High production of hyaluronic and lactic acids by *Streptococcus zooepidemicus* in fed-batch culture using commercial and marine peptones from fishing by-products.

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ABSTRACT

The combined production of biomass, hyaluronic acid (HA) and lactic acid (LA) in a glucose fed-batch system was studied. The complex culture media used were formulated with commercial and residual peptones from fish by-products. In all cases, fed-batch fermentations increased the productive period of HA and LA. Tryptone led to the highest productions but with the peptones from shark by-products similar LA concentrations and prominent HA levels were reached. Moreover, with this residual peptone higher molecular weight of HA were achieved. On the other hand, the equations proposed adjusted with accuracy and high statistical robustness the experimental kinetic profiles.

INTRODUCTION

Hyaluronic acid (HA) is a high molecular mass glycosaminoglycan composed by repeating units of D-glucuronic acid and N-acetyl-D-glucosamine linked by β(1-3) and β(1-4) glycosidic bonds. This biopolymer is present in many animal tissues (skin, combs, umbilical cord, cartilage, vitreous humour and sinovial fluid), as well as in the cell wall of bacteria such a *Streptococcus zooepidemicus* [1-3]. HA has numerous and increasing applications in clinical and cosmetic areas including, among others, plastic surgery, treatment of arthritis, major burns and intra-ocular surgery [3,4]. As a consequence of these wide applications, in recent years HA production from microbial fermentation is receiving increased attention for avoiding the risk of cross-species viral infection, to have lower production cost and to favour a more efficient purification [5,6].

On the other hand, lactic acid (LA) and its derivatives are largely used in pharmaceutical, food, textile and polymers industry. These utilities have led to a continuous and increasing demand in the

last decade [7,8]. Comparatively with the chemical synthesis, the microbiological production of LA offers the advantages in both utilization of renewable raw materials and production of pure isomers D- or L-lactic acid depending on the microorganism used [8].

In both cases, production costs are greatly influenced by the price of the culture media, mainly protein sources. Use of broths with commercial peptones is not economical due to their higher charges. However, these costs could be reduced if residual materials were used as nutrients in the media formulations and a friendly environmental approach would also be reached. Though there are a lot of reports for the LA production by lactic acid bacteria using residual sources of carbohydrates [9-14], HA formation in waste materials with high protein concentration remain unstudied. Marine peptones from fish by-products could be a plausible alternative because they have yielded excellent results in different biotechnological productions [15-22].

In the present work, the suitability for HA and LA productions in fed-batch fermentations using commercial and marine peptones from fish by-products (shark and thornback ray) was studied. Furthermore, a set of simple and biphasic equations based on the logistic model were used to fit the experimental data and to describe the kinetics cultures.

MATERIALS AND METHODS

Microorganisms and culture media

The micro-organism used was *Streptococcus equi* subsp. *zooepidemicus* ATCC 35426. Stock cultures were stored at –80ºC in complex medium (defined in table 1) with 25% glycerol. The inocula were prepared following the methodology described by Armstrong et al. [23]. Thus, cellular suspensions of *S. zooepidemicus* from 16 h aged in complex medium, were transferred in serial tenfold dilutions of peptone-buffered solution, 0.1 mL samples were plated on sheep blood agar

plates (SBA) and incubated overnight at 37ºC. Pure mucoid colonies were then added into the initial inoculum in a flask with 30 mL of M17G medium [24]. After 3.5 hours of growth at 37ºC and 100 rpm of orbital shaker, the whole of this initial inoculum was transferred into the secondary inoculum in a flask with 70 mL of VIG medium. After 4 hours of growth in the same conditions, this secondary inoculum was gathered into the tertiary inoculum in a flask with 250 mL of VIG and was incubated 4 hours at 37ºC and 100 rpm. Finally, the tertiary inoculum was added to the batch and fed-batch cultures at 10% (v/v).

The compositions of the media are summarized in Table 1. Each marine peptone was used at a level that replaced the Lowry protein concentration present in the tryptone used for the complex medium. The preparation and composition of the peptones solutions from shark and thornback ray was described in a previous work [25]. Moreover, in order to reduce the hyaluronidase activity releases by *S. zooepidemicus* we have included 15 mg/L of polystyrene (M_w=990 kDa, Sigma) in the culture media. In all cases, the initial pH was adjusted to 6.7 and the media were sterilised at 121ºC for 15 min. Cultures were carried out in duplicate using a glass 2L-bioreactor with a working volume of 1.8 L. All fermentations were performed without aeration at 37ºC, with agitation of 500 rpm and the pH was automatically controlled with sterile 5 M NaOH. In the fed-batch cultures, the reducing sugars profiles were always maintained above 10 g/L by repeated added of sterile glucose solution of 500 g/L.

Analytical methods

At pre-established times, each sample from the bioreactor were first incubated with a 10% volume of 5% (w/v) SDS for 10 min with the purpose to separate the cells and to liberate the capsular HA [26,27]. The biomass was removed by centrifugation at 5,000 *g* for 30 min and the sediment washed and resuspended in distilled water to the adequate dilution for measuring the optical density (OD) at 700 nm. The dry weight can then be estimated from a previous calibration curve. The

supernatant was divided into two aliquots. The first aliquot was used for the measure of reducing sugars, glucose, LA and proteins. In the second aliquot, HA was precipitated by mixing with three volumes of ethanol and centrifuged at 5,000 *g* for 10 min. The sediment was resuspended with 1 volume of NaCl (1.5M) and 3 volumes of ethanol and precipitated by centrifugation at 5,000 *g* for 10 min. Finally, this last sediment was redissolved in distilled water for HA analysis.

HA assay was a slight modification of the method of Blumenkrantz and Asboe-Hansen [28] following the proposal and mathematical corrections defined by Murado et al. [29]. Additional analyses (in duplicate) were, Proteins: method of Lowry et al. [30]. Reducing sugars: 3,5-dinitrosalicylic reaction [31]. LA and glucose: HPLC, after membrane filtration (0.22 µm Millex-GV, Millipore, USA) of the samples, using an ION-300 column (Transgenomic, USA) with 6 mM sulphuric acid as mobile phase (flow=0.4 mL/min) at 65ºC and a refractive-index detector. HA molecular weight (M_w): was determined by size-exclusion chromatography on HPLC by means of an Ultrahydrogel Linear column (Waters, USA) with 0.1 M $NaNO_3$ as mobile phase (flow=0.6 mL/min) and a refractive-index detector. The column was calibrated with polystyrene standards (Sigma) of varying molecular weights (32, 77, 150, 330, 990 and 2600 kDa). The M_w results were expressed as mean ±confidence interval (α=0.05).

Mathematical models

The mathematical model used to describe the sigmoid profiles of *S. zooepidemicus* growth (*X*), in all the cultures, was the logistic equation [32] (see symbol notation table 2 for the definition of the parameters and their units):

$$X = \frac{K}{1+\exp\left[2+\frac{4\cdot v_x}{K}\cdot\left(\lambda_x - t\right)\right]} \qquad (1)$$

Similar models were used to adjust the sigmoid trends of hyaluronic acid (*H*) and lactic acid (*L*) productions in complex medium and batch fermentations:

$$H = \frac{H_1}{1+\exp\left[2+\frac{4\cdot v_{h1}}{H_1}\cdot\left(\lambda_{h1}-t\right)\right]} \quad (2)$$

$$L = \frac{L_1}{1+\exp\left[2+\frac{4\cdot v_{l1}}{L_1}\cdot\left(\lambda_{l1}-t\right)\right]} \quad (3)$$

These productions in the fed-batch cultures were fitted to the biphasic logistic [33]:

$$H = \frac{H_1}{1+\exp\left[2+\frac{4\cdot v_{h1}}{H_1}\cdot\left(\lambda_{h1}-t\right)\right]} + \frac{H_2}{1+\exp\left[2+\frac{4\cdot v_{h2}}{H_2}\cdot\left(\lambda_{h2}-t\right)\right]} \quad (4)$$

$$L = \frac{L_1}{1+\exp\left[2+\frac{4\cdot v_{l1}}{L_1}\cdot\left(\lambda_{l1}-t\right)\right]} + \frac{L_2}{1+\exp\left[2+\frac{4\cdot v_{l2}}{L_2}\cdot\left(\lambda_{l2}-t\right)\right]} \quad (5)$$

where $H_m=H_1+H_2$ and $L_m=L_1+L_2$ are the maximum HA and LA concentrations, respectively.

Numerical methods

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 (quasi-Newton) method provided by the macro 'Solver' of the Microsoft Excel XP spreadsheet. Statistica 6.0 (StatSoft, Inc. 2001) and Mathematica 6 (Student

Version, Wolfram Research, Inc.) were 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

Batch and Fed-batch cultures in a complex medium

Batch and fed-batch fermentations of *S. zooepidemicus* were performed in a medium formulated with tryptone and under the conditions described previously. Figure 1 shows the experimental data and the profiles predicted by the equations (1-5). The parametric estimations and statistical analysis of the mathematical models proposed are shown in Table 3. According to these results, after 3.4 and 4.1 hours of lag phase (λ_x) for batch and fed-batch culture, respectively, the cells entered the exponential growth phase until the asymptotic phase at 8 hours with a maximum biomass concentration of 5.2 and 5.4 g/L (K). The highest concentrations of LA and HA (69.37 g/L and 4.85 g/L, respectively) as well as both maximum rate productions were reached in the fed-batch system.

However, the yields of biomass, LA and HA formation per glucose consumed ($Y_{x/s}$=0.11 g/g; $Y_{l/s}$=0.74 g/g; $Y_{h/s}$=0.07 g/g, respectively) were higher in batch culture than fed-batch ($Y_{x/s}$=0.04 g/g; $Y_{l/s}$=0.59 g/g; $Y_{h/s}$=0.04 g/g). With regard to the proteins, the ratios between metabolites production and protein uptakes favoured to the fed-batch culture ($Y_{l/p}$=18.3 g/g; $Y_{h/p}$=1.25 g/g) in comparison to batch control ($Y_{l/p}$=11.2 g/g; $Y_{h/p}$=1.01 g/g). On the other hand, the average HA M_w throughout the time-course of both cultures were $(1.54\pm0.11)\times10^3$ kDa and $(2.32\pm0.32)\times10^3$ kDa in batch and fed-batch, respectively.

The results obtained in batch fermentation were in agreement with the previous report of Armstrong et al. [34]. These authors used different experimental conditions (broth, aeration, agitation) achieved

HA productions of 4.2 g/L with M_w of 3.1×10^3 kDa and $Y_{h/s}$=0.07 g/g. Recently, Liu et al. [35] obtained concentrations of 6 g/L of HA but with a final M_w of 45 kDa by using 0.15 g/L of hyaluronidase in the culture media.

Fed-Batch cultures in residual media with marine peptones

Based on the results using fed-batch fermentation, the next step consisted of replacing the commercial peptone (tryptone) of the complex medium by marine peptones from fish by-products and to carry out kinetics of culture with intermittent glucose fed-batch. Therefore, two different broths were prepared (table 1), SM with protein source from shark viscera and RM with peptones from thornback ray.

Figure 2 shows the fed-batch fermentation in SM and the numerical parameters from the mathematical models are summarized in table 3. In this medium, the maximum biomass concentration (K=3.59 g/L) was obtained after approximately 6 h (asymptotic phase). The lag phase was the shortest (2.93 h) and the maximum HA production rates (v_{h1}=0.43 and v_{h2}=0.54 g $L^{-1}h^{-1}$) were the lowest in all conditions tested. Afterwards 12 h of culture the maximum HA concentration was achieved (H_m=2.52 g/L). This result was inferior than that obtained in CM but the average M_w was higher $(3.15\pm0.95)\times10^3$ kDa by using residual peptone from shark.

Furthermore, all the yields of biomass and HA production per glucose and protein consumed were lower in SM ($Y_{x/s}$=0.03 g/g; $Y_{h/s}$=0.02 g/g; $Y_{x/p}$=0.94 g/g and $Y_{h/p}$=0.66 g/g). It must also be noted that the biphasic profiles in the HA production were similar in both media. This sort of profiles is very common in a diauxic growth, but in our case this behaviour can not be attributed to metabolize a mixture of two sugars. No evidences of differential peptides or amino acids uptakes are also supported. On the other hand, the differences between the results of LA formation in CM and SM were no significant (L_m=66.9 g/L; $Y_{l/s}$=0.61 g/g; $Y_{l/p}$=17.8 g/g).

Very similar trends of production were observed in RM when compared with the results in SM (figure 3 and table 3). The maximum growth and final LA concentration were slightly lower 2.47 g/L and 58.56 g/L, respectively. However, the yields of LA production were the highest in all fed-batch cultures investigated ($Y_{l/s}$=0.69 g/g; $Y_{l/p}$=22.6 g/g) and the yields of HA were higher than SM ($Y_{h/s}$=0.03 g/g; $Y_{h/p}$=0.99 g/g). On the contrary, the average M_w of HA was the lowest $(0.97\pm0.22)\times10^3$ kDa.

These significant differences in the molecular weight of HA could be due to the peptones used in the fermentations since they were cultivated under the same experimental conditions and no other system variable was modified. The organic nitrogen sources are considered essential for growth and metabolite formation in lactic acid bacteria [36,37]. In streptococci these components also supply a large proportion of the carbon for cellular and metabolite biosynthesis [23,38]. There have been no many works on the specific nutritional requirements of *S. zooepidemicus* and in particular for HA production. Armstrong et al. [23] found that 11 amino acids were essential for *S. zooepidemicus* growth. Moreover, the media formulated with commercial peptone or with well-known concentration of amino acids led to different kinetics of HA.

In our results, a different composition of amino acids and peptides in the peptones could provoke a different efficiency in the peptide or amino acids transmembrane transport. Guirard and Snell [39] proposed that peptide transport (e.g. di, tri or tetrapeptides) could be more efficient than transport of the individual amino acids. These conclusions are in the same way than those obtained for the bacteriocin production by lactic acid bacteria [40-44].

On the other hand, although the HA productivity with commercial tryptone duplicated the outcomes obtained with peptones from marine wastes, the reduction of total costs using residual media was of

a 34%. Furthermore, the economical gain per gram of lactic acid performed with peptones from shark was 1.5 times larger than commercial tryptone.

Finally, from a statistical point of view, the proposed equations showed a high accuracy to predict the production profiles of *S. zooepidemicus*. In all cases, the fitting of results was graphically and statistically satisfactory. The mathematical equations were consistent (Fisher's F test) and the parametric estimations were significant (Student's t test). Furthermore, all the values foreseen in the non-linear adjustments produced high coefficients of linear correlation with the values really observed ($r > 0.997$).

CONCLUSIONS

The main contribution of this paper is the demonstration that the peptones from eviscerates of fishing by-products have excellent capability for promoting the production of HA and LA by *S. zooepidemicus*. To our knowledge, it is the first time that residual peptones are used as nitrogen source for HA formation. Moreover, the fermentations were performed in fed-batch increasing the time course of the cultures and the final concentration of HA and LA. Shark peptones led to highest molecular weight of HA. Consequently, these results allow replacement of the high-cost commercial peptones used for these bioproductions.

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FIGURE CAPTIONS

Figure 1: *Streptococcus zooepidemicus* fermentations in complex medium using batch culture (open symbols) and fed-batch culture with glucose (closed symbols). Continuous lines represent the fitting functions corresponding to the experimental results (points), according to the equations (1-5). X: biomass; L: lactic acid; S: reducing sugars (○,●); P: proteins (□,■); H: hyaluronic acid. The corresponding confidence intervals of independent experiments are not shown (α=0.05, n=2), since these did not transcend in practically any case, the 10% of the experimental mean value.

Figure 2: *Streptococcus zooepidemicus* fermentations in residual medium SM using batch culture (open symbols) and fed-batch culture with glucose (closed symbols). Continuous lines represent the fitting functions corresponding to the experimental results (points), according to the equations (1-5). X: biomass; L: lactic acid; S: reducing sugars (●); P: proteins (■); H: hyaluronic acid. The corresponding confidence intervals of independent experiments are not shown (α=0.05, n=2), since these did not transcend in practically any case, the 10% of the experimental mean value.

Figure 3: *Streptococcus zooepidemicus* fermentations in residual medium RM using batch culture (open symbols) and fed-batch culture with glucose (closed symbols). Continuous lines represent the fitting functions corresponding to the experimental results (points), according to the equations (1-5). X: biomass; L: lactic acid; S: reducing sugars (●); P: proteins (■); H: hyaluronic acid. The corresponding confidence intervals of independent experiments are not shown (α=0.05, n=2), since these did not transcend in practically any case, the 10% of the experimental mean value.

TABLE 1

Table 1. Composition of culture media used in batch and fed-batch fermentations (g/L).

	SM	RM	CM
Glucose	50.0	50.0	50.0
Yeast extract[1]	5.0	5.0	5.0
Tryptone[1]	-	-	15.0
KH_2PO_4	2.0	2.0	2.0
K_2HPO_4	2.0	2.0	2.0
$MgSO_4.7H_2O$	0.5	0.5	0.5
$(NH4)_2SO_4$	0.5	0.5	0.5
Polystyrene (Mw=990 kDa)[1]	0.015	0.015	0.015
Marine peptone protein (Lowry)	8.00	8.00	-

SM: Shark medium (using peptone from viscera by-products of shark).
RM: Thornback ray media (using peptone from viscera by-products of ray).
CM: Complex medium.
[1]Yeast Extract and Tryptone were provided by Cultimed (Panreac Química, Spain) and Polystyrene by Sigma.

TABLE 2

Table 2: Symbolic notations used.

X :	Biomass production, g/L
t :	Time, hours (h)
K :	Maximum biomass production, g/L
v_x :	Maximum biomass production rate, g L^{-1} h^{-1}
λ_x :	Biomass production lag phase, h
H :	HA production, g/L
H_1 :	Maximum HA production in the first part of the curve profile, g/L
v_{h1} :	Maximum HA production rate in the first part of the curve profile, g L^{-1} h^{-1}
λ_{h1} :	HA production lag phase in the first part of the curve profile, h
H_2 :	Maximum HA production in the second part of the curve profile, g/L
v_{h2} :	Maximum HA production rate in the second part of the curve profile, g L^{-1} h^{-1}
λ_{h2} :	HA production lag phase in the second part of the curve profile, h
H_m :	Maximum HA production at the end of the culture, g/L
L :	LA production, g/L
L_1 :	Maximum LA production in the first part of the curve profile, g/L
v_{l1} :	Maximum LA production rate in the first part of the curve profile, g L^{-1} h^{-1}
λ_{l1} :	LA production lag phase in the first part of the curve profile, h
L_2 :	Maximum LA production in the second part of the curve profile, g/L
v_{l2} :	Maximum LA production rate in the second part of the curve profile, g L^{-1} h^{-1}
λ_{l2} :	LA production lag phase in the second part of the curve profile, h
L_m :	Maximum LA production at the end of the culture, g/L
$Y_{x/s}$:	Biomass production/sugar consumption, g biomass/g reducing sugars
$Y_{h/s}$:	HA production/sugar consumption, g HA/g reducing sugars
$Y_{l/s}$:	LA production/sugar consumption, g LA/g reducing sugars
$Y_{x/p}$:	Biomass production/protein consumption, g biomass/g protein
$Y_{h/p}$:	HA production/protein consumption, g HA/g protein
$Y_{l/p}$:	LA production/protein consumption, g LA/g protein

TABLE 3

Table 3: Parametric estimations corresponding to the equations (1-5), applied to the production of biomass, hyaluronic and lactic acids by *Streptococcus zooepidemicus*. CI: confidence intervals (α=0.05). *F*: F-Fisher test (df_1=model degrees freedom and df_2=error degrees freedom). r=correlation coefficient between observed and predicted data. NS: not significant

VARIABLES	CM-Batch	CM-Fed Batch	SM-Fed Batch	RM-Fed Batch
BIOMASS (*X*)	values ± CI	values ± CI	values ± CI	values ± CI
K	5.18 ± 0.16	5.37 ± 0.07	3.59 ± 0.08	3.20 ± 0.05
v_x	1.55 ± 0.22	1.97 ± 0.20	1.41 ± 0.27	1.01 ± 0.13
λ_x	3.44 ± 0.26	4.05 ± 0.16	2.93 ± 0.28	2.99 ± 0.22
F (df_1=3, df_2= 9-15; α=0.05)	2960.48	11434.62	3934.7	7212.26
p-value	<0.001	<0.001	<0.001	<0.001
r (obs-pred)	0.999	0.999	0.997	0.999
HYALURONIC ACID (*H*)	values ± CI	values ± CI	values ± CI	values ± CI
H_1	3.04 ± 0.05	4.22 ± 0.15	1.98 ± 0.20	2.02 ± 0.12
v_{h1}	1.25 ± 0.11	1.42 ± 0.08	0.43 ± 0.05	0.72 ± 0.09
λ_{h1}	4.59 ± 0.12	4.32 ± 0.09	3.70 ± 0.26	3.33 ± 0.20
H_2	-	0.63 ± 0.16	0.54 ± 0.21	0.47 ± 0.14
v_{h2}	-	1.89 (NS)	1.78 (NS)	1.78 (NS)
λ_{h2}	-	11.43 ± 0.16	11.37 ± 0.15	11.42 ± 0.14
F (df_1=3-6, df_2= 9-15; α=0.05)	9543.72	8936.76	2028.64	2088.73
p-value	<0.001	<0.001	<0.001	<0.001
r (obs-pred)	0.999	0.999	0.997	0.997
LACTIC ACID (*L*)	values ± CI	values ± CI	values ± CI	values ± CI
L_1	33.89 ± 0.63	40.45 ± 18.05	44.90 ± 3.97	23.65 ± 21.45
v_{l1}	10.33 ± 0.83	10.76 ± 1.67	8.06 ± 0.72	6.05 ± 2.19
λ_{l1}	3.89 ± 0.15	4.29 ± 0.53	3.22 ± 0.26	3.54 ± 1.64
L_2	-	28.92 ± 0.76	22.00 ± 5.19	34.91 ± 22.41
v_{l2}	-	2.95 ± 0.63	3.32 ± 0.82	2.89 ± 1.13
λ_{l2}	-	7.37 ± 5.57	11.70 ± 1.85	5.76 (NS)
F (df_1=3-6, df_2= 9-15; α=0.05)	8401.00	5569.67	7237.34	1376.13
p-value	<0.001	<0.001	<0.001	<0.001
r (obs-pred)	0.999	0.999	0.999	0.998

FIGURE 1

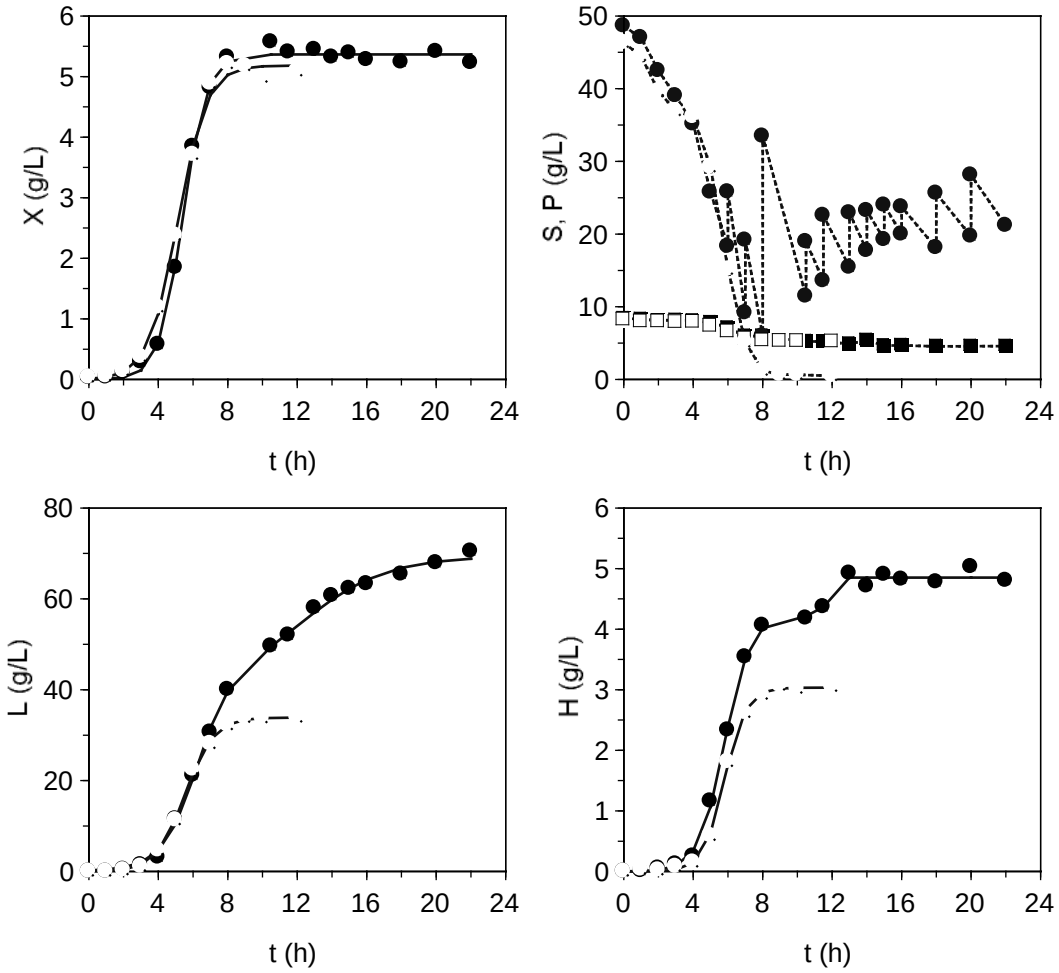

FIGURE 2

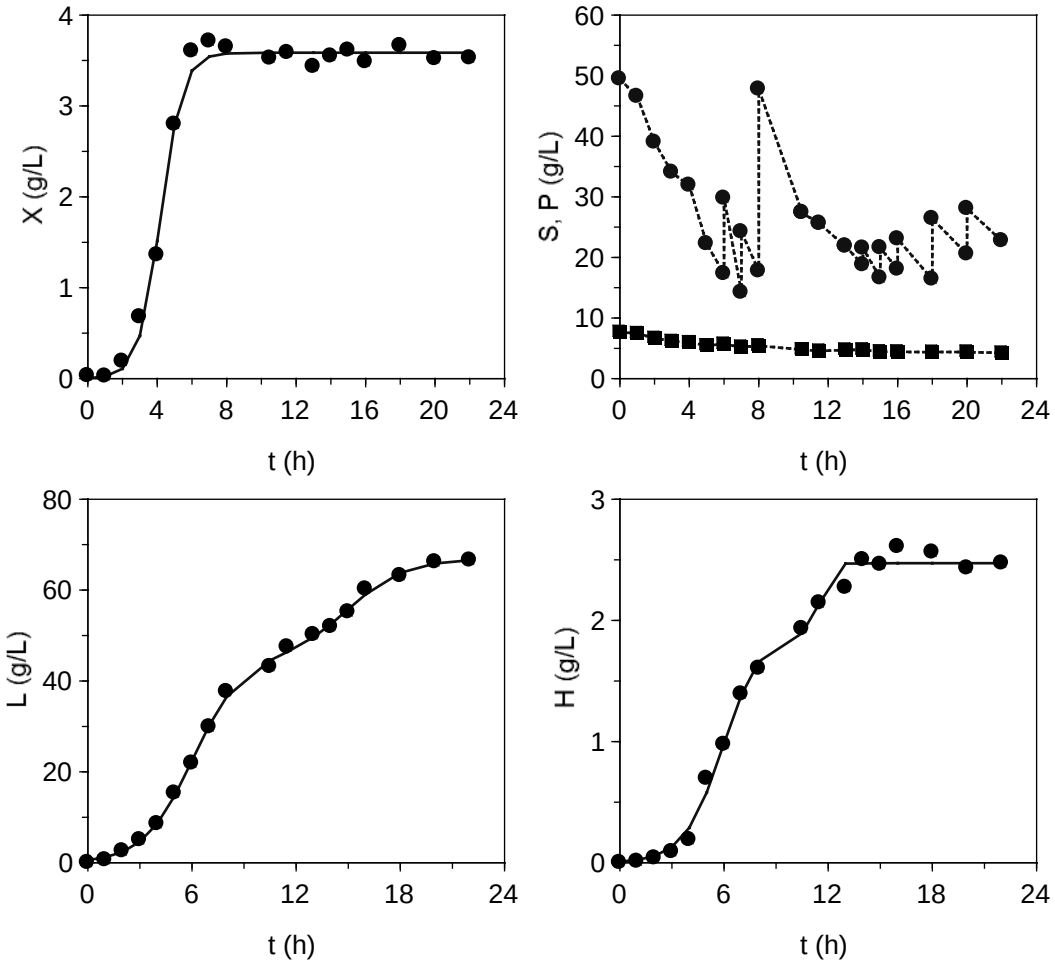

FIGURE 3