Bio-silage of mussel work-processing wastes by lactobacilli on semi-solid culture.

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ABSTRACT

The aim of this work was to evaluate the fermentability of mussel work-processing wastes by lactic acid bacteria in order to remove and to upgrade a material that generate important focuses of pollution in coastal areas. With this perspective, three lactobacilli (*Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus buchneri*) were employed, and the production of metabolites, as well as the nutrient uptake, was evaluated. The effects of inoculum concentration and previous sterilization process were also studied. The kinetic tests were performed in semi-solid cultures and the results indicated the high feasibility of these materials as substrate for bio-silage production. Cultivations of 24 h led to productions of more than 90 g L^{-1} of lactic acid and 9 g L^{-1} of final protein. All the fermentation assays were stable for various days without contaminations by other bacteria.

Keywords: mussel wastes; by-products upgrading; lactobacilli; bio-silage; lactic acid production.

INTRODUCTION

Mussel culture production is an economical activity of great importance in Galicia (NW, Spain), since in this region there is currently 3360 tray-mussel farms distributed in 50 cultivation areas along the coast. In 2006, 247000 tons of mussels were obtained what it represents 99% of Spanish production and 45% of EU. This extensive production generates a large volume of wastes associated with the different steps from mussel work-processing, collection,

transformation and canning. Thus, around 80000 tons per year of shells are wasted from these farms and foodstuff companies. This by-product presents a high fraction of calcium carbonate that is management by means of thermal process with the obtaining of a calcareous material of high purity (Barros et al., 2009a). However, another type of residuals appear in the mussel-production chain. These are mainly originated in the primary works of harvest and processing on the tray-farms what from now we will call mussel work-processing (MWP). Due to their high composition in organic material (rests of mussels, epifauna, zooplankton, phytoplankton), heterogeneity of size and high volume (~35000 tons per year) they provoke serious reductions in the efficiency and in the yield of the thermal process for the treatment of the inorganic material of shell wastes (Barros et al., 2009a). Therefore, it is necessary to develop a complementary and environmental friendly process to allow a global use of the different fractions derived from mussel production (Barros et al., 2009a; Barros et al., 2009b).

In the last years the most relevant alternative for the use of these materials has been associated with their application as amending and nutrient supplement of degraded grounds (mines, forest burnt). Nevertheless, the results have not been positive and satisfactory due to the high cost of transport for these materials. Thus, these wastes are currently being deposited in non-controlled landfill or, more frequently, dumped directly to the sea. Another possible and more realistic alternative could be obtained by means of biological silage using lactic acid bacteria (Pagarkar et al., 2006). This is an easy and low cost process that generates an acid fermentative product with good nutritious qualities,

antimicrobial features against pathogen bacteria and high stability for a long time that can be used as protein supplement for animal feeding (Goddard and Perret, 2005). Different lactic acid bacteria (LAB) have been used for obtaining bio-silage of marine by-products: *Lactobacillus plantarum* (Fagbenro and Jauncey, 1995; Lassen, 1995; Pagarkar et al., 2006), *Lactobacillus brevis* (Uchida et al., 2004), yoghurt-bacteria as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Yoon et al., 1997), *Lactobacillus delbruecki* spp. *bulgaricus* and *Streptococcus salivarius* spp. *thermophilus* (Martínez-Valdivieso et al., 1996) as well as *Lactobacillus buchneri* and *Lactobacillus casei* (Vázquez et al., 2008a). In all cases, an additional source of carbohydrates was necessarily added in the form of molasses (Fagbenro and Jauncey, 1995; Hammoumi et al., 1998) or dextrose (Vázquez et al., 2008a). Though the fermentative capacity of many organic nitrogen source from marine waste materials has been tested with excellent results (Dufossé et al., 2001; Ellouz et al., 2001; Vázquez et al., 2004a; Vázquez et al., 2004b; Martone et al., 2005; Aspmo et al., 2005a; Aspmo et al., 2005b; Vázquez et al., 2006; Gao et al., 2006; Vázquez et al., 2008b) as well as wastewaters from thermal process of mussel (González et al., 1992; Pastrana et al., 1993; Murado et al., 1993; Guerra and Castro, 2002; Vázquez et al., 2003; Vázquez et al., 2004c; Guerra et al., 2005), the present work is the first one that studies the useful of by-products from MWP as substrate for bio-silage formulations.

Based on these considerations, in this manuscript a preliminary study of fermentability of MWP wastes by lactic acid bacteria is reported. A quick and

easy fermentative process similar to the bio-silage is established in order to propose an environmental friendly solution for this contaminant material.

MATERIALS AND METHODS

1: Preparation and composition of material from mussel wastes

A representative sample of MWP wastes (10 kg) was collected from mussel-tray farm placed in Boiro (Ría de Arousa, Galicia, Spain). Its composition was principally shells (45%), remnants of meat (20%), epifauna (15 %), algae (15%) and mud (5%). This sample was subsequently homogenized by milling until obtaining a material with particle size of < 3 mm and maintained (15 d at most) at –20 ºC until use. Its chemical characterization was: moisture (61.6%), ash (28.8%) and organic matter (9.6%). The composition of this organic matter was 45 % of soluble protein-Lowry and 42 % of total sugars (basically glycogen and glucose).

2: Microbiological methods

The micro-organisms used were *Lactobacillus casei* ssp. *casei* CECT 4043 (abbreviated key Lb 3.04), *Lactobacillus plantarum* CECT 220 (Lb 8.01) and *Lactobacillus buchneri* CECT 4111 (Lb 10.01). Stock cultures were stored at –75 ºC in MRS medium (Hispanlab) with 25% glycerol (Cabo et al., 2001). Inocula (0.1 g of LAB in final medium, that is, 0.6 % w/w of material to silage) consisted of cellular suspensions from 24-h aged cultures on MRS medium, concentrated by centrifugation (4000. *g*, 10 min) until cell number required (~10^{10}-10^{11} cfu mL^{-1}).

Cultures were carried out in duplicate, using 30 mL Pirex tubes with 7.5 g of homogenised waste material, 7.5 mL of a glucose solution of 200 g L^{-1} and 1 mL of the corresponding inoculum. The experimental conditions were temperature at 30 ºC and orbital shaking at 200 rpm. In all cases, initial pH was adjusted to 7.0 with 5 N NaOH and media were sterilised at 101 ºC for 60 min.

3: Analytical methods

At pre-established times, each experimental unit was divided into two aliquots. The first aliquot of 1 g was used for the quantification of viable cells by means of a plate count technique on MRS agar media. Serial tenfold dilutions were prepared in peptone-buffered solutions, and 0.1 mL samples were plated in quadruplicate, incubated at 30 ºC for 48-72 h, and manually counted. Results were expressed as colony-forming units per g (cfu g^{-1}). The second aliquot (the rest of the culture) was centrifuged at 6,000 *g* for 12 min, and the sediment resuspended with 15 mL of distilled water for a second centrifugation at the same previous conditions. Both supernatants obtained from these two centrifugations were mixture for analytical determinations. These determinations were corrected taking into account the dilution generate by the sediment resuspension. The second sediment was also used to determine protein concentration.

Additional analyses (in duplicate) were: *Proteins*: method of Lowry et al. (1951). For sediments, this method was applied to the samples with a previous alkaline treatment using NaOH (1M) for 24 h at 30ºC as well as the standard of bovine serum albumin. *Total sugars*: measured by means of the phenol-sulphuric

144 reaction (Dubois et al., 1956) according to the application of Strickland and
145 Parsons (1968) with glucose as a standard. *Reducing sugars*: 3,5-
146 dinitrosalicylic reaction (Bernfeld, 1951). *Lactic acid*: HPLC, after membrane
147 filtration (0.22 μm Millex-GV, Millipore, USA) of samples, using an ION-300
148 column (Transgenomic, USA) with 6 mM sulphuric acid as a mobile phase (flow
149 = 0.4 mL min^{-1}) at 65 ºC and a refractive-index detector.
150
151 ***4: Mathematical equations and numerical methods***
152 The profile of *pH* was modelled by means of von Bertalanffy equation (Vázquez
153 et al., 2005):
154
155 $$pH = pH_f + a \cdot \exp(-ct) \quad \text{with} \quad a = pH_0 - pH_f \qquad (1)$$
156
157 where, t is the time-course (h), pH_f is the final value of *pH*, a is the *pH* drop, pH_0
158 is the initial value of pH, c is the specific maximum rate of *pH* drop (h^{-1}).
159
160 The mathematical model used to describe kinetically the sigmoid production of
161 lactic acid was as follows (Vázquez et al., 2008c):
162
163 $$L = \frac{L_m}{1 + \exp\left(2 + \frac{4\nu_m}{L_m}(\lambda - t)\right)} \qquad (2)$$
164
165 where, L is the lactic acid (g L^{-1}), L_m is the maximum production of lactic acid (g
166 L^{-1}), ν_m is the maximum rate of lactic acid production (g L^{-1} h^{-1}) and λ is the lag-
167 phase of lactic acid production (h).

An additional calculation of the yields in the formation of lactic acid (*L*) referred to both the consumption of reducing sugars and of total proteins (in the supernatant + in the sediment) was obtained in the following terms:

$$Y_{L/RS} = \frac{\Delta L}{\Delta RS} = \frac{L_f - L_i}{RS_i - RS_f} \quad (3)$$

$$Y_{L/P} = \frac{\Delta L}{\Delta P} = \frac{L_f - L_i}{P_i - P_f} \quad (4)$$

where, $Y_{L/RS}$ is the yield of lactic acid production on reducing sugars (g of lactic acid g^{-1} of reducing sugars) and $Y_{L/P}$ is the yield of lactic acid production on total proteins (g of lactic acid g^{-1} of total proteins).

On the other hand, fitting procedures and parametric estimations calculated from the results were carried out by minimisation of the sum of quadratic differences between observed and model-predicted values, using the non linear least-squares (Levenberg-Marquadt) method provided by Statistica 8.0 (StatSoft, Inc. 2007). This software was also used to evaluate the significance of the parameters estimated by the adjustment of the experimental values to the proposed mathematical models and the consistency of these equations.

RESULTS AND DISCUSSION

1: Fermentability of MWP by lactobacilli

Initially, the selection of LAB used in the present work (Lb 3.04, Lb 8.01 and Lb 10.01) was done in relation to the fermentative features that these bacteria showed in the valorisation of fish viscera (Vázquez et al., 2008a). Using wastes

193 from ray, swordfish and shark as protein source for the formulation of complex
194 microbiological media, high lactic acid (more than 80% of yield as function of
195 glucose uptake) and biomass productions were obtained.
196
197 Figure 1 shows experimental result of lactobacilli fermentations on the MPW
198 waste with supplemental glucose. The time course trends were very similar for
199 three bacteria tested here. In all cases, the drop of *pH* was ~2.5 units with pH_f
200 values down to 4.51. The highest specific maximum rate of *pH* drop was
201 obtained in Lb 8.01 (c= 0.235±0.059 h^{-1}) followed by Lb 10.01 (c= 0.224±0.044
202 h^{-1}) and Lb 3.04 (c= 0.166±0.037 h^{-1}). Maximum lactic acid productions were up
203 to 80 g L^{-1} and more than 85% of glucose added was consumed. The maximum
204 rate of lactic acid production were also higher with Lb 8.01 (v_m= 8.64±3.50 g L^{-1}
205 h^{-1}) than Lb 3.04 (v_m= 7.52±5.05 g L^{-1} h^{-1}) and Lb 10.01 (v_m= 6.30±3.74 g L^{-1} h^{-1}). No other metabolites like acetic acid or ethanol were generated. Yields of
207 lactic acid formation on glucose as substrate were up to 0.81 g of lactic acid g^{-1}
208 of reducing sugars and up to 8.70 g of lactic acid g^{-1} of total proteins for the
209 three lactobacilli. As it has been early commented, similar results were obtained
210 when other marine wastes were used as culture media (Vázquez et al., 2008a).
211 Nevertheless, heterofermentative behaviour was observed with these
212 substrates; from 20 h of culture, conversion of lactic acid in acetic acid was
213 developed. Furthermore, Lb 3.04 led to important ethanol concentrations as
214 response to the stress conditions of *pH* gradient in fed-batch cultures with
215 successive re-alkalisations (Vázquez et al., 2005). In our work, lactic acid was,
216 however, the only metabolite synthesized what could be due to the different

217 formulation of culture broth in relation to the source of proteins and mineral
218 salts.
219
220 On the contrary, the dynamics of LAB biomass did not show the common
221 sigmoid profiles that are habitually generated by the growth of *Lactobacillus* in
222 batch cultivation (Horn et al., 2005; Kedia et al., 2008; Charalampopoulos et al.,
223 2009). The bacteria counts increased at the first 8 hours of culture (1
224 logarithmic unit, log-unit) and subsequently fall until the value of the inocula. For
225 Lb 10.01 this drop was superior to 1.5 log-units from the maximum growth.
226 Regarding the consumption of organic nitrogen source more than 10 g L^{-1} of
227 final protein (sum of supernatant and sediment concentrations) is not consumed
228 at the end of the kinetic. This fermented material with probiotic properties
229 (Planas et al., 2004; Guerra et al., 2007; Bernárdez et al., 2008a; Bernárdez et
230 al., 2008b) could be used as feed for poultry farming where high concentrations
231 of calcium carbonate in the alimentary substrate are easily assimilable for the
232 animals. Moreover, this formulation was stable to the microbial contamination
233 for 10 days at 20 ºC.
234
235 In all cases, the fitting of pH and lactic acid production were satisfactory
236 statistically. The mathematical equations were consistent (Fisher's *F* test) and
237 the parametric estimations were significant (Student's *t*-test). All the values
238 foreseen in the non-linear adjustments produced high coefficients of linear
239 correlation with the experimental values ($r > 0.97$).
240

2: Effect of inoculum in the fermentability of MWP by Lactobacillus plantarum

In order to reduce the necessity of inoculum, the next step consisted in the study of the effect of initial Lb 8.01 concentration in the MWP fermentation. This strain was selected due to the highest values of kinetic parameters obtained for lactic acid production and *pH* drop. Time course of semi-solid cultures on mussel-based media at different concentrations of Lb 8.01 are depicted in figure 2. The results revealed that with the decrease in the initial LAB employed lower (L_m= 60.92±14.57 and 34.71±7.64 g L^{-1} with 0.075 and 0.01 g L^{-1} of initial Lb 8.01, respectively) and slower production of lactic acid is obtained. The *pH* drop followed the same tendency as well as the maximum specific rate of *pH* drop (c= 0.306±0.078, 0.205±0.032, 0.055 h^{-1}) for 0.075, 0.05, and 0.01 g L^{-1} of Lb 8.01 inoculum, respectively. Comparing with the results from previous section, the reduction of one order of magnitude in the initial concentration of LAB entailed a fall of 50 g L^{-1} in the maximum production of lactic acid. In this case, the yields of lactic acid production on glucose were lower with less inoculum: $Y_{L/RS}$= 0.70 and 0.43 g of lactic acid g^{-1} of reducing sugars for 0.075 and 0.01 g L^{-1}, respectively. Similar differences were obtained for $Y_{L/P}$ with values of 7.93 and 5.60 g of lactic acid g^{-1} of total proteins.

On the other hand, similar trends of LAB dynamics and protein evolution throughout the time with previous experiment were observed. An initial rise of bacteria until a maximum growth was reached and then decreased gradually until values comparable with the inoculum. Likewise, correlative profiles of biomass production in relation with the initial LAB concentration were obtained.

3: Effect of non-sterilisation in the fermentability of MWP by Lactobacillus plantarum

In the early sections, the tests were carried out using MWP thermally sterilized with the purpose of evaluating their capacity to be fermented by LAB. However this possibility to industrial scale is less realistic than employing raw material with an added LAB-inoculum to lead the lactic acid fermentation since the cost of sterilization process could make the bio-silage too expensive. Therefore, effect of non previous sterilization was assayed.

In Figure 3 the outcomes obtained for fermentations in non thermal processing substrate are shown. When compared to Figure 2, not very significant differences between pH profiles and numerical parameters in sterilized and non sterilized media (c= 0.272, 0.210, 0.054 h^{-1}) were noted. Similar lactic acid productions and glucose consumptions were obtained for both initial conditions of wastes and for different LAB initial concentration. These productions and consumptions were significantly dependent with the inoculum, hence, higher productions (L_m= 67.55±2.85, 61.51±15.72 and 42.21±6.07 g L^{-1}) and uptakes were obtained at higher Lb 8.01 inoculum. Moreover, the specific maximum rate of growth was much higher with 0.075 g L^{-1} than 0.05 and 0.01 g L^{-1} of initial LAB. However, in these three cases the parabolic profiles showed in Figures 1 and 2 were not developed and more common kinetic trends were got.

As in previous cultures the proposed equations were statistically robust (Fisher´s F-test and p-values < 0.005), and the parametric estimations were

291 significant (Student´s *t*-test α= 0.05). The coefficients of linear correlation
292 between predicted and observed values were in all cases > 0.97.
293
294 Further experiments should be done in order to study the possibility of
295 incorporation of the fermented material in the formulation of fodder for poultry
296 feeding.
297
298 **CONCLUSIONS**
299 The main conclusion of the present study is that MWP wastes can be fermented
300 with LAB, even under non-sterility conditions whenever it is supplemented with
301 an enough amount of fermentable sugar. Thus, with a no longer culture (20-28
302 h) of lactobacilli a material likely suitable for animal feed is obtained. The
303 process developed in this preliminary work could suggest an easy protocol to
304 reduce impact pollution of MWP on marine ecosystem.
305
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FIGURE CAPTIONS

Figure 1: Biological silage of MWP wastes by Lb 3.04 (●), Lb 8.01 (○) and Lb 10.01 (□). L: lactic acid, N: colony forming units per gram (cfu g^{-1}), RS: reducing sugars, Pr: protein-Lowry in supernatant, Pr sed: protein-Lowry in sediment. Continuous lines show the fits of the experimental data (points) to the equations (1) and (2); discontinuous lines only represent the experimental profiles. The corresponding confidence intervals of independent experiments are not shown (α=0.05, n=2), since these were below 10% of the experimental mean value in all cases.

Figure 2: Bio-silage of MWP wastes by Lb 8.01 with different inocula concentrations (●: 0.075 g L^{-1}, ○: 0.05 g L^{-1}, □: 0.01 g L^{-1}) and previous thermal sterilisation of media. Keys as in figure 1.

Figure 3: Bio-silage of MWP wastes by Lb 8.01 with different inocula concentrations (●: 0.075 g L^{-1}, ○: 0.05 g L^{-1}, □: 0.01 g L^{-1}) and without sterilisation of media. Keys as in figure 1.

FIGURE 1

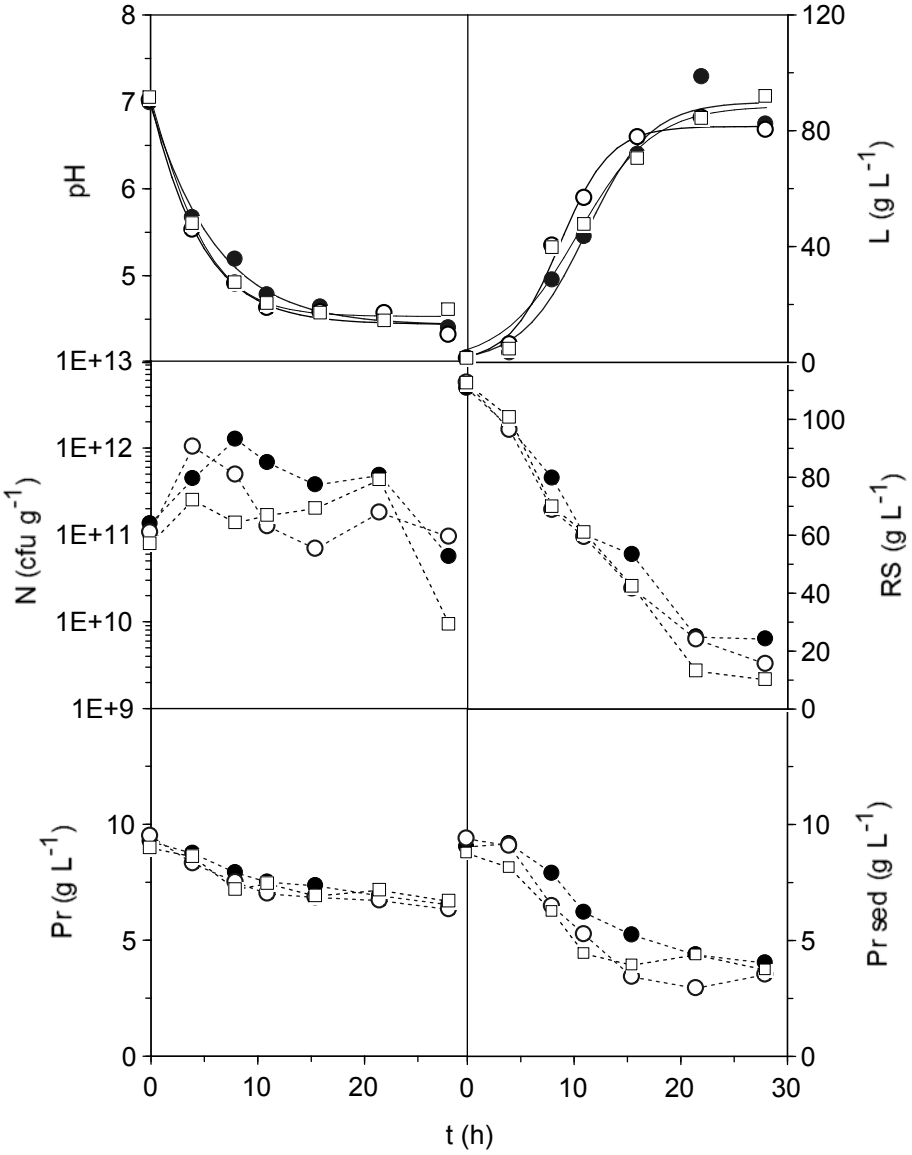


FIGURE 2

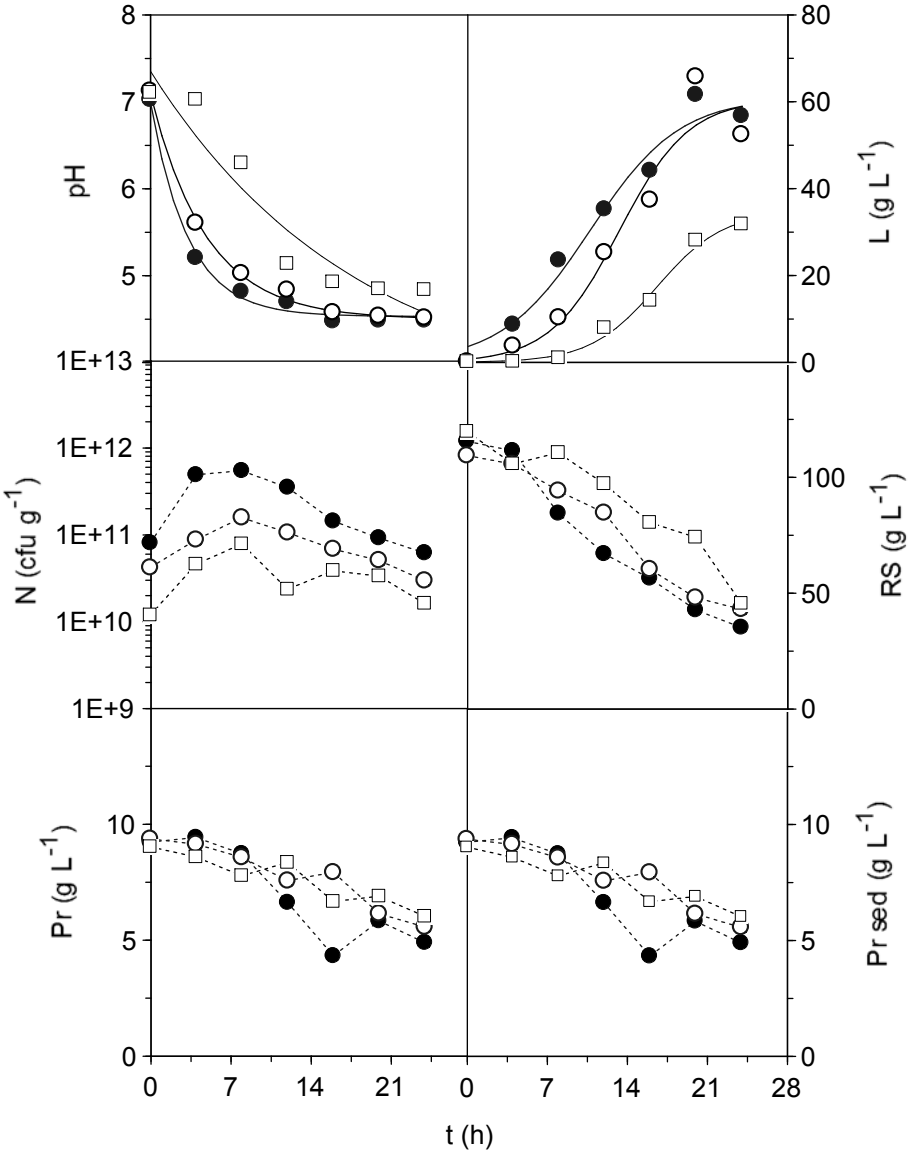
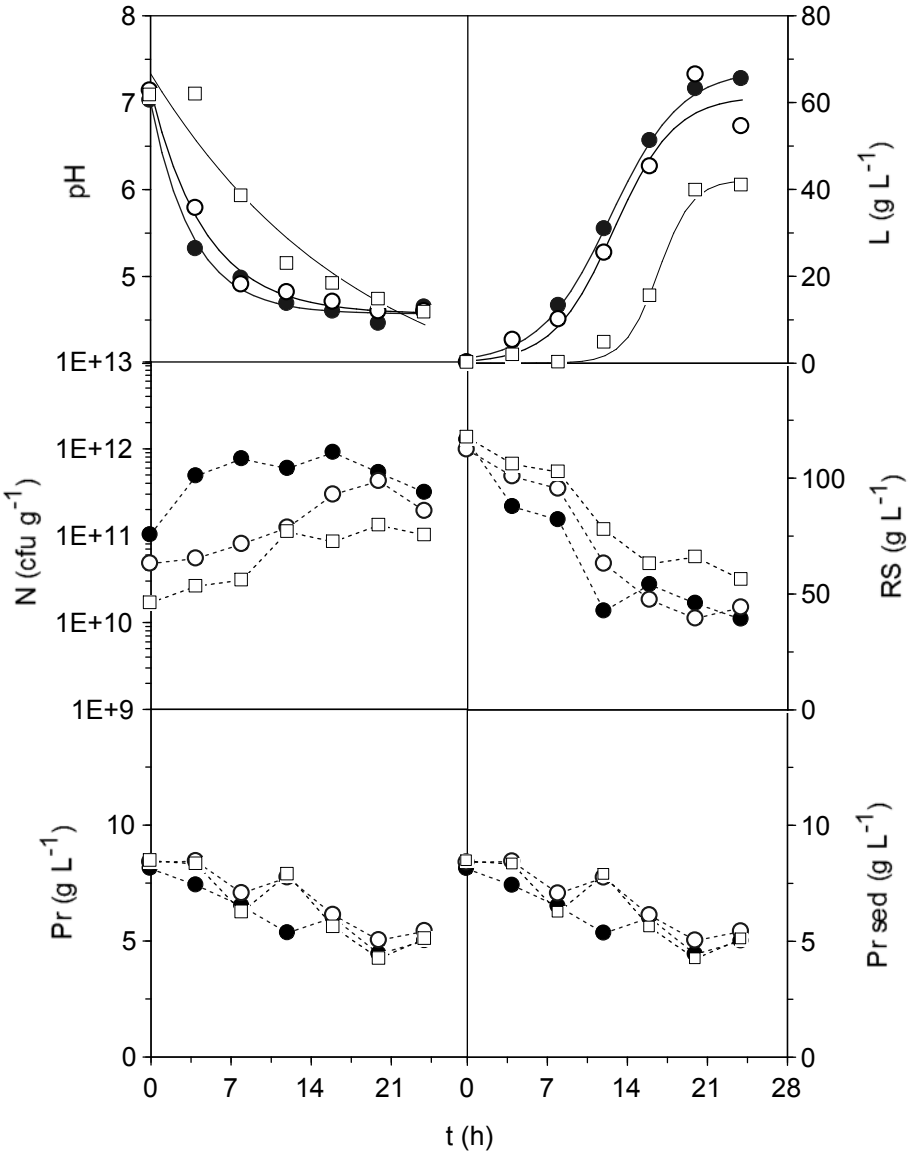


FIGURE 3


pH
L (g L-1)
N (cfu g-1)
RS (g L-1)
Pr (g L-1)
Pr sed (g L-1)
t (h)
8
7
6
5
1E+13
1E+12
1E+11
1E+10
1E+9
80
60
40
20
0
100
50
10
0
7
14
21
28