.1 ± 0.6	3.2 ± 0.2	1.57 ± 0.07	0.4 ± 0.0	5.1 ± 0.0
3	47.4 ± 6.6	35.6 ± 1.8	4.3 ± 0.3	1.21 ± 0.08	0.4 ± 0.2	4.9 ± 0.0
4	67.6 ± 3.3	57.4 ± 4.2	4.2 ± 0.1	0.79 ± 0.03	0.5 ± 0.2	4.9 ± 0.0

These results are similar to others reported in the literature, even though the medium composition and the carbon source were different. Kyung Young *et al.* (2006) cultivated a L. casei strain using a culture medium based on cabbage juice (45.6 g/L initial sugar), reaching 11 $\times 10^8$ CFU/ mL after 48 h of fermentation at 30 °C. After 72 h of fermentation, 80% of the initial sugar content remained.

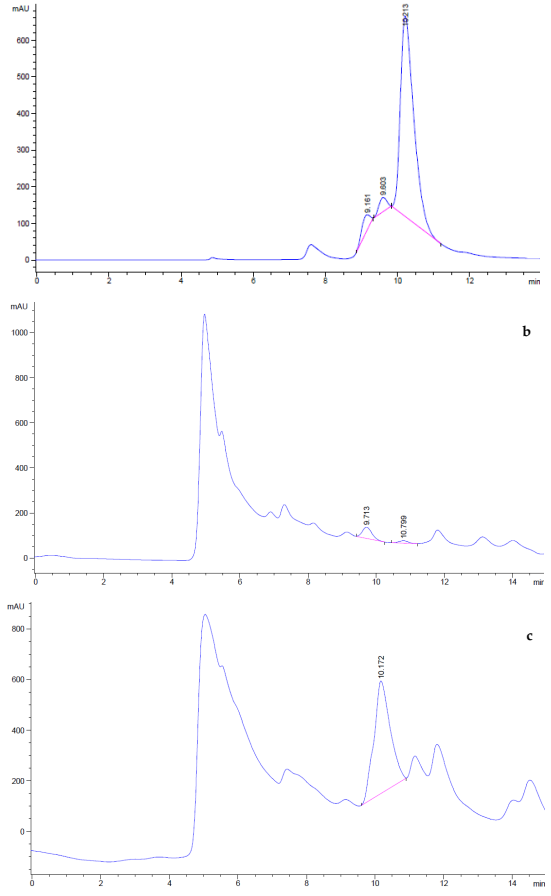

Fig. 6. HPLC profiles for a) standard of lactic acid, b) ASH-based medium alone (no fermentation occurred) and c) 48 h-fermentation sample. The lactic acid peak is identified by its retention time, 10.2 min.

Other authors have grown *L. rhamnosus* using glucose as the only carbon source (40 g/L) in a continuous bioreactor, resulting in a maximum specific growth rate (μmax) of 0.40 h^{-1}. Also, the maximum cell viability and biomass dry weight was estimated at 1.3 $\times 10^{10}$ CFU/mL and 5 g/L, respectively, achieved at dilution rates ranging from 0.28 h^{-1} and 0.35 h^{-1} (Liew *et al.*, 2006). Fig. 6 shows the chromatographic profile of the lactic acid produced during the fermentation, with a retention time of around 10.17 minutes.

The peak of the lactic acid standard is shown in Fig. 6 (a); in Fig. 6 (b), the peaks of the ASH culture medium can be observed without the presence of lactic acid (no fermentation occurred); finally, in Fig. 6 (c), the chromatogram of a 48 h-fermentation sample is presented, corresponding to the highest lactic acid concentration achieved. It would be interesting to search for possible presence of inhibitory compounds in the ASH-based medium as it could help to elucidate if the low lactic acid production is due to the strain used in the experiments or the composition of the medium. Moreover, if the inhibitory compounds had a high commercial value, the option of recovering such molecules from the hydrolysate, before the lactic acid fermentation takes place, could bring up the idea of a biorefinery with multiple products as an outcome.

In fact, Domínguez *et al.* (2014) reported the presence of minor constituents in avocado seeds (Hass variety) like catechol (602.5 ± 278.5 mg/100 g dry matter basis-d.m.b.) and tannic acid (332.8 ± 61.5 mg/100 g d.m.b.) which are regarded to have microbial growth inhibition potential as well as commercial applications (Papuc *et al.*, 2017).

Table 7. Flask and bioreactor kinetic results.

Parameter	Flask	Bioreactor
Specific growth rate, μ (h^{-1})	0.50 (R-sq = 0.89)	0.52 (R-sq = 0.98)
Doubling time, t_d (h)	1.39	1.33
YP/S (g/g)	0.3 ± 0.1	0.43 ± 0.08
Reducing sugar consumption (%)	28.6 ± 4.2	39.0 ± 1.2

Table 8. Costs associated with the medium components, excluding the carbon-source. ASH: Avocado Seed Hydrolysate. Month of analysis: March 2017.

Medium component	Unit cost ($US)	Unit	Amount (per L)	Cost (MRS medium) ($US/L)	Cost (ASH-based medium) ($US/L)	Supplier
Peptone	0.035	g	10	0.354	0.354	Sigma-Aldrich
Meat extract	0.173	g	8	1.382	1.382	bioWORLD
Yeast extract	0.045	g	4	0.181	0.181	Sigma-Aldrich
Polysorbate (Tween 80)	0.040	mL	1	0.040	N/A	bioWORLD
Dipotassium phosphate	0.094	g	2	0.188	N/A	Sigma-Aldrich
Sodium acetate	0.017	g	5	0.084	0.084	Sigma-Aldrich
Ammonium citrate	0.081	g	2	0.162	N/A	bioWORLD
Magnesium sulfate	0.029	g	0.2	0.006	N/A	Sigma-Aldrich
Manganese sulfate	0.082	g	0.05	0.004	N/A	Sigma-Aldrich
	Total			2.402	2.001	

3.5 Medium cost reduction (initial estimation)

As part of the study, a comparison of the component costs between the standard commercial MRS medium and the ASH-based medium for lactic acid production can be seen in Table 8. The latter medium was found to be beneficial from an economic point of view since it could be up to 17% cheaper than the conventional MRS medium. Note that this statement is without considering the cost of the carbon-source, in which case the ASH-based medium would be presumably more economical as the hydrolysis process can produce high reducing sugar concentrations (up to 68 g/L). Still, the impact of the production cost of the hydrolysate is not considered. Modelling and simulation approaches could be used to estimate the overall economic viability of the process; that is, not only including medium preparation but also fermentation and downstream processing steps. A good example of this approach is the work of Anaya-Reza and López-Arenas (2018), in which they assessed the technoeconomic viability of a biorefinery to produce lactic acid from sugarcane molasses.

It is noticeable that the most expensive nutrients in the analysis were the nitrogen sources (~80% cost in the MRS medium and ~95% cost in the ASH-based medium, excluding the carbon-source). Therefore, finding alternative nitrogen-sources is necessary for the development of an even more competitive medium. For example, some authors have reported the utilization of the hydrolysate of the insoluble protein content (gluten) of wheat in lactic acid fermentations with promising results (Hetenyi *et al*., 2008). Others have evaluated inexpensive organic nitrogen supplements such as flour of pigeon pea, red lentil, black gram, bengal gram, green gram, soya bean, and baker's yeast, finding that red lentils and baker's yeast cells were the best alternatives (Altaf *et al*., 2005; Altaf *et al*., 2007).

Conclusions

The avocado seed hydrolysate-based medium, supplemented with the statistically-selected nutrients, could support biomass growth and lactic acid production by *Lactobacillus* sp. Furthermore, an initial economic estimation showed that this alternative medium could be at least 17% cheaper than the conventional MRS medium for lactic acid production. However, the strain used in this study presented poor yields and produced low concentrations of lactic acid when compared to other processes based on starchy biomass materials. As far as the authors know, this study is the first one reported in the literature which explores the lactic acid production from the hydrolysate of avocado seed, and may serve as a basis for further improvement and optimization research.

Acknowledgements

Authors thank Alejandro Meléndez-Villalobos for his assistance with the fermentation experiments, and Berenice Cueva and Yolanda Rodríguez for their help with the HPLC quantifications.

References

Alsaheb, R., Aladdin, A., Othman, N., Malek, R., Leng, O., Aziz, R. and Enshasy, H. (2015). Lactic acid applications in pharmaceutical and cosmeceutical industries. *Journal of Chemical and Pharmaceutical Research 7*, 729-735.

Altaf, M., Naveena, B. and Ready, G. (2005). Screening of inexpensive nitrogen sources for production of L(+)

lactic acid from starch by amylolytic *Lactobacillus amylophilus* GV6 in single step fermentation. *Food Technology and Biotechnology 43*, 235-239.

Altaf, M., Naveena, B. and Reddy, G. (2007). Use of inexpensive nitrogen sources and starch for L(+) lactic acid production in anaerobic submerged fermentation. *Bioresource Technology 98*, 498-503.

Anaya-Reza O. and López-Arenas T. (2018). Design of a sustainable biorefinery for the production of lactic acid from sugarcane molasses. *Revista Mexicana de Ingeniería Química 17*, 243-259.

Bozell, J. and Petersen, G. (2010). Technology development for the production of biobased products from biorefinery carbohydrates−the US Department of Energy's "Top 10" revisited. *Green Chemistry 12*, 539-554.

Blanco, E., Loridant, S. and Pinel, C. (2016). Valorization of lactic acid and derivatives to acrylic acid derivatives: review of mechanistic studies. In: *Reaction Pathways and Mechanisms in Thermocatalytic Biomass Conversion II: Homogeneously Catalyzed Transformations, Acrylics from Biomass, Theoretical Aspects, Lignin Valorization and Pyrolysis Pathways*, (Schlaf, M. and Zhang, Z. C., eds.), Pp. 39-62. Springer, Singapore.

Brizuela, M., Serrano, P. and Ferez, Y. (2001). Studies on probiotics properties of two lactobacillus strains. *Brazilian Archives of Biology and Technology 44*, 95-99.

Coelho, L., De Lima, C., Rodovalho, C., Bernardo, M. and Contiero, J. (2011). Lactic acid production by new *Lactobacillus plantarum* LMISM6 grown in molasses: optimization of medium composition. *Brazilian Journal of Chemical Engineering 28*, 27-36.

Chauhan, K., Trivedi, U. and Patel, K. (2007). Statistical screening of medium components by Plackett-Burman design for lactic acid production by *Lactobacillus* sp. KCP01 using date juice. *Bioresource Technology 98*, 98-103.

Derkx, P.M.F., Janzen, T., Sørensen, K.I., Christensen, J.E., Stuer-Lauridsen, B. and Johansen, E. (2014). The art of strain improvement of industrial lactic acid bacteria without the use of recombinant DNA technology. *Microbial Cell Factories 13(Suppl 1)*, S5.

Djuki, A., Mojovi, L., Nikoli, S. and Pejin, J. (2013). Distillery stillage as a new substrate for lactic acid production in batch and fed-batch fermentation. *Chemical Engineering Transactions 34*, 97-102.

Domínguez, M., Araus, K., Bonert, P., Sánchez, F., San Miguel, G. and Toledo, M. (2014). The avocado and its waste: an approach of fuel potential/application. In: *Environment, Energy and Climate Change II*, (Lefebvre G., Jiménez E. and Cabañas B., eds.), Pp. 199-223. Springer, Cham.

FAO (2016). Food and Agriculture Organization of the United Nations. Available at: www.faostat3.fao.org/download/FB/FBS/E. Accessed: 03 March 2018.

Gao, C., Ma C. and Xu, P. (2011). Biotechnological routes based on lactic acid production from biomass. *Biotechnology Advances 29*, 930-939.

Ghaffar, T., Irshad, M., Anwar, Z., Aqil, T., Zulifqar, Z., Tariq, A., Kamran, M., Ehsan, N. and Mehmood, S. (2014). Recent trends in lactic acid biotechnology: a brief review on production to purification. *Journal of Radiation Research and Applied Sciences 7*, 222-229.

Goyal, S., Esposito, M., and Kapoor A. (2016). Circular economy business models in developing economies: lessons from India on reduce, recycle, and reuse paradigms. *Thunderbird International Business Review*, doi:10.1002/tie.21883.

Grand View Research (2017). Lactic acid market & polylactic acid (PLA) market, industry report 2025. Available at: www.grandviewresearch.com/industry-analysis/lactic-acid-and-poly-lactic-acid-market. Accessed: 03 March 2018.

Hayek, S. and Ibrahim, S. (2013). Current limitations and challenges with lactic acid bacteria: a review. *Food and Nutrition Sciences 4*, 73-87.

Hetenyi, K., Nemeth, A. and Sevella, B. (2008). Examination of medium supplementation for lactic acid fermentation. *Hungarian Journal of Industry and Chemistry 36*, 49-53.

Iino, T., Uchimura, T. and Komagata, K. (2003). The effect of sodium acetate on the activity of L− and D−lactate dehydrogenases in *Lactobacillus sakei* NRIC 1071T and other lactic acid bacteria. *The Journal of General and Applied Microbiology 49*, 51-58.

John, R., Nampoothiri, K. and Pandey, A. (2009). Direct lactic acid fermentation: focus on simultaneous saccharification and lactic acid production. *Biotechnology Advances 27*, 145-152.

Jong, E., Higson, A., Walsh, P. and Wellisch, M. (2012). Biobased chemicals - value added products from biorefineries. Publisher: IEA Bioenergy−Task 42 Biorefinery.

Kahn, V. (1987). Characterization of Starch Isolated from Avocado Seeds. *Journal of Food Science 52*, 1646-1648.

Kyung, Y., Edward E. and Yong D. (2006). Production of probiotic cabbage juice by lactic acid bacteria. *Bioresource Technology 97*, 1427-1430.

Lasprilla, A., Martinez, G., Lunelli, B., Jardini, A. and Filho, R. (2012). Poly-lactic acid synthesis for application in biomedical devices – a review. *Biotechnology Advances 30*, 321-328.

Liew, S., Rosfarizan M., Raha A., Ho Y. and Arbakariya B. (2006). Improved production of live cells of *Lactobacillus rhamnosus* by continuous cultivation using glucose-yeast extract medium. *Journal of Microbiology 44*, 439-446.

Martínez Antonio A., Minchaca Acosta A., Hernández Orihuela A., Rosales Colunga L. Estévez Palmas J. and Espinel Ríos S. (2016). Culture medium derived from avocado seed material. Patent pub. no. WO/2016/079568.

McCormick, K. and Kautto, N. (2003). The bioeconomy in Europe: an overview. *Sustainability 5*, 2589-2608.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry 31*, 426-428.

Nadra, M. (2007). Nitrogen metabolism in lactic acid bacteria from fruits: a review. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology 1*, 500-510.

Nancib, A., Nancib, N., Meziane, D., Boubendiral, A., Fick, M. and Boudrant, J. (2005). Joint effect of nitrogen sources and B vitamin supplementation of date juice on lactic acid production by *Lactobacillus casei* subsp. *rhamnosus*. *Bioresource Technology 96*, 63-67.

Pandey, S. and Garg, F. (2013). Preparation of spiced sauerkraut by using lactic acid bacteria and by natural fermentation. *International Journal of Science and Research 14*, 2319-7064.

Papuc, C., Goran, G. V., Predescu, C.N., Nicorescu, V. and Stefan, G. (2017). Plant polyphenols as antioxidant and antibacterial agents for shelf-life extension of meat and meat products: classification, structures, sources, and action mechanisms. *Comprehensive Reviews in Food Science and Food Safety 16*, 1243-1268.

Philippidis, G., Robert, M. and Ferrari, E. (2016). Drivers of the bioeconomy in Europe towards 2030 - short overview of an exploratory, model-based assessment. Publisher: Publications Office of the European Union.

Pieterse, B., Leer, R.J., Schuren, F.H.J. and van der Werf, M.J. (2005). Unravelling the multiple effects of lactic acid stress on *Lactobacillus plantarum* by transcription profiling. *Microbiology 151*, 3881-3894.

Samansoranakun, C. and Adthalungrong, C. (2012). Statistical screening of medium components for lactic acid production from tapioca starch hydrolysate by *Lactobacillus casei* TISTR 453 using Plackett-Burman design. *KKU Research Journal 17*, 754-761.

Saqib, A. and Whitney, P. (2011). Differential behaviour of the dinitrosalicylic acid (DNS) reagent towards mono- and di-saccharide sugars. *Biomass & Bioenergy 35*, 4748-4750.

Sigma-Aldrich 69966 MRS Broth (*Lactobacillus* Broth acc. to De Man, Rogosa and Sharpe) Available online: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Datasheet/1/69966dat.pdf (accessed on Feb 21, 2017).

Tzintzun-Camacho O., Sánchez-Segura L., Minchaca-Acosta A., Rosales-Colunga L., Hernández-Orihuela A. and Martínez-Antonio A. (2016). Development of bacterial culture medium from avocado seed. *Revista Mexicana de Ingeniería Química 15*, 831-842.