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Optimization of Lactic Acid Production Using Immobilized *Lactobacillus Rhamnosus* and carob Pod waste from the Lebanese Food Industry

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Abstract

The valorization of a solid carob waste from the Lebanese industry was investigated by optimizing the production of lactic acid using immobilized *Lactobacillus rhamnosus* in alginate beads and response surface methodology. The results showed that pH and alginate concentration had a significant effect on the production of lactic acid. The fermentation of non-enriched carob waste juice needed an additional nitrogen source to improve lactic acid production and yield. From extracts with 65 g/L sugars, the optimum conditions were found to be 2% for the concentration of alginate, 4% bacteria cells entrapped in beads, 80 rpm agitation speed and pH 6.4. Lactic acid concentration obtained under these conditions was 22 g/L with a yield of 76.9 g/g consumed sugar and a productivity of 1.22 g/L/h. The use of invertase pretreatment increased lactic acid concentration from 22 to 40 g/L, but reduced yield at 66.6%. Finally, cells immobilized in alginate beads could be used for at least five successive cycles.

Keywords: carob by-product; cell immobilization; lactic acid; *Lactobacillus rhamnosus*; response surface methodology

1. Introduction

The carob tree (*Ceratonia siliqua*) belongs to the family of leguminous plants that are grown in the Mediterranean regions (ZENGİN et al., 2008). This is often used as an ornamental plant and in the reforestation of areas damaged by erosion or desertification, given its low cultivation requirements and its tolerance of poor soils (Haddarah et al., 2013). The carob fruit consists of pulp and seeds. With a content of 64%, the carob pulp is particularly rich in sugar, richer than sugar cane and sugar beet (Haankuku et al., 2015; Limtong et al., 2007), and is currently used as a substitute for cacao. In recent years carob pulp has been used to produce value-added products such as mannitol, citric acid and pullulan by fermentation, while the seeds are used for the extraction of galactomannan to obtain locust bean gum (Haddarah et al., 2013).

Lactic acid is a valuable chemical platform that has extensive applications in food, cosmetics, pharmaceutical and chemical industries. Demand for lactic acid increases each year by 5 to 8% (Yadav et al., 2011). One of the main applications of lactic acid is its capacity to form polylactic acid (PLA), a polymer of great interest (Tian et al., 2018). The demand for PLA increases, while its production is only 450,000 tons per year (Okano et al., 2010). Lactic acid can be produced by chemical or fermentation routes. The chemical pathway leads to the production of a racemic mixture of DL-lactic acid, which increases the cost of separation, while fermentation leads to the production of D- or L-lactic acid depending on the organism used, and opens the opportunity to use cheaper substrates with low energy consumption and to develop a more environment-friendly process (Zhao et al., 2016). In general, fermentation is carried out using free cells, but the immobilization of cells sometimes improves productivity (Zhao et al., 2016). In addition, in relation with free cells, immobilization offers several advantages: namely, a reduced susceptibility to contamination, a decrease in substrate/product inhibition, and cell reuse in recycling steps. Calcium alginate is the most commonly used immobilization matrix (Zhao et al.,

2016) . Idris and Suzanna (Idris and Suzana, 2006) investigated the production of lactic acid from pineapple waste using *Lactobacillus delbrueckii* immobilized in alginate. Similarly, Sirisansaneeyakul et al.(Sirisansaneeyakul et al., 2007) studied the optimization of lactic acid production by immobilization of *Lactococcus lactis*. On the other hand, several alternative immobilization matrices are available, such as calcium pectate and chemically-modified chitosan gels (Kourkoutas et al., 2005). Zhao et al.(Zhao et al., 2016) found that the immobilization of *L. rhamnosus* in mesoporous silica-based material increased the fermentation efficiency by 4.1% over free cells.

In Lebanon, carob molasses, called “dibs”, is industrially produced from carob pods and widely consumed as a sweetener. The carob bean juice used for the preparation of molasses is obtained by two successive steps of maceration of carob pods cut into pieces of size close to 5 mm. The solid waste from this preparation is either discharged or used for animal feed. This waste has demonstrated its interest due to its high sugar content (44% of dry matter), and its ability to be fermented in bioethanol of second generation by *Saccharomyces cerevisiae* (Bahry et al., 2017). The only work investigating lactic acid production from carob juice has been carried out by Turhan et al.(Turhan et al., 2010a). Conversely, no data can be found on the lactic acid production from carob waste which is less rich in sugar. In addition many nutrients could be removed in the dibs preparation.

Thus, the purpose of this work is to explore another efficient route to valorize the residual carbohydrates of this carob waste by producing lactic acid using immobilized *Lactobacillus rhamnosus* in alginate beads, which differs from Turhan et al.(Turhan et al., 2010a) who worked directly on pods, using *Lactobacillus casei* in the liquid phase. The factors influencing the production, such as pH, alginate concentration, pre-culture volume and agitation speed were optimized using response surface methodology (RSM), as well as the number of possible

fermentation cycles using the same beads.

2. Materials and methods

2.1. Strain

The strain *Lactobacillus rhamnosus* ATCC 53103 was grown at 37°C for 48 hours in a medium containing 20 g of glucose, 4 g of yeast extract, 0.2 g of $MgSO_4.7H_2O$, 0.05 g of $MnSO_4.7H_2O$, 0.5 g of K_2HPO_4, 5 g of sodium acetate, and 0.5 g of KH_2PO_4 per liter of deionized water. The culture was stored at 4°C and subcultured bi-weekly in order to maintain viability. For a long-term storage, stock cultures were maintained in 20% glycerol at -80°C.

2.2. Extraction of sugars from carob waste

Carob waste (2.5 cm size) issued from molasses preparation was used in this work. Residual sugar extraction was obtained by maceration at room temperature for a liquid/solid ratio (L/S=3), without stirring for 90 minutes (Bahry et al., 2017). The concentration of total sugars in the juice was 65 g/L (54 ± 2% saccharose, and 46 ± 2% hexose, among which glucose and fructose are the most abundant).

2.3. Immobilization of Lactobacillus rhamnosus

Inoculum was prepared in the MRS medium (de Man, Rogosa and Sharpe) 24 hours before the fermentation step. Three volumes of pre-culture relative to the volume of work in the reactor (2, 4 and 6%) were used for the immobilization in alginate beads. After centrifugation in 50 mL sterile tubes at 3800 rpm and 4°C for 20 min, the pellet was mixed with 20 mL of alginate solution prepared in three concentrations (2, 3 and 4%). The bacteria were encapsulated in

alginate beads by the method of extrusion/gelation of the previously prepared solution using a pump (Gilson-Minipuls 2, France) and a syringe needle (type 20G, 0.9x40 mm) in 4% (w/w) $CaCl_2$ solution, leading to the production of beads of 5 mm approximate diameter. Then, the beads were left for 4 hours, and washed with a sterile 0.85% NaCl solution to remove non-adherent cells and the excess of calcium and chloride ions.

2.4. Batch fermentation

The fermentation was carried out in a 500-mL volume reactor, with a working volume of 400 mL. The carob juice was either used alone as unenriched carob waste (UECW), or supplemented with yeast extract, 10 g/L; MgSO4 7H2O, 0.5 g/L; MnSO4 H2O, 0.03 g/L; K2HPO4, 3 g/L; CH3COONa H2O, 2g/L and Tween 80, 1 mL/L, then denoted enriched carob waste (ECW). The fermentation medium was sterilized in the reactor at 121°C for 20 minutes. The alginate beads were transferred to the reactor in a sterile manner. Temperature was set at 37°C for all the tests and pH was adjusted by automatic addition of 5M NaOH. Lactic acid fermentation was studied for 24h and followed by sampling every 2h during the first 12h and then every 6h. Samples were analyzed for the determination of lactic acid and residual sugars.

In order to test the ability of reuse alginate beads, several batch fermentation runs were carried out in the reactor using the same beads. After 24h, the fermentation broth was removed and replaced by the same volume of sterile carob juice. Samples were taken for the analysis of lactic acid and residual sugars. To convert sucrose extracted from carob waste into monosaccharides, technical invertase from baker's yeast (*S. cerevisiae*) of 60 U/mg enzyme activity was employed at 55°C and pH 5.5.

2.5. Chemical analysis

Lactic acid concentration was determined using an HPLC device. The apparatus (HPLC 1260 Infinity Quaternary LC system, from Agilent Technology, USA) was equipped with two ion

exclusion columns connected in series (Rezex ROA 300X7.8 mm, Phenomenex, USA). The mobile phase was a 2 mM sulfuric acid solution, in ultrapure water (Millipore, MilliQmore), continuously degassed, and flow rate was fixed at 0.7 mL/min. Lactic acid was detected using a refractometer (HP 1100 series, Agilent Technologies, USA). Before analysis, samples were deproteinized to prevent clogging of the column. This was carried out as follows: 250 µL of barium hydroxide solution ($BaOH_2$, 0.3M) and 250 µL of zinc sulfate solution ($ZnSO_4.7H_2O$, 5% w/v) were added to a 2 mL sample, which caused the precipitation of proteins; after centrifugation for 5 min at 10,000 rpm, the supernatant was finally filtered using a cellulose acetate syringe filter (0.45µm).

Total sugars were determined using the method of Dubois et al.(Dubois et al., 1956), and reducing sugars were analyzed by the DNS method of Miller (Miller, 1959).

2.6. Experimental design and statistical analysis

Experiments were defined using the Design of Experiments (DOE) methodology. For the optimization of fermentation conditions, response surface methodology (RSM) was applied. Four factors were studied:

- Alginate concentration (A): the range chosen was between 2 and 4%, according to Idris and Suzana (Idris and Suzana, 2006), because for a concentration lower than 1%, the beads are easily broken, whereas a too high concentration causes a delay in sugar consumption by bacteria.
- pH (B): the range was fixed between 4.5 and 6.5 which is the optimal pH range for the growth of *Lactobacillus* (Nancib et al., 2001).
- Agitation speed (C): the range was between 50 and 150 rpm to maintain sufficient mass

transfer, without breaking the beads.

- Amount of bacteria Cells Entrapped in Beads, referred to as ACEB (D): this amount ranged between 2 and 6%, which is classical in alginate beads (Ercan et al., 2013).

The minimum and maximum values of these four main factors and their coded levels are summarized in Table 1. A four-factor Box–Behnken design was applied to examine the relationship between one or more response variables and for process optimization (Box and Behnken, 1960). This included 27 runs, including 3 replications of the center point. The response variables were the amount of lactic acid produced (P, g/L), the yield (YP/S, %), and the maximum production rate (QP, g/L/h).

The free software environment R (Team, 2014) was used for the creation and analysis of experimental designs and multiple comparison. These were analyzed using ANOVA. Parameters with a p-value (p) of 0.05 or less were considered significant.

Table 1

3. Results and discussion

3.1. Preliminary experiments

Preliminary experiments involving the carob waste highlighted that *Lactobacillus* was able to use saccharose or glucose as the substrate when a single carbon source was used. Conversely, when saccharose and glucose were added simultaneously, only the hexose was consumed. As carob waste extracts contains 35 g/L and 30 g/L saccharose and hexoses, respectively, this shows that *L. rhamnosus* prefers monosaccharides as the substrate and that metabolic adaptation from glucose to saccharose is not rapid.

3.2. Analysis of the experimental design

3.2.1. ANOVA and model assessment

197 The experimental results of Box–Behnken design are summarized in Table 2. This clearly
198 displays a significant evolution of lactic acid production P (from 15 to 22 g/L), yield YP/S (from
199 65.0 to 77%) and productivity Q_P (from 0.45 to 1.22 g/L/h) as a function of the four main factors.
200 A quadratic model with interactions was applied to fit experimental data. The ANOVA tables for
201 the three response variables are summarized in Table 3. The determination coefficient R^2 is
202 satisfactory for P and QP, R^2=0.941, 0.947, respectively. Conversely, it is weak for YP/S, about
203 0.77. In addition, the lack-of-fit of the model is statistically significant only for YP/S, which
204 implies that the linear model is not adapted for fitting the evolution of this parameter. Another
205 information that arises from Table 3 is that many predictors do not play a significant role among
206 the interaction and the quadratic terms, so that the models can be significantly reduced to a few
207 influential predictors. A descending method was applied: this consists in removing iteratively the
208 predictor with the highest p-value until all the predictors are statistically significant.

209 **Table 2**

210 The reduced models are summarized in Table 4 for P and QP, while no significant model could
211 be deduced for YP/S, as expected from Table 3. R^2 was 0.87 and 0.92 for P and QP, respectively,
212 which is satisfactory. Finally, A, B, the quadratic term B^2 and the interaction AD (interaction
213 between alginate concentration and the Amount of bacteria Cells Entrapped in Beads) exhibited
214 a significant effect on P, while D and the same predictors were significant on QP.

215 The final model equation for acid lactic production based on coded levels was:

216 $$P = 20.3 - 1.38\times A + 2.12\times B + 0.9\times AD - 0.9\times B^2$$

217 In parallel, the reduced model for QP was:

218 $$QP = 1.01 - 0.15\times A + 0.21\times B + 0.05\times D + 0.08\times AD - 0.12\times B^2$$

219 The good agreement between the observed values and those predicted by these equations
220 arises from Figure 1. The optimum conditions determined from these models for both
221 variables were very close, so that it was not necessary to define a desirability function for
222 multiple responses: the minimum value of alginate concentration (A) studied, 2%, with a pH (B)
223 of 6.4, close to the maximum value studied. For C and D factors, which played a secondary role,
224 intermediate values of 80 rpm for agitation speed (C) and 4% for ACEB (D) were retained. The
225 value of lactic acid production corresponding to these parameters was 22.2 g/L, while
226 productivity was 1.24 g/L/h. Experimental runs with these optimal conditions were carried out
227 and the experimental values of 22.1± 0.2 g/L and 1.22± 0.08 g/L/h for production and
228 productivity were observed, respectively, which was in very good accordance with the model.

229 **Table 3**

230 In comparison to Turhan et al.(Turhan et al., 2010a), the initial sugar concentration was between
231 100–105 g/L sugar because these authors used directly carob pods for sugar extraction, while a
232 waste was used in this work. This explains why fermentation started only with 65 g/L sugar, but
233 shows that the waste remains very rich in extractable sugars. As a result, (Turhan et al., 2010a)
234 achieved 31.35 g/L lactic acid with a supplemented medium, while it was only 22.1 g/L in this
235 work. However, YP/S was only 68.6% in their study, while 77% was reached in the present
236 work. As the medium and the microorganism both differ, it is not possible to conclude
237 definitively on the role of immobilization. In addition, sugar utilization was about 44% in both
238 studies, which shows that immobilization does not significantly change this parameter.

239 **Figure 1**

240 **Table 4**

241 However, it arises clearly from Table 2 that *L. rhamnosus* immobilized in alginate beads is able

242 to produce effectively lactic acid. But as in Turhan et al.(Turhan et al., 2010a) with *L. casei*, it
243 seems that *L. rhamnosus* is only able to use monosaccharides as the substrate, which could
244 explain the high amount of unconsumed sugar at the end of each run in both studies.

245 *3.2.2. Effect of alginate concentration (A)*

246 Experimental results clearly demonstrate that the increase in alginate concentration has a
247 negative effect on the yield of lactic acid (Fig.2). They show that 2% leads to a lactic acid
248 concentration of 22 g/L and a yield of 77% (Table 2). Higher concentrations than 2% alginate may
249 lead to a delay in the consumption of glucose and fructose because it would be more difficult for
250 sugars to percolate into the cell pores (Ercan et al., 2013). As already mentioned, for an or
251 alginate concentration lower than 1%, beads were too soft and were, therefore, easily broken due
252 to their low mechanical strength, thus causing bacteria to leak throughout the beads. These
253 conclusions are consistent with those found by Idris and Suzana(Idris and Suzana, 2006) on the
254 production of lactic acid by *Lactobacillus delbrueckii* using pineapple waste as substrate.
255 Similarly, Givry et al.(Givry et al., 2008) found a significant difference in the yield of lactic acid
256 obtained by *Lactobacillus bifermentans* on hemicellulose hydrolyzates with 2 to 4% alginate.
257 The same results have also been reported by Najafpour et al. (Najafpour et al., 2004)and Ercan et
258 al. (Ercan et al., 2013), but in the production of ethanol. As a conclusion, the results of Table 2
259 fully agree with the literature and *L. rhamnosus* displays the same behavior as other
260 microorganisms when immobilization in alginate beads is applied.

261 *3.2.3. Effect of pH (B)*

262 pH is a very important parameter in fermentation, as it affects at least two aspects of
263 microbial metabolism: the metabolism of enzymes and the transport of nutrients into the cells
264 (Panesar et al., 2010). In Table 2 and from ANOVA in Table 3, it emerges that it affects
265 significantly lactic acid production, which also emerges in Figure 2. Contrary to alginate

concentration, both B and B^2 predictors are statistically significant and the coefficient estimates are of opposite sign, which means that a parabolic shape with a maximum is expected. With *lactobacilli*, the critical pH value is 4 and the optimal pH of growth is between 4.5 and 6.4 (Nancib et al., 2001). It is known that when the pH approaches the pK_a of lactic acid (about 3.9), the undissociated form of lactic acid plays a more inhibitory effect than the dissociated lactate form. At a pH lower than 5, the undissociated acid form cannot be neglected, whereas a pH greater than 6 leads to almost complete dissociation of lactic acid, which explains why the optimal pH of fermentation around 6 (Pal et al., 2009). This is in agreement with Figure 2 in which the effect of pH is stronger between 4.5 and 5.5 than between 5.5 and 6.5. A pH of 5.5 was found by Ghaly et al.(Ghaly et al., 2004) as for the optimum production of lactic acid by *L. helveticus*. From the results obtained, a pH of 6.4 was found to be the optimal value. This result is close to that of Idris and Suzana (Idris and Suzana, 2006) in which 6.5 was reported to maximize the production of lactic acid by *L. delbrueckii*. Similarly, Panesar et al.(Panesar et al., 2010) found a maximum conversion rate of lactose and an optimum lactic acid production of 33.5 g/L with a pH value of 6.5. Krischke et al.(Krischke et al., 1991) also reported that a pH interval between 6.0 and 6.5 is optimal for lactic acid production by *Lactobacillus casei.* As a conclusion, the optimum pH observed for immobilized *L. rhamnosus* is close to the optimum values reported in the literature for similar microorganisms in liquid phase, as expected.

Figure 2

3.2.4. Effect of agitation rate (C)

Agitation speed is a key parameter for the efficient transfer of nutrients from the bulk to the cells

290 in the fermentation process (Liu and Shen, 2008). In this work, Table 3 clearly shows that this
291 factor is not statistically significant. Contradictory results can be found in the literature on this
292 topic. Bai et al.(Bai et al., 2003) reported that lactic acid production increased by 42% when
293 stirring was increased from 0 to 300 rpm. The same conclusion was drawn in Roukas (Roukas,
294 1994), where alcoholic fermentation on carob juice was enhanced by higher agitation speed.
295 Ercan et al.(Ercan et al., 2013) selected 150 rpm as the optimum agitation rate to produce ethanol
296 from carob bean juice by immobilized *Saccharomyces cerevisiae* cells. However, Panesar et
297 al.(Panesar et al., 2010) found that there was no difference in the pro- duction of lactic acid
298 between fermentation with and without agitation, in agreement with this work. Finally, this work
299 highlights that contrary to expectations, mass transfer is not the limiting step, even when
300 agitation speed is 50 rpm. This shows that immobilization does not slow down lactic
301 fermentation because of mass transfer limitation.

302 *3.2.5. Effect of ACEB (D)*

303

304 Based on the results of the experimental design, this parameter does not exhibit a significant
305 effect on the production of lactic acid and has a weak role on productivity (Table 4). However,
306 the interaction AD with alginate concentration is always significant, which shows that this factor
307 may have a weak non-linear behavior which is not accounted for by the model. In this work,
308 4% was retained for the volume of inoculum, which does not exactly fit the optimized values
309 because the optimum is not the same when P and QP are considered. This behavior differs
310 significantly from the other main factors, A and B. ACEB has a slight positive effect on QP,
311 but this is compensated by the AD interaction: when QP is maximized at low pH (i.e. when A
312 tends towards -1), the AD and the D terms are, therefore, of opposite sign in the QP expression
313 and their sum plays a weak role in the model. Finally, 4% agrees with the work of Panesar et

al.(Panesar et al., 2010) in which the maximum concentration of lactic acid was obtained with an inoculum size between 2 and 4%. The increase in inoculum size is supposed to induce an increase in biomass concentration and consumption and production rates, which explains why D has a stronger influence on QP in this work. The reason of an optimum inoculum, volume can be found in Laluce et al.(Laluce et al., 2009) who reported that a high cell density can negatively affect ethanol production due to a lack of nutrients, a limit of space and too strong cell interactions. Similarly, 3% was the optimal inoculum size for ethanol production by *S. cerevisiae* in Izmirlioglu and Demirci (Izmirlioglu and Demirci, 2012) and in Turhan et al.(Turhan et al., 2010b).

3.2.6. Effect of the AD (Alginate×ACEB) interaction

The role of the alginate×ACEB interaction has been slightly studied in the literature. Figure 3 illustrates AD interaction for P and QP. As already mentioned, the AD interaction compensates the effect of D in P. For QP, it appears clearly that the effect of D becomes insignificant when A= -1 in Figure 3. More generally, it also arises from this figure that when A is decreased, D should also be decreased to enhance lactic acid production. Actually, concentrations of alginate higher than 2% may delay the diffusion of sugars in the beads. So, an increase of alginate concentration requires an increase of the inoculum to maintain the nutrient accessibility to biomass. This explains why the AD interaction is significant both on P and QP in Table 4.

Figure 3

3.3. Further improvements of the fermentation process

3.3.1. Batch fermentation using UECW

In order to study whether carob waste juice can be considered as a complete fermentation

337 medium, a negative control has been achieved on carob juice without enrichment. Experimental
338 results display a significant difference for the kinetic parameters between the enriched carob
339 waste (ECW) and the unenriched carob waste (UECW) in Table 5. The concentration of lactic
340 acid produced on ECW media was 22 g/L, whereas this value was only 14 g/L for the UECW
341 medium. In addition, there was also a significant difference in maximum production rate: for
342 ECW, QP was 1.22 g/L/h, whereas this value was reduced to 0.43 g/L/h for UECW. Similarly,
343 the fermentation of ECW displayed a yield of 77%, while the yield YP/S was decreased to
344 48.5% for UECW. This result agrees with the data from Turhan et al.(Turhan et al., 2010a) in
345 which YP/S fell at 41% without enrichment. This result highlights that the fermentescibility of
346 the carob waste does not differ significantly from that of the fruit, which means that similar
347 nutrients must be added to enhance lactic acid production. As in Turhan et al.(Turhan et al.,
348 2010a), the issue is that carob and, consequently, the carob waste does not contain an adequate
349 nitrogen source. Both contain only proteins (Bahry et al., 2017), while among the nutritional
350 elements, yeast extract was considered essential for *Lactobacillus* to obtain efficient production
351 of lactic acid (Aeschlimann and Von Stockar, 1990). In the study of Göksungur and Güvenç
352 (Göksungur and Güvenç, 1997), the presence of nitrogen source improved lactic acid production
353 from 10 g/L to 60 g/L. Similarly, Nancib et al.(Nancib et al., 2001) reported that the
354 concentration of lactic acid rose from 30 ± 2.4 g/L without yeast extract, to 46 g/L with yeast
355 extract. In addition, manganese ions, a constituent of lactate dehydrogenase, have been shown to
356 have a strong effect on lactic acid production. On date juice, supplementation by yeast extract
357 has been shown to improve production yield, compared to non-enriched date juice (Nancib et al.,
358 2001). Similar conclusions were drawn by Turhan et al. (Turhan et al., 2010a) who compared
359 five nitrogen sources among which the yeast extract maximized the production of lactic acid.
360 Thus, all the kinetic parameters show the need to enrich the culture medium with a source of

nitrogen and elements that are necessary for the growth of *Lactobacillus* bacteria. This conclusion can be generalized for Carob pods and carob waste, as similar results were reported concerning the importance of enrichment of culture medium during the production of bioethanol (Bahry et al., 2017; Germec et al., 2015).

Table 5

3.3.2. Reusability of alginate beads

Among the objectives of immobilization, the ability to re-use beads with immobilized cells after replacement of the culture medium is one of the most relevant. For that purpose, repeated-batch fermentation was carried out to assess the number of possible cycles performed by the immobilized cells while maintaining the stability of the alginate beads. The same optimum fermentation conditions defined in section 3.2 were always applied (2% alginate, pH 6.4, stirring rate 80 rpm and ACEB 4%; total initial sugar concentration: 65 g/L).

From the results obtained in Table 5, cells immobilized in alginate beads can be used for five successive cycles with only a small reduction in yield. At the fifth cycle, the yield decreased by up to 72± 2%, with a small leakage of the cells into the culture medium; this can be explained by the possible combination of lactic acid with phosphate, citrate and calcium ions leading to the disintegration of calcium alginate. However, the P and QP did not significantly change among the fifth run, which highlights that more cycles could probably be applied. This result agrees with Ganguly et al.(Ganguly et al., 2007) who investigated the production of lactic acid with loofa sponge immobilized in *Rhizopus oryzae* RBU2-10 could be carried out for 10 cycles with a high productivity (1.66–1.84 g/L/h) during the first 5 cycles. Similar results were also reported on ethanol production using *Saccharomyces cerevisiae* immobilized in alginate by Ercan et al.(Ercan et al., 2013). As a conclusion, this result highlights the interest of *L. rhamnosus*

385 immobilization for lactic acid production.

386 *3.3.3. Pretreatment of carob juice by invertase enzyme*

387 In the extracts, the major sugars are glucose, fructose and sucrose. During fermentation with
388 *Lactobacillus rhamnosus*, glucose and fructose were consumed by the microorganism, whereas
389 sucrose was not metabolized. These results are similar to those found by Nancib et al.(Nancib et
390 al., 2001) using *L. rhamnosus* and date juice as the substrate. During lactic acid fermentation on
391 pineapple syrup, sucrose was not easily metabolized by *Lactococcus lactis* because bacteria
392 preferentially used glucose and fructose (Ueno et al., 2003). The same conclusion was drawn by
393 Turhan et al. (Turhan et al., 2010a) using carob juice and *L. casei*. This may be explained by the
394 fact that enzymes for sucrose catabolism are not synthesized when glucose and fructose exist in
395 the medium, indicating that glucose and fructose have probably caused the repression of sucrose
396 catabolism. In a study involving the simultaneous fermentation of D-xylose and glucose by
397 *Candida shehatae*, Kastner et al.(Kastner et al., 1998) concluded that glucose inhibits the
398 catabolism of D-xylose by suppressing the induction of D-xylose enzymes. For this purpose, a
399 conversion of sucrose was necessary. As a result, only a pretreatment of the extracts by an
400 invertase enzyme, as in Turhan et al.(Turhan et al., 2010a), was able to lead to the total
401 conversion of sucrose into glucose and fructose and enhance lactic fermentation. Thus, the
402 fermentation was replicated on carob waste juice with a total concentration of reducing sugars of
403 65 g/L.

404

405 The carob waste juice was treated with an invertase prior to the addition of pre-culture, under
406 optimum conditions previously described. A DNS analysis was performed to validate the total
407 conversion of sucrose into simple sugars. After the pre-culture was added, the fermentation
408 was started by keeping the same optimum conditions of fermentation found by the

409 experimental design. The results show that, after treatment with the invertase enzyme, sugar
410 utilization, and lactic acid production and productivity increased up to 92%, 40 g/L and 1.66
411 g/L/h, compared to 44%, 22 g/L and 1.2 g/L/h without enzymatic treatment, respectively (Table
412 6). The yield decreased from 77% to 66.6%, which shows that increasing sugar concentration
413 promotes biomass growth at the expense of lactic acid productivity. Similar conclusions were
414 drawn by Turhan et al. (Turhan et al., 2010a). Similarly, (Ueno et al., 2003) have improved the
415 production of lactic acid from pineapple waste by *Lactococcus lactis* after using another
416 invertase enzyme extracted from grape.

417 **Table 6**

418 As a conclusion, low cost invertase can be used to subsequently enhance sugar utilization,
419 lactic acid production and productivity, but at the expense of yield. This stems from the sugar
420 composition of extracts from carob waste which contains both mono- and disaccharides. The
421 inability of lactic bacteria to ferment at the same time these mono- and disaccharides seems
422 general in the literature, which means that it is favored or repressed neither by the waste, nor by
423 immobilization. Similarly, *L. casei* and *L. rhamnosus* display apparently the same behavior, but
424 the reusability of immobilized cells remains a key advantage of this study.

425 **4. Conclusions**

426 The solid carob waste resulting from molasses production in the Lebanese food industry is an
427 attractive source to produce lactic acid. Extraction process is able to provide extracts
428 containing 65 g/L sugars. However, the enrichment of the culture medium, especially in yeast
429 extract as a nitrogen source, is necessary to significantly increase the yield of lactic acid
430 production at constant sugar utilization. The optimum conditions deduced from response
431 surface methodology lead to a final concentration of lactic acid of 22 g/L, a yield of 76.9% and a
432 productivity of 1.22 g/L/h. Further improvements included a pretreatment with invertase, so

that a final concentration of 40 g/L and a productivity of 1.66 g/L/h could be achieved, but with a reduced yield of 66.6%. Immobilization was shown to be an efficient process for the reuse of microorganisms, which allowed an easy separation of the biomass from the liquid phase. The reusability of the beads up to five cycles was demonstrated. A techno-economic analysis must be carried out, now, to optimize the process from sugar extraction to lactic acid purification and determine whether a pre-concentration step of sugar is necessary.

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REFERENCES

Aeschlimann, A., Von Stockar, U., 1990. The effect of yeast extract supplementation on the production of lactic acid from whey permeate byLactobacillus helueticus. Appl. Microbiol. Biotechnol. 32, 398–402.

Bahry, H., Pons, A., Abdallah, R., Pierre, G., Delattre, C., Fayad, N., Taha, S., Vial, C., 2017. Valorization of carob waste: Definition of a second-generation bioethanol production process. Bioresour. Technol. 235, 25–34. https://doi.org/10.1016/j.biortech.2017.03.056

Bai, D.-M., Jia, M.-Z., Zhao, X.-M., Ban, R., Shen, F., Li, X.-G., Xu, S.-M., 2003. (+)-lactic acid production by pellet-form Rhizopus oryzae R1021 in a stirred tank fermentor. Chem. Eng. Sci. 58, 785–791.

Box, G.E.P., Behnken, D.W., 1960. Simplex-sum designs: a class of second order rotatable designs derivable from those of first order. Ann. Math. Stat. 31, 838–864.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P. t, Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Ercan, Y., Irfan, T., Mustafa, K., 2013. Optimization of ethanol production from carob pod extract using immobilized Saccharomyces cerevisiae cells in a stirred tank bioreactor. Bioresour. Technol. 135, 365–371.

Ganguly, R., Dwivedi, P., Singh, R.P., 2007. Production of lactic acid with loofa sponge immobilized Rhizopus oryzae RBU2-10. Bioresour. Technol. 98, 1246–1251.

Germec, M., Turhan, I., Karhan, M., Demirci, A., 2015. Ethanol production via repeated-batch fermentation from carob pod extract by using Saccharomyces cerevisiae in biofilm reactor. Fuel 161, 304–311.

Ghaly, A.E., Tango, M.S.A., Mahmoud, N.S., Avery, A.C., 2004. Batch propagation of Lactobacillus helveticus for production of lactic acid from lactose concentrated cheese whey with microaeration and nutrient supplementation. World J. Microbiol. Biotechnol. 20, 65–75.

Givry, S., Prevot, V., Duchiron, F., 2008. Lactic acid production from hemicellulosic hydrolyzate by cells of Lactobacillus bifermentans immobilized in Ca-alginate using response surface methodology. World J. Microbiol. Biotechnol. 24, 745–752.

Göksungur, Y., Güvenç, U., 1997. Batch and continuous production of lactic acid from beet molasses by Lactobacillus delbrueckii IFO 3202. J. Chem. Technol. Biotechnol. Int. Res. Process Environ. Clean Technol. 69, 399–404.
Haankuku, C., Epplin, F.M., Kakani, V.G., 2015. Industrial sugar beets to biofuel: field to fuel production system and cost estimates. Biomass Bioenergy 80, 267–277.
Haddarah, A., Ismail, A., Bassal, A., Hamieh, T., Ioannou, I., Ghoul, M., 2013. Morphological and chemical variability of Lebanese carob varieties. Eur. Sci. J. ESJ 9.
Idris, A., Suzana, W., 2006. Effect of sodium alginate concentration, bead diameter, initial pH and temperature on lactic acid production from pineapple waste using immobilized Lactobacillus delbrueckii. Process Biochem. 41, 1117–1123.
Izmirlioglu, G., Demirci, A., 2012. Ethanol production from waste potato mash by using Saccharomyces cerevisiae. Appl. Sci. 2, 738–753.
Kastner, J.R., Jones, W.J., Roberts, R.S., 1998. Simultaneous utilization of glucose and D-xylose by Candida shehatae in a chemostat. J. Ind. Microbiol. Biotechnol. 20, 339–343.
Kourkoutas, Y., Xolias, V., Kallis, M., Bezirtzoglou, E., Kanellaki, M., 2005. Lactobacillus casei cell immobilization on fruit pieces for probiotic additive, fermented milk and lactic acid production. Process Biochem. 40, 411–416.
Krischke, W., Schröder, M., Trösch, W., 1991. Continuous production of l-lactic acid from whey permeate by immobilizedLactobacillus casei subsp. casei. Appl. Microbiol. Biotechnol. 34, 573–578.
Laluce, C., Tognolli, J.O., De Oliveira, K.F., Souza, C.S., Morais, M.R., 2009. Optimization of temperature, sugar concentration, and inoculum size to maximize ethanol production without significant decrease in yeast cell viability. Appl. Microbiol. Biotechnol. 83, 627–637.
Limtong, S., Sringiew, C., Yongmanitchai, W., 2007. Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated Kluyveromyces marxianus. Bioresour. Technol. 98, 3367–3374.
Liu, R., Shen, F., 2008. Impacts of main factors on bioethanol fermentation from stalk juice of sweet sorghum by immobilized Saccharomyces cerevisiae (CICC 1308). Bioresour. Technol. 99, 847–854.
Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.
Najafpour, G., Younesi, H., Ismail, K.S.K., 2004. Ethanol fermentation in an immobilized cell reactor using Saccharomyces cerevisiae. Bioresour. Technol. 92, 251–260.
Nancib, N., Nancib, A., Boudjelal, A., Benslimane, C., Blanchard, F., Boudrant, J., 2001. The effect of supplementation by different nitrogen sources on the production of lactic acid from date juice by Lactobacillus casei subsp. rhamnosus. Bioresour. Technol. 78, 149–153.
Okano, K., Zhang, Q., Yoshida, S., Tanaka, T., Ogino, C., Fukuda, H., Kondo, A., 2010. D-lactic acid production from cellooligosaccharides and β-glucan using L-LDH gene-deficient and endoglucanase-secreting Lactobacillus plantarum. Appl. Microbiol. Biotechnol. 85, 643–650.
Pal, P., Sikder, J., Roy, S., Giorno, L., 2009. Process intensification in lactic acid production: a review of membrane based processes. Chem. Eng. Process. Process Intensif. 48, 1549–1559.
Panesar, P.S., Kennedy, J.F., Knill, C.J., Kosseva, M., 2010. Production of L (+) lactic acid using Lactobacillus casei from whey. Braz. Arch. Biol. Technol. 53, 219–226.

Roukas, T., 1994. Kinetics of ethanol production from carob pods extract by immobilizedSaccharomyces cerevisiae cells. Appl. Biochem. Biotechnol. 44, 49–64.
Sirisansaneeyakul, S., Luangpipat, T., Vanichsriratana, W., Srinophakun, T., Chen, H.H.-H., Chisti, Y., 2007. Optimization of lactic acid production by immobilized Lactococcus lactis IO-1. J. Ind. Microbiol. Biotechnol. 34, 381.
Team, R.C., 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2013.
Tian, X., Wang, Y., Chu, J., Mohsin, A., Zhuang, Y., 2018. Exploring cellular fatty acid composition and intracellular metabolites of osmotic-tolerant mutant Lactobacillus paracasei NCBIO-M2 for highly efficient lactic acid production with high initial glucose concentration. J. Biotechnol. 286, 27–35.
Turhan, I., Bialka, K.L., Demirci, A., Karhan, M., 2010a. Enhanced lactic acid production from carob extract by Lactobacillus casei using invertase pretreatment. Food Biotechnol. 24, 364–374.
Turhan, I., Bialka, K.L., Demirci, A., Karhan, M., 2010b. Ethanol production from carob extract by using Saccharomyces cerevisiae. Bioresour. Technol. 101, 5290–5296.
Ueno, T., Ozawa, Y., Ishikawa, M., Nakanishi, K., Kimura, T., 2003. Lactic acid production using two food processing wastes, canned pineapple syrup and grape invertase, as substrate and enzyme. Biotechnol. Lett. 25, 573–577.
Yadav, A.K., Chaudhari, A.B., Kothari, R.M., 2011. Bioconversion of renewable resources into lactic acid: an industrial view. Crit. Rev. Biotechnol. 31, 1–19.
ZENGİN, E., SAĞLIKER, H.A., Darici, C., 2008. Carbon mineralization of Ceratonia siliqua L. soils under different temperature and humidity conditions. Turk. J. Bot. 32, 123–127.
Zhao, Z., Xie, X., Wang, Z., Tao, Y., Niu, X., Huang, X., Liu, L., Li, Z., 2016. Immobilization of Lactobacillus rhamnosus in mesoporous silica-based material: An efficiency continuous cell-recycle fermentation system for lactic acid production. J. Biosci. Bioeng. 121, 645–651.

Figure legends

Figure 1: Plot of predicted vs. observed P and QP values.

Figure 2: Illustration of the influence of alginate concentration and pH on lactic acid production (Fig.2A) and productivity (Fig. 2B).

Figure 3: Interaction plots between alginate concentration and ACEB (D). Effect on: (Fig.3A) Lactic acid production *p* (g/L); (Fig.3B) productivity Q*p* (g/L/h).

Figure 1

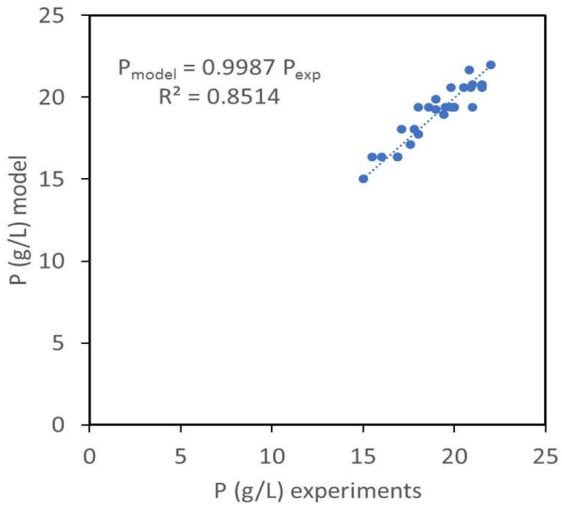


Fig 1.A

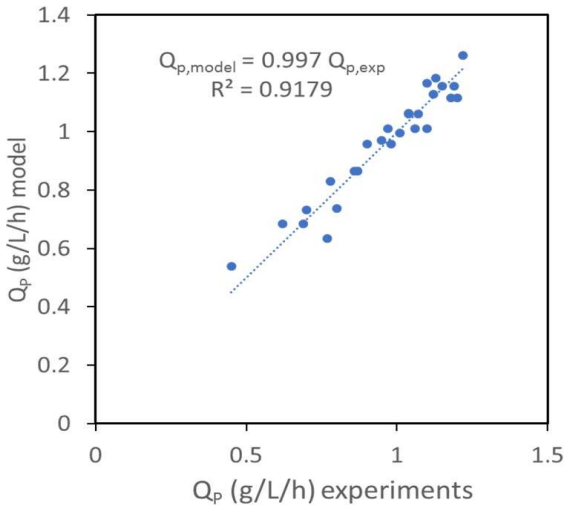
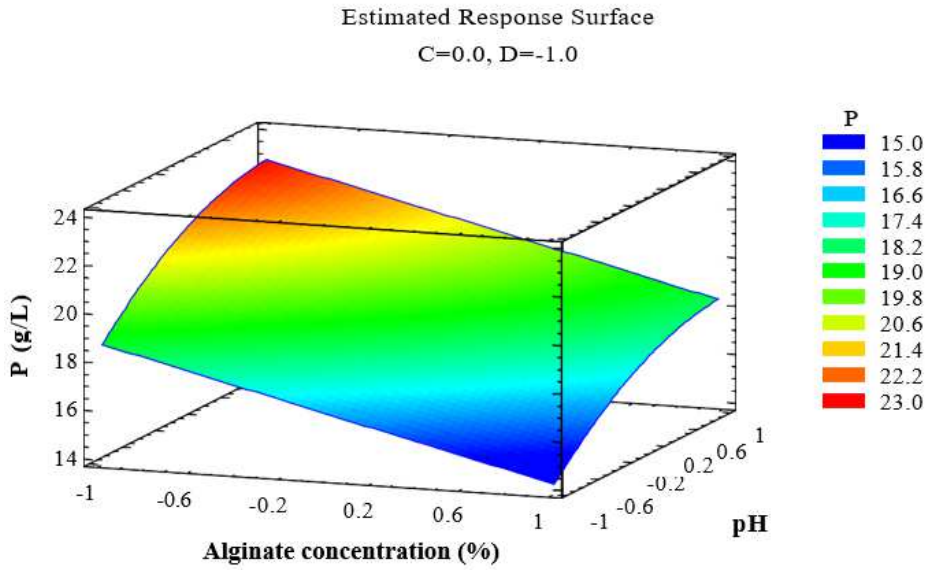


Fig 1.B

577 **Figure 2**



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579 **Fig 2.A**

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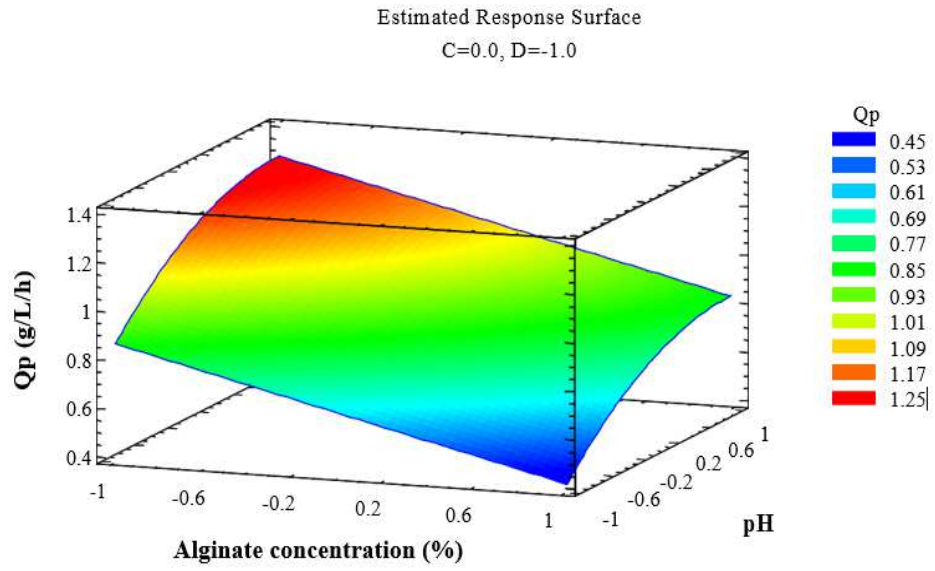

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582 **Fig 2.B**

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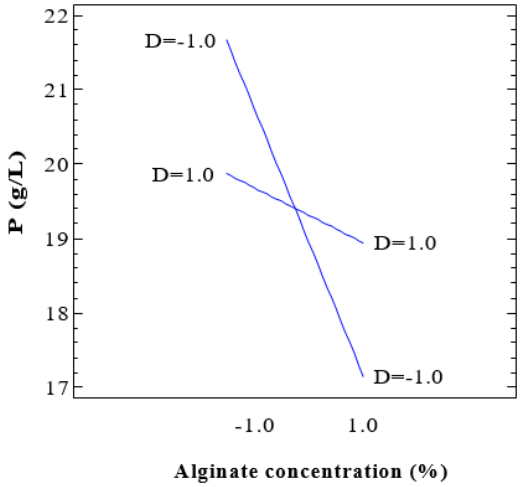
Figure 3



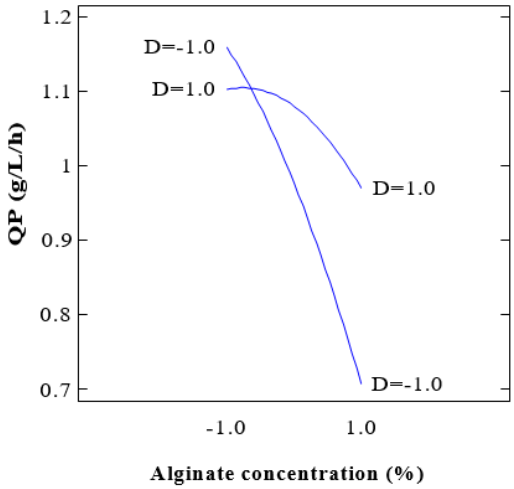


Fig 3.A **Fig 3.B**

Tables

Table 1. Range and levels of the independent variables in the Box–Behnken design.

Factor	Code	-1	0	+1
Alginate concentration (%)	A	2	3	4
pH	B	4.5	5.5	6.5
Agitation speed (rpm)	C	50	100	150
ACEB (%)	D	2	4	6

Table 2. Experimental data (lactic acid production P, yield YP/S, and maximum productivity QP) collected in the runs of the Box–Behnken design as a function of the four main factors.

collected in the runs of the Box–Behnken design as a function of the four main factors.

Run	Alginate concentration (%)	pH	Agitation speed (rpm)	ACEB (%)	P (g/L)	YP/S (%)	QP (g/L/h)
1	0	0	0	0	20	73.6	1.06
2	0	0	0	0	21	74	1.10
3	0	0	0	0	19.8	73.3	0.97
4	-1	-1	0	0	18	72.0	0.78
5	+1	-1	0	0	15	65.2	0.45
6	-1	+1	0	0	22	76.9	1.22
7	+1	+1	0	0	19	76.6	0.95
8	0	0	-1	-1	18	69.2	0.90
9	0	0	+1	-1	18.6	70.0	0.98
10	0	0	-1	+1	19.52	72.3	1.04
11	0	0	+1	+1	20	74.0	1.07
12	-1	0	0	-1	20.8	74.3	1.13
13	+1	0	0	-1	17.6	73.4	0.70
14	-1	0	0	+1	19	67.9	1.12
15	+1	0	0	+1	19.4	77.6	1.01
16	0	-1	-1	0	15.5	62.0	0.62
17	0	+1	-1	0	21.5	75.5	1.18
18	0	-1	+1	0	16.9	70	0.69
19	0	+1	+1	0	20.5	78.8	1.20
20	-1	0	-1	0	21	76.4	1.15
21	+1	0	-1	0	17.1	70.6	0.86
22	-1	0	+1	0	21.5	77.7	1.19
23	+1	0	+1	0	17.8	69.5	0.87
24	0	-1	0	-1	16.9	70.7	0.77
25	0	+1	0	-1	19.8	75.5	1.04
26	0	-1	0	+1	16	64.0	0.80
27	0	+1	0	+1	20.9	76.8	1.10

Table 3. ANOVA analysis for the three responses (significant p-values are in bold).

	P (g/L)		$Y_{P/S}$ (%)		Q_P (g/L/h)	
Predictor	Sum of Squares	p-value	Sum of Squares	p-value	Sum of Squares	p-value
A	22.4	0.0179	12.3	0.0100	0.255	0.0169
B	53.7	0.0076	262	0.0005	0.555	0.0079
C	0.599	0.3520	16.4	0.0075	0.00521	0.3917
D	0.811	0.2962	0.0234	0.7076	0.0320	0.1150
A^2	1.104	0.2438	1.75	0.0647	0.0102	0.2684
AB	0.000	1.0000	10.6	0.0116	0.0009	0.6964
AC	0.0100	0.8907	1.45	0.0765	0.000225	0.8427
AD	3.24	0.1074	28.6	0.0044	0.0256	0.1382
B^2	8.06	0.0476	7.42	0.0165	0.0800	0.0512
BC	1.440	0.2029	5.45	0.0222	0.000625	0.7434
BD	1.00	0.2601	16.0	0.0077	0.000225	0.8427
C^2	1.280	0.2205	4.67	0.0258	0.000075	0.9084
CD	0.0036	0.9342	0.216	0.3193	0.000625	0.7434
D^2	2.36	0.1395	4.95	0.0244	0.0012	0.6547
Lack-of-fit	4.979	0.5361	113.393	0.0110	0.0504	0.5552
Pure error	0.826		0.2504		0.00886	
Total error	97.54		487.32		1.026	

673 **Table 4.** Summary of ANOVA and coefficient estimates of the reduced models for P and QP,

674 including df (degree of freedom), p (p-value) and the standard error of the estimates.

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P (g/L)							
Source	Sum of Squares	df	Mean Square	F	p	Coefficient	Error
A	22.4133	1	22.4133	34.03	0.0000	-1.38	0.23
B	53.7633	1	53.7633	81.63	0.0000	2.12	0.23
AD	3.24	1	3.24	4.92	0.0436	0.9	0.40
B^2	5.49643	1	5.49643	8.35	0.0119	-0.9	0.31
Lack-of-fit	3.41657	8	0.427072	0.65	0.7265		
Pure error	9.2202	14	0.658586				
Total (corr.)	97.5499	26					
QP(g/L/h)							
Source	Sum of Squares	df	Mean Square	F	p	Coefficient	Error
A	0.255208	1	0.255208	127.47	0.0000	-0.146	0.013
B	0.5547	1	0.5547	277.06	0.0000	0.216	0.013
D	0.0320333	1	0.0320333	16.00	0.0039	0.0517	0.013
AD	0.0256	1	0.0256	12.79	0.0072	0.08	0.023
B^2	0.0806667	1	0.0806667	40.29	0.0002	-0.122	0.017
Lack-of-fit	0.0620417	13	0.00477244	2.38	0.1107		
Pure error	0.0160167	8	0.00200208				
Total (corr.)	1.02627	26					

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693 **Table 5.** Evolution of kinetic parameters of lactic acid production when recycling alginate beads
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Kinetic parameters	Optimum condition (ECW)	Cycle 1 (ECW)	Cycle 2 (ECW)	Cycle 3 (ECW)	Cycle 4 (ECW)	Cycle 5 (ECW)	Culture on UECW
Sugars Consumed (g/L)	29^{a} ±1.2	28^{a} ±4	28.0^{a} ±0.9	29^{a} ±3	28^{a} ±2	29^{a} ±4	29^{b} ±4
Lactic acid (g/L)	22^{a} ±0.9	22^{a} ±2	21.4^{a} ±0.5	22^{a} ±4	21^{a} ±2	20^{a} ±3	14^{b} ±3
Yield (%)	77^{a} ±2	79^{a} ±4	76.0^{a} ±0.9	75^{a} ±5	75^{a} ±4	72^{b} ±3	49^{c} ±3
Production rate (g/L/h)	1.22^{a} ±0.08	1.16^{a} ±0.02	1.2^{a} ±0.6	1.1^{a} ±0.1	1.2^{a} ±0.9	1.2^{a} ±0.3	0.43^{b} ±0.08

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696 In each line, the same superscript letter within columns indicates homogeneous subset.

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713 **Table 6.** Kinetic parameters of lactic acid production from carob waste (CW)
714 and carob waste treated with invertase enzyme (CWI).

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Kinetic parameters	CW	CWI
Initial sugar concentration (g/L)	65[a]	65 [a]
Sugars consumed (g/L)	28.6[a]	60[b]
Lactic acid produced, P (g/L)	22[a]	40[b]
Yield, P (%)	76.9[a]	66.6[b]
Max production rate, QP (g/L/h)	1.22[a]	1.66[b]

716 In each line, the same superscript letter within columns indicates homogeneous subset.

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