KINETIC PARAMETERS OF *Gracilaria chilensis* SEAWEED FERMENTATION

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SUMMARY

Gracilaria chilensis *were batch fermented with different initial carbohydrate concentrations and inoculated with an initial fixed optical density of the yeast* Saccharomyces cerevisiae*. To determine the kinetics and effects on the kinetic parameters of the bioethanol production process, the fermentations were performed under controlled oxygen, pH and temperature conditions. When high carbohydrate concentrations were used as substrate, a rapid decline in their concentration was observed, but the development of the yeast's metabolic activity was very slow. Besides, the saturation constants (Ks) were high and the maximum specific growth rate (μ_{max}) of the yeast remained within the same order of magnitude, without significant variation, although the ethanol yield was low. When an initial low substrate concentration was used, an increase in ethanol production (Yp/s) and in cellular growth (Yx/s) was observed. The positive impact on the development of the microorganism's metabolic activity was evidenced by a decrease in μ_{max} from 0.47 to 0.29h^{-1} and in Ks from 15.19 to 1.07g·l^{-1}. The development of the microorganism's metabolic activity is influenced by the carbohydrate concentration of* G. chilensis *when other physicochemical parameters of the fermentation process are kept constant.*

Introduction

Biomass obtained from soil-cultivated raw materials has played an important role in bioenergy production. Ethanol biofuels have been produced by countries such as Brazil, Canada and the United States; however, the issue of using agricultural land for this purpose has been widely discussed. (Somma *et al.*, 2009).

Macroalgae as raw material have many advantages compared to the biomass obtained from food or cellulosic material because macroalgae can grow in different environments (seawater, blackwater, wastewater and saltwater) and have high growth rates and greater mass productivity per area unit compared to terrestrial plants (22.46 dry weight/m^2/year *vs* 0.5-4.4kg dry weight/m^2/year (Wi *et al.*, 2009), since macroalgae may be cultivated in three dimensions rather than in two, as is the case on land. Additionally, the nutrients that macroalgae require for development are found in marine habitats; thus, their cultivation does not require fertilizers (Buck y Buchholz, 2004; Jung *et al.*, 2013). The water content of macroalgae is between 80 to 90% of that of terrestrial plants, making them a very reactive substrate to the action of microorganisms. Furthermore, macroalgae have high carbohydrate concentrations, reaching values above 70% (Cardozo *et al.*, 2007; Sebaaly *et al.*, 2012), which can be easily depolymerized by different microorganisms or bacteria for conversion into bioenergy.

KEYWORDS / Bioethanol / Fermentation / *Gracilaria chilensis* / Kinetic Parameters / Macroalgae /

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PARÁMETROS CINÉTICOS DE FERMENTACIÓN DE LA MACROALGA *Gracilaria chilensis*

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RESUMEN

Se realizaron fermentaciones en batch de Gracilaria chilensis *con diferentes concentraciones iniciales de carbohidratos e inoculados con una densidad óptica inicial fija of* Saccharomyces cerevisiae *yeast. Para la determinación de las cinéticas y los efectos en los parámetros cinéticos en el proceso de obtención de bioetanol, las fermentaciones se llevaron a cabo en condiciones controladas de oxígeno, pH y temperatura. Cuando se utilizó como sustrato altas concentraciones de carbohidratos se observó un rápido decaimiento de la concentración de éstos, sin embargo el desarrollo de la actividad metabólica de la levadura fue muy lento. Similarmente, las constantes de saturación (Ks) fueron altas y la velocidad específica máxima de crecimiento (μmax) de la levadura se mantuvo dentro del mismo orden de magnitud, sin variación significativa, aunque el rendimiento de etanol es bajo. Cuando se utilizó una concentración inicial de sustrato baja se observó un incremento tanto en la producción de etanol (Yp/s) como del crecimiento celular (Yx/s). El impacto positivo en el desarrollo de la actividad metabólica del microorganismo se manifiestó en la disminución de los valores de los parámetros μmax de 0,47 a 0,29 h^{-1} y Ks de 15,19 a 1,07g·l^{-1}. El desarrollo de la actividad metabólica del microorganismo es influenciado por la concentración de los carbohidratos de* G. chilensis *cuando otros parámetros fisicoquímicos del proceso de fermentación se mantienen constantes.*

PARÂMETROS CINÉTICOS DE FERMENTAÇÃO DA MACROALGA *Gracilaria chilensis*

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RESUMO

Realizaram-se fermentações em batch de Gracilaria chilensis *com diferentes concentrações iniciais de carboidratos e inoculados com uma densidade óptica inicial fixa de levedura* Saccharomyces cerevisiae*. Para a determinação das constantes cinéticas, os efeitos nos parâmetros cinéticos no processo de obtenção de bioetanol, as fermentações se realizaram em condições controladas de oxigênio, pH e temperatura. Quando altas concentrações de carboidratos foram utilizadas como substrato, foi observada uma rápida queda nessas concentrações, no entanto o desenvolvimento da atividade metabólica da levedura foi muito lento. Similarmente, as constantes de saturação (Ks) foram altas e a velocidade específica máxima de crescimento (μmax) da levedura se manteve dentro da mesma ordem de magnitude, sem variação significativa, embora o rendimento de etanol é baixo. Quando se utilizou uma baixa concentração inicial de substrato, se observou um incremento tanto na produção de etanol (Yp/s) como do crescimento celular (Yx/s). O impacto positivo no desenvolvimento da atividade metabólica do microrganismo se manifestou na diminuição dos valores dos parâmetros μmax de 0,47 a 0,29 h^{-1} e Ks de 15,19 a 1,07g·l^{-1}. O desenvolvimento da atividade metabólica do microrganismo é influenciado pela concentração dos carboidratos de* G. chilensis *quando outros parâmetros físico-químicos do processo de fermentação se mantêm constantes.*

Therefore, macroalgae are considered an excellent alternative source of renewable and sustainable biomass for the production of liquid biofuels such as ethanol and butanol. Among the different macroalgae divisions, red macroalgae are characterized by their high concentration of carbohydrates, composed of monomeric units of glucose that form cellulose and monomeric units of galactose that form agar (galactans) and carrageenans (primarily sulphated galactose) (Yoon *et al*., 2010; Park *et al*., 2012). Red macroalgae are particularly noted as biomass for bioethanol production because their carbohydrates are easily hydrolysable compared to the carbohydrates in brown and green algae (Kim *et al*., 2012; Lee *et al*., 2013; Seguel *et al*., 2015).

Based on a number of studies on the production of bioethanol, bioenergy production from algae is still in the initial development stage and the search for more efficient and economically viable processes continues (Horn *et al*., 2000; Wei *et al*., 2013). Among these, the influence of different stress factors on the quality and effectiveness of the fermentation process are emphasized. These factors include the lack of micro- and macronutrients, very low pH, which affects the ionic equilibrium, generation of inhibitory microorganisms, secondary reactions, and inadequate temperature and oxygen concentrations (Teixeira *et al*., 2009; Zinnai *et al*., 2013).

The production of bioethanol using *Saccharomyces cerevisiae* as a fermenting microorganism and *Gracilaria* sp. as a sole carbon source has been recently studied (Kumar *et al*., 2013). However, there are no studies on the kinetic parameters of this yeast with *Gracilaria chilensis* for ethanol production. In this study, the main fermentation kinetic growth parameters, including specific growth rate, maximum specific growth rate, affinity constant, biomass yield and product yield of fermentation processes for the production of bioethanol were evaluated.

Methods

All fermentations processes were carried out using different concentrations of carbohydrates of *Gracilaria chilensis* (red macroalgae) which were inoculated with a defined optical density of *Saccharomyces cerevisiae*, under controlled oxygen, pH and temperature conditions.

Microorganisms and inoculum preparation

A yeast solution was prepared suspending 1g of commercial lyophilized *Saccharomyces cerevisiae* (baker's yeast, Lefersa, Chile) in 1% (w/v) glucose solution. The sterilized solution was activated at 40ºC with constant shaking for 30-40min. The activated yeast was cultured in 100ml of solid YEPD (2g of glucose, 2g of peptone, 2g of agar, and 1g of yeast extract). For inoculum preparation the microorganism was cultured in 100ml of liquid yeast extract peptone dextrose (YEPD) media (2g of glucose, 2g of peptone, and 1g of yeast extract) on a rotary shaker at 200rpm. The media were

prepared using distilled water, pH was adjusted to 5.0 and the cells of *S. cerevisiae* were grown at 30ºC overnight.

Batch fermentation

Batch fermentations were carried out in 500ml E-flasks with 40, 30 and 20g·l^{-1} of the initial reducing sugars from *G. chilensis* as the sole carbon source. The different solutions were inoculated with 50ml of yeast inoculums (equivalent to an optical density of 1.7g·l^{-1}) and the pH was adjusted to 5.0. The experiments were carried out for 15h at 27ºC under limited oxygen at 250rpm. The monitoring of individual fermentations was accomplished by taking 5ml samples at time 0 and every 1h. The glucose and the cell growth quantitation were determined using previously described methods (Seguel *et al*., 2015). Ethanol concentration was measured by gas chromatography (7890A GC-System, Agilent Technologies) equipped with an FID detector, Restek Stabiwax-DA (30m, 0.32mmID, 0.25µm df column). The injection volume was 1µl with an inlet split ratio of 30:1. Initial and maximum oven temperatures were 35 and 225ºC.

Calculation of kinetic parameters

The time-dependent evaluation of the fermenting microorganism *S. cerevisiae* on *G. chilensis* carbohydrate substrates was conducted by determining the specific growth rate (µ), maximal specific growth rate (μ_{max}) and the saturation constant Ks (substrate affinity). These parameters were obtained by linearization of the Monod equation (Eq. 1). The calculation of µ was carried out by means of Eq. 2 and that of μ_{max} and Ks by using the Lineweaver-Burke transformation (Eq. 3). The Monod equation relates the maximal microbial growth rate to the concentration of the limiting substrate (Waites *et al*., 2001). The Ks value is usually low when the microorganism has a high affinity for the limiting substrate.

$$\mu = \mu_{max}\left(\frac{S}{ks+S}\right) \quad (1)$$

$$\mu = \left(\frac{x_o x_i}{t_o t_i}\right)\cdot\frac{1}{x_o} \quad (2)$$

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{ks}{\mu_{max}}\cdot\frac{1}{s} \quad (3)$$

where µ: specific growth rate (h^{-1}), μ_{max}: maximal specific growth rate (h^{-1}), Ks: saturation constant (g·l^{-1}), S: substrate concentration (g·l^{-1}), X: *S. cerevisiae* biomass in g·l^{-1}, and t: sampling time (h). Subscripts i and o: initial and final, respectively.

Results and Discussion

It has been well-documented that the ability of *Saccharomyces cerevisiae* to metabolize hexoses depends on several variables, including pH, temperature, yeast immobilization, anaerobic conditions, and initial microorganism and substrate concentrations, among other (Bai *et al*., 2008). Since the substrate conversion rate depends on the concentration of the microorganism population in the medium. The effects in the fermentation kinetics were analysed in relation to different concentrations of carbohydrates from *G. chilensis* (40, 30 and 20g·l^{-1}, corresponding to fermentations A, B and C, respectively), which were inoculated with *S. cerevisiae* at an initial optical density (OD) equivalent to 1.7g·l^{-1}. All fermentation was performed in triplicate under anaerobic, temperature and pH conditions as described previously, during an incubation time of 15h. Fermentations A and B, corresponding to the highest carbohydrate concentrations (Figures 1 and 2), displayed a relatively normal substrate decrease rate, but low bioethanol concentrations were obtained (2.3 and 3.8g·l^{-1}, respectively). The lack o efficiency in carbohydrate conversion is demonstrated by the low product yields (Yp/s) (Table I). However, when a lower substrate concentration was used (fermentation C; Figure 3), the yield (Yp/s) increased from 0.08 to 0.47, which agrees with the high percentage of carbohydrates (91.80%) that were metabolized by the yeast

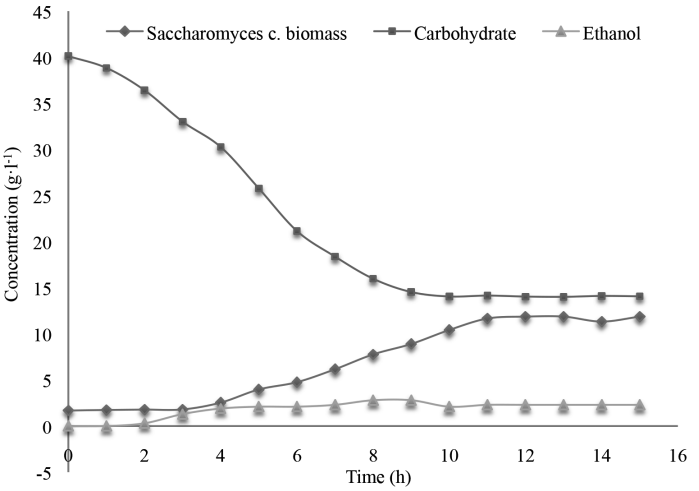

Figure 1. Fermentation process A.

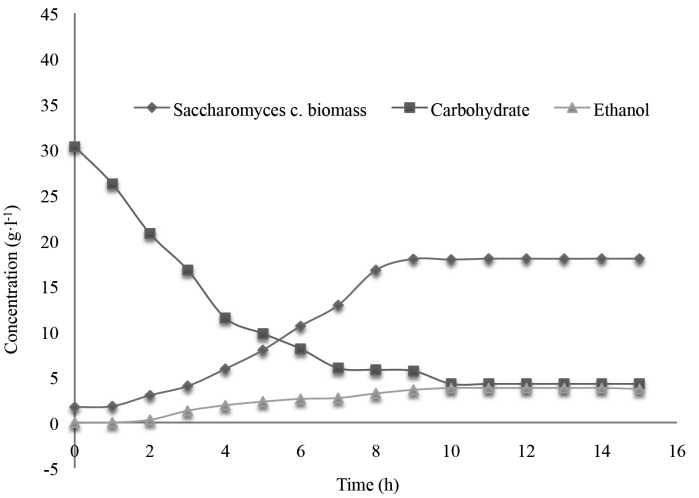

Figure 2. Fermentation process B.

TABLE I
KINETIC PARAMETERS VALUE OF *Gracilaria chilensis* FERMENTATION PROCESSES WITH *Saccharomyces cerevisiae*

Parameters	Fermentation processes		
	A	B	C
Initial sugar concentration (g·l^{-1})	40	30	20
Initial yeast biomass concentration (g·l^{-1})	1.7	1.7	1.7
Final yeast biomass concentration (g·l^{-1})	11.87	18.01	18.8
Ethanol production (g·l^{-1})	2.3	3.8	8.9
Maximum specific growth rate: μ_{max} (h^{-1})	0.47	0.44	0.29
Substrate saturation constant: K_S (g·l^{-1})	15.19	4.55	1.07
Biomass yield (Yx/s)	0.39	0.63	0.90
Product yield (Yp/s)	0.08	0.15	0.47

μ_{max}: maximal specific growth rate (h^{-1}), Ks: substrate concentration (g·l^{-1}), Yx/s: yield g *S. cerevisiae*/g carbohydrate, Yp/s: yield g ethanol/g carbohydrate of *G. chilensis*.

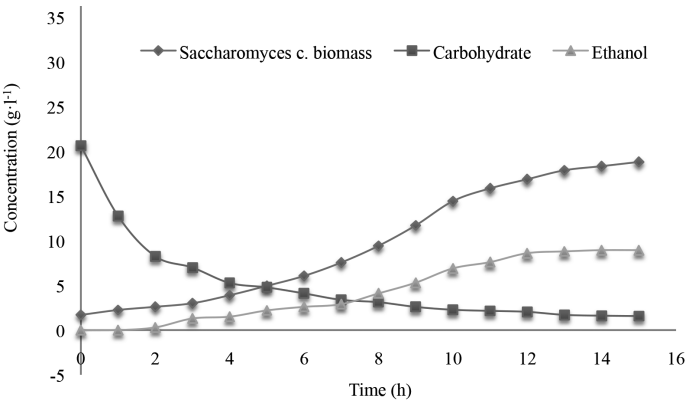

Figure 3. Fermentation process C.

(calculated based on 0.51g ethanol/g glucose, which is generated in the stoichiometric conversion of glucose into ethanol) (Borines *et al.*, 2013). The start of the microorganism's stationary growth phase occurred within the same time period in all fermentations (10 to 11h). Nonetheless, the biomass yield as a function of the substrate (Yx/s) shows that it increased significantly from 0.39 to 0.90 when the substrate concentration decreased from 40 to 20g·l^{-1}. Figure 4 shows the specific growth rate μ (h^{-1}) for fermentations A, B and C; the highest values for each were 0.46, 0.44 and 0.28h^{-1}, respectively. These experimental results are similar to the maximum specific growth rate μ_{max} (h^{-1}) calculated by the Lineweaver-Burk equation. A significant influence of the initial substrate concentration on the maximum specific growth of fermentations A and B was not observed. However, a substantial reduction of μ_{max} was obtained in fermentation C (Table I).

Studies on the kinetic parameters of *Saccharomyces* spp. with different substrates (Bauer y Pretorius, 2000; Brethauer y Wyman, 2010; Basso *et al.*, 2011) showed diverse values for the saturation constant (Ks). However, according to the Monod model, Ks is inversely related to the affinity of the microorganism for the substrate (Hernández, 2003). The experimental values obtained for this parameter (Table I) show a clear trend towards a decrease as the initial carbohydrate concentration decreases. These results agree with the idea that lower Ks values are indicative of better substrate/microorganism affinity. The data obtained show that high initial carbohydrate concentrations can decrease the efficiency of conversion into ethanol; these are consistent with other sugar fermentation studies with *Saccharomyces* spp. (Helle *et al.*, 2003; Lin *et al.*, 2012). One of the reasons for low ethanol production during fermentation with high initial carbohydrate concentrations may be the accumulation of sub-products that can modify the ionic equilibrium of the fermentation medium (change in pH). Also, high substrate concentrations can generate a respiratory deficiency in the yeast, inhibiting its metabolic activity. An increase in the osmotic pressure gradient through the cell membrane when the concentrations of some solutes are very high may be another factor determining the low ethanol production (Thomas y Ingledew, 1992).

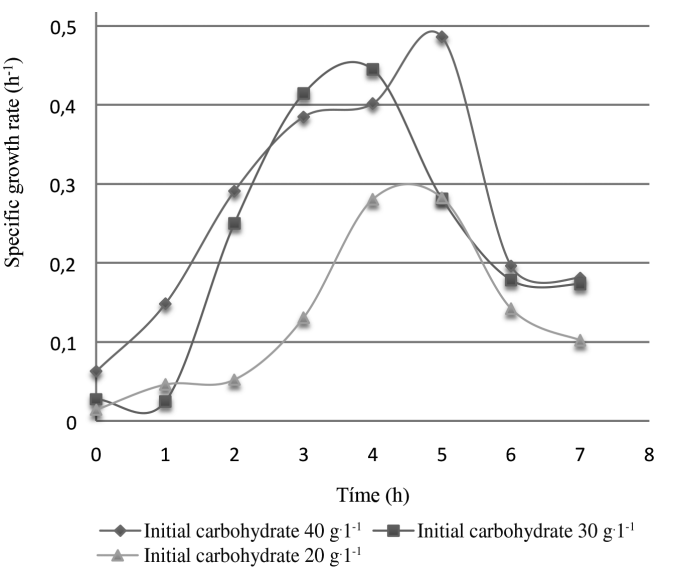

Figure 4. Time courses of specific growth rate of *Saccharomyces cerevisiae*.

Conclusions

This study is the first to report the kinetic parameters obtained from the fermentation of *Gracilaria chilensis* carbohydrates by *Saccharomyces cerevisiae.* The collected data demonstrate the influence of initial substrate concentration on the kinetic parameters under constant oxygen, temperature and pH conditions. The maximum specific growth rate of the yeast is influenced by the initial substrate concentration. High initial carbohydrate concentrations increase the maximum specific growth rate of the yeast. However, the substrate's inhibitory effects are also accelerated, decreasing both cell growth and ethanol production (Yx/s, Yp/s). At lower substrate concentrations, lower Ks values were obtained, indicating better affinity of the yeast to the substrate. Positive effects on cell activity and ethanol production can be observed when the yeast/substrate ratio reaches values close to 10% of the microorganism cell concentration (Thomas y Ingledew, 1992).

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