Kinetic modelling of bioethanol production using agro-industrial by-products

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***Abstract*—** This work aims to evaluate a sustainable bioethanol production by a *Saccharomyces cerevisiae* and using, agro-industrial by-products as carbon source for fermentation process. The influence of several carbon sources and their concentrations was studied using carob pod extract (CPE), citrus waste pulp (CWP) and beet molasses (BM) and compared with glucose and sucrose as conventional carbohydrates at different concentrations, 15, 20 and 30 g/l. Kinetics parameters were determined by Langmuir–Hanes equation, based in the linearization of the Monod equation. The agro-industrial by-products presented similar values of μmax and Ks to the conventional carbohydrates.

No significant difference was found between maximum ethanol production obtained with CPE, CWP, BM, glucose and sucrose fermentations profiles.

***Keywords*—** agro-industrial residues, bioethanol production, Monod model, *Saccharomyces cerevisiae*

I. Introduction

Bioethanol has an increased attention over the last few years, mainly due to its potential as a substitute for fossil fuels and the need to reduce global economics dependence on fossil resources [1] - [5].

Actually Brazil and the USA are the world's largest producers of bioethanol, counting with approximately 62% of world production [6, 7]. The major feedstocks used by these countries are sugar cane and corn, respectively. In Europe ethanol production, based in beet molasses, is still very sharp due to the lack of available feedstocks that can support local ethanol productions plants [7].

The European Union has established a goal of 5.75% biomass-derived transportation fuels by December 2010. The use of fuel ethanol has been quite successful in Brazil, where it is produced at a very low cost by fermentation of sugarcane. In the United States corn is the dominant biomass feedstock for production of ethanol, and in the EU straw and other agricultural wastes are the preferred types of biomass for ethanol production.

Several research approaches are being carried out in order to assess the possibility of increasing ethanol yields from alternative and available feedstocks [2, 3, 8, 9]. Ethanol produced from lignocellulose and agri-industrial wastes can be seen as the most promising ones with the great advantage of a bioenergy not competing with food resources and yet a broader spectrum of feedstocks used when compared to traditional processes [7, 8, 10]. Some of these residues such has, beet molasses, citrus waste pulp or carob pulp, represent an abundant, cheap and readily available source of raw-material to be converted into fuel [11] – [13].

Carob is the fruit of an evergreen (*Ceratonia siliqua*) cultivated in the Mediterranean basin and Southwest Asia, requiring little maintenance and producing a range of products from the seeds and the pod [14, 15]. Carob pod contains about 50% of its weight in mono and disaccharides which are ready extractable by water. It's extensively used as a raw material for the production of syrups or as a cocoa substitute for the food industry [15, 16]. The carob pod 2005 world production was approximately 315 000t and Portugal being responsible for about 10% of that production [15, 17].

Sugar beet molasses is the noncrystallizable residue after most of the sucrose has been crystallized in the purification process of sugar from sugar beet. Molasses has been used in several industries such as animal feed, baking yeast and ethanol production, mainly because it is a relatively inexpensive material and readily available. [18] – [21].

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In previous studies it was reported by different authors the use of conventional carbon sources and industrial residues for ethanol production using the yeast *Saccharomyces cerevisiae* [12, 13, 18, 19, 22]. In Table II are summarized bioethanol productivities and yields coefficients obtained in batch cultures, from recent studies [19, 21, 22, 24, 25] and compared with the coefficients obtained in this work.

Various mathematical models have been proposed to describe quantitatively microbial growth kinetics. Kinetic models for microbial growth are classified in unstructured and structured models. The use of unstructured models is completely adequate in those cases where the substrate concentration is high compared to the saturation constant in the major part of a batch fermentation. The unstructured model includes the most fundamental observations concerning microbial growth processes: (i) the rate of the cell mass production is proportional to biomass concentration and (ii) there is an upper (saturation) limit for growth rate on each substrate [26].

The Monod model (equation 1) is considered the basic equation of an unstructured model [27, 28]. This model introduced the concept of growth-controlling (limiting) substrate, relating the growth rate to the concentration of a single growth-controlling substrate [μ = f (S)] via two parameters, the maximum specific growth rate (μmax), and the substrate affinity constant (Ks). This model exhibits the typical hyperbolic shape for growth rate express in function of substrate concentration.

$$\mu = \frac{\mu_{max} \cdot S}{K_S + S} \qquad (1)$$

We aim to develop a sustainable 2nd generation bioethanol production, using agri-industrial residues like carob pod extract, citrus waste pulp and beet molasses which are rich in sugar and cheap feedstocks. The kinetic study of the microbial growth process is of great relevance for the upscale of the bioethanol production.

II. Methods

A. Microbial growth and pre-inoculum

A laboratory isolate of the yeast *Saccharamyces cerevisiae* was used throughout the process. The yeast strain was maintained on solid NYDA medium (Nutrient broth 8g/l, Yeast extract 6 g/l, Dextrose 10 g/l, Agar 20 g/l) distributed on sterile petri dishes.

Pre-inoculum was prepared by growing 4 days old culture on solid NYDA medium for 18h at a 250 ml erlenmeyer with 50 ml of liquid YEPD medium (Yeast Extract 10g/l, Peptone 20g/l, Glucose 20g/l), in an orbital shaker with temperature controller (Neifo Pentlab, Portugal) at 25ºC and 150 rpm.

B. Fermentation conditions

Growth medium was based on YEPD medium with a variation on carbon source and carbon source concentration according to the by-product under study, beet molasses (BM), carob pod extract (CPE) or citrus waste pulp (CWP).

Carbon source concentration effect was studied using three different concentrations, 15, 20 and 30 g/l of total sugar available. All studies were performed in triplicate for 28h, on 250 ml erlenmeyers with 50 ml of medium, in an orbital shaker with temperature controller at 25ºC and 150 rpm.

C. Analytical techniques

Samples were collected throughout fermentation cycle. Absorbance at λ = 554 nm (Genisys 10 vis., Thermo Electron Corporation) and pH were measured (Crison GLP21, Portugal) Samples were then centrifuged, filtered and analyzed. HPLC analyses were performed on a Beckman System Gold HPLC (Beckman, USA) equipped with a Jasco Refractive Index model 1530 (Jasco, Japan). Sugar analyses of the carob pod extract (CPE), beet molasses (BM), glucose and sucrose were performed using a Purospher STAR NH_2 column (Merck KGaA, Germany) in a isocratic system, Acetonitrile:Water (75:25) at 1 ml/min and 35ºC. Ethanol quantification used an OH AY column (Merck KGaA, Germany), in an isocratic system, with H_2SO_4 0,002N at 0.5 ml/ml and room temperature.

D. Kinetic Parameters

Growth rate measurement

The growth rate of culture is given by the equation 2

$$\ln\left(\frac{X}{X_0}\right) = \mu \cdot t \qquad (2)$$

where X_0, is the initial biomass concentration (g/l), X is the biomass concentration at time t (g/l), and μ is the specific growth rate (h^{-1}) and was determined by plotting the natural logarithm of cell biomass concentration (X) versus time. The slope of the line is the growth rate μ.

Ks and μ_{max} determination

The maximum specific growth rate (μ_{max}) and the substrate saturation concentration (Ks) for the different carbon sources tested, was calculated by Langmuir–Hanes equation (equation

3), based in the linearization of the Monod equation (equation 4).

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

$$\mu = \frac{\mu_{max} \cdot S}{K_S + S} \quad (4)$$

The data generated in this study are linearly fitted with the model, as a function of concentration produced during the exponential phase versus time. From the plot, the maximum specific growth rate and Monod constant were determined.

III. Results and Discussion

This study was performed using agro-industrial by-products, as carob pod extract (CPE), citrus waste pulp (CWP) and beet molasses (BM) in a *Saccharomyces cerevisiae* batch culture, in a perspective of optimal yields for bioethanol production. A comparison between these carbon sources and conventional carbon sources, glucose and sucrose, was performed.

The kinetic characterization of *S. cerevisiae* growth, using those carbon sources was done, by the determination of maximum specific growth rate (μ_{max}) and the substrate saturation concentration (K_s).

For each carbon source used, the variation of the specific growth rates with substrate concentrations, showed that the cell growth fits with the classical Monod kinetics. The maximum specific growth rate and the substrate saturation concentration for the different carbon sources, was calculated by the linearization of the Monod equation based in the Langmuir–Hanes representation (equation 3). The values presented in table I show that maximum specific growth rate has similar values for the different carbon sources used with a slight high value for sucrose.

Studies performed by Nath et al. [29] show that the maximum specific growth rate depends on temperature and pH of the medium. In this study, temperature and initial pH were kept constant during growth experiment. Only sucrose presents a higher specific growth rate, comparatively with the others substrates. This fact may be justified by the high affinity of the *S. cerevisiae* cells to this disaccharide and is corroborated by the lower value of Monod constant, 8.98 g/l, (table I).

Table I. Values of maximum specific growth rate (μ_{max}) and Monod constant (K_s), determined by Langmuir–Hanes, based in the linearization of the Monod equation, for *S. cerevisiae* grown with different carbon sources. CWP - citrus waste pulp, CPE - carob pod extract, BM - beet molasses.

Substrate	μ_{max} (h^{-1})	K_s (g/l)
CWP	0.35	10.69
CPE	0.33	12.47
GLUCOSE	0.38	9.40
SUCROSE	0.55	8.98
BM	0.35	5.66

The saturation constant (K_s) reflects the fact that large values of K_s imply that there is a weak affinity for the bacterial strain to 'bind' the substrate. In these studies, the K_s values were different for the different carbon sources assayed. CPE was the substrate with the higher value of K_s and BM present the lower value i.e., this *S. cerevisiae* culture presents more affinity for the beet molasses extract than to carob pod extract. Beet molasses shows a lower K_s, comparatively with the others carbon sources. Probably is due to the high content of carbohydrates, mostly sucrose (90 % p/p) and this substrate also evidences a high affinity to this yeast strain culture (table 1)

The results obtained with citrus waste pulp and glucose are similar, as the composition of CWP is predominantly glucose and fructose, reducing sugars, easily metabolisable and a very small quantity of sucrose (results not shown).

Results reported by other authors [26, 29, 30] are concordant with the results obtained in this work for conventional sugars used. In the literature, as far as we know it could not be found values of K_s or μ_{max} to the beet molasses, citrus waste pulp or carob pod extract.

The kinetic constants obtained are very relevant if used as design parameters for bioethanol large scale producing bioprocesses. In the case of beet molasses (BM), as the K_S value 5.66 g/l represents the substrate concentration required to achieve 50% of the maximum growth rate, it can become a criteria for adjusting the most efficient beet molasses concentration in reactor.

Ethanolic production of *Saccharomyces cerevisiae*, grown in batch culture with different carbon sources concentrations (15 g/l, 20 g/l, 30 g/l) glucose, sucrose, beet molasses, citrus waste pulp and carob pod extract are presented in figure 1.

Ethanol production was significantly improved at 30 g/l

initial carbon source concentration, for any of the assayed raw-materials, except for beet molasses that showed a slight decrease, comparatively with the others concentrations.

At 15 g/l carbon source (figure 1A), a maximum of ethanolic production was obtained, in general after 24 hour inoculation, but glucose promoted an ethanolic maximum after 20 hour inoculation. Probably this occurs due to *S. cerevisiae* higher affinity to glucose than to others carbohydrates. In these conditions maximum concentration of ethanol (6 g/l) was achieved for 15 g/l of carob pod extract and 9 g/l of ethanol for 20 g/l beet molasses growth.

For cultures grown at 30 g/l carbon source (figure 1C), values of ethanol formation are between 8 and 10 g/l and the maximum ethanol formation achieved within the first 20 hours of culture for any of the studied carbon sources, at a less period of time than for the others carbon sources concentrations used.

Table II depicts results for ethanol production, product yields ($Y_{P/S}$) and productivities achieved in this study and establishing a comparison with results already described by other authors.

Atiyeh & Duvnjak [19] and Roukas [20] reported fermentations of *S. cerevisiae* with beet molasses, in which the sugar concentration varied between 0.98 to 276.2 g/L, with a maximum ethanol of 0.48 and 3.5 g/l respectively, which are lower than the obtained in this work. For initial sugar concentration at 30g/L, CPE fermentation profile achieves an ethanol production, productivity and yields very similar to the assayed carbon sources, glucose and sucrose.

Although a higher yield is achieved with half the concentration (0.43 with 15 g/l) it requires almost two fold the amount of time to produce nearly 70% of the ethanol produced with 30 g/l (10.30 g/l). Mishima *et al* [21] report 14.9 g/l ethanol for water hyacinth (30g/l) as substrate. However, higher carbon source concentrations, 200 g/ sucrose and 220 g/l glucose can produce 96.7 g/l and 82.1 g/l ethanol concentration respectively, as verified by ζaylak and Sukan [22] and Borzani [24].

S. cerevisiae is able to get high rates of glycolysis and production of ethanol when optimal conditions are presented, by producing 2.5 g/l more ethanol per h and per g of cellular protein. However, this high rate is kept only by brief periods of time during the batch fermentation and decreases gradually while ethanol accumulates in the nutrient medium [18]. Although the yield is slightly higher with a lower substrate concentration, it is relevant the fact that when the carbon source increases ethanol production also increases and the maximum peak of ethanol appears earlier in the fermentation.

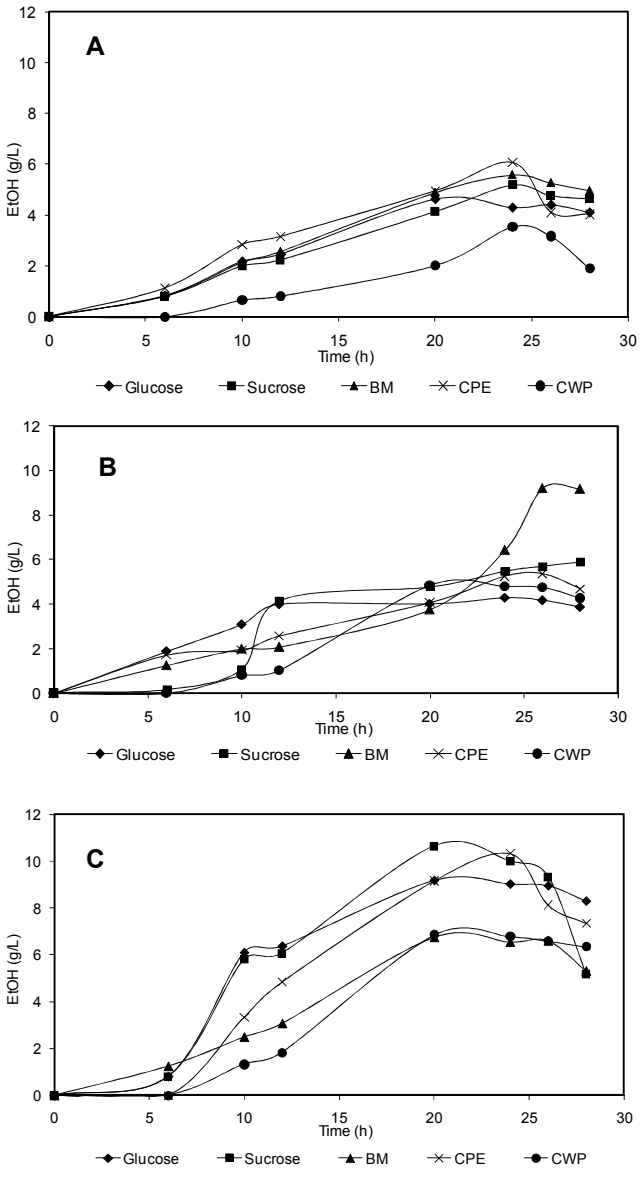

Figure 1. Ethanol production, using *S. cerevisiae* BBE-1 in batch system for different carbon sources, glucose, sucrose, beet molasses (BM), citrus waste pulp (CP) and carob pod extract (CPE) at different concentrations: A – 15 g/l, B – 20 g/l and C – 30 g/l.

Table II. Ethanolic production by *Saccharomyces cerevisiae* in batch culture, with different substrates

Substrate	Microorganism	Substrate (g/l)	Ethanol Concentration (g/l)	Productivity (g/l.h)	Yp/s (g ethanol/g subst)	Reference
Glucose	*S. cerevisiae*	15	4.63	0.25	0.31	
		20	4.28	0.17	0.21	This work
		30	9.16	0.50	0.31	
Sucrose	*S. cerevisiae*	15	5.19	0.26	0.34	This work
		20	5.92	0.24	0.31	
		30	10.65	0.57	0.35	
Sucrose	*S. cerevisiae*	220	96.71	1.01	0.44	[22]
Glucose	*S. cerevisiae*	200	82.1	---	0.41	[24]
Beet molasses (BM)	*S. cerevisiae*	15	5.57	0.25	0.37	This work
		20	9.21	0.31	0.46	
		30	6.75	0.34	0.22	
Carob pod extract (CPE)	*S. cerevisiae*	15	6.08	0.25	0.43	This work
		20	5.36	0.20	0.31	
		30	10.30	0.48	0.34	
Citrus waste pulp (CWP)	*S. cerevisiae*	15	3.53	0.15	0.26	This work
		20	4.86	0.24	0.26	
		30	6.84	0.35	0.25	
Mahula (Madhuca latifolia L.)	*S. cerevisiae*	fermentable sugars (28.1– 36.3 g /100 g)	31.84	0.33	0.54	[25]
Beet molasses	*S. cerevisiae*	242 – 276		0.48 – 2.97	0.59 – 0.76	[19]
Water hyacinth	*S. cerevisiae*	30.1 g /l glucose	14.4	---	---	[21]
Water lettuce	*S. cerevisiae*	33.3 g/l glucose	14.9	---	---	[21]
Potato starch	*Aspergillus Niger* + *S. cerevisiae* (SSF)	180 g/l glucose	92	---	0.4	[27]

In fermentations performed with carob pod extract and beet molasses it was observed that maximum ethanol production increased with sugar concentration as reported by several others authors (Table II). However, Ks value was slight high, CPE, as feedstock showed the overall best results for product yield at 15 g/l and 30 g/l of total sugar available and similar to the conventional traditional sources, like glucose and sucrose. We believe that kinetics parameters depend of various factors, like pH, temperature, cell physiologic stage and growth conditions and not only the type of carbon source.

The ethanol productivities obtained (g/l.h), in this work, at different concentrations are in the same range of values of results referred by other authors (table II).

Presently, other microorganisms have been investigated as potential for the production of bioethanol. The use of agri-food waste, as possible low cost sources of carbon has also been subject of research, what may be determinant for the production of bioethanol becoming economically competitive. Table III presents examples of some microorganisms used in ethanol concentration and high yields when compared with *Saccharomyces cerevisae* values (Table II).

Wilkins *et al.* [31] reported the work done with two ethanologenic yeasts, *S. cerevisiae* and *Kluyveromyces marxianus*, that were used to ferment hydrolyzed sugars extracted from Valencia orange peel waste. In these conditions *S. cerevisiae* produced more ethanol than *K. marxianus* at 24, 48, and 72 h of culture. With these results and for this reason, *S. cerevisiae* was preferred over *K. marxianus* to get more ethanol and higher growth rates than *K. marxianus*. The results reported showed that ethanol and cell mass yields were inhibited by the presence of limonene in orange peel waste. We believed that the same can happen with citrus waste pulp (CWP), where the ethanol concentration was slightly lower than that obtained with other carbon sources tested (table II).

Table III. Microorganisms used in ethanol production, with different substrates

Microrganism	Substrate	Initial reducing sugars (g/l)	Ethanol Concentration (g/l)	Yp/s (g ethanol/g subst)	Reference
Zymomonas mobilis + *Candida tropicalis*	Fruit and vegetable residues	122 – 36	50 – 14	---	[32]
Zymomonas mobilis	agro-industrial waste (thippi)	153	65.3	0.42	[33]
Candida tropicalis	agro-industrial waste (thippi)	153	61.2	0.39	[33]
Zymomonas mobilis + *Candida tropicalis*	agro-industrial waste (thippi)	153	72.8	0.48	[33]
Zymomonas mobilis	sugar cane molasses	200	55.8	0.34	[34]
Kluyveromyces marxianus	sugar cane juice + sucrose	220	87.0	---	[35]
Kluyveromyces marxianus	Cheese whey powder	25 – 150	----	0.35 – 0.54	[36]

Among many microorganisms that have been exploited for ethanol production, *Saccharomyces cerevisiae* still remains as the prime species. *Zymomonas mobilis*, when compared with *S.*

cerevisiae, present an ethanol yield and productivity higher, because less biomass is produced and has a higher metabolic rate in conversion of glucose in to ethanol [37]. However, due to its specific substrate spectrum as well as the undesirability of its biomass to be used as animal feed, this species cannot readily replace *S. cerevisiae* in ethanol production. Although, Cazetta *et al*. [34] report ethanol production by *Z. mobilis* in high concentration of sugar cane molasses. The results obtained comparatively with the results presented in table II, showed that *S. cerevisiae* is a successful case, at high concentrations of carbon source. This yeast seems to have the best ethanol yields.

Further experiments will be done to explore the potential use of these industrial by-products at higher concentration and in a process of carbon source enrichment with the objective of maximizing ethanol production. Kinetics determination of parameters by Monod model, demonstrated that the affinity for those industrial by-products is high, similar to the conventional carbon sources, which reinforces its potential application in the production of bioethanol on a large scale.

IV. Conclusion

The industrial residues, CPE, CWP and BM, used as carbon sources, seem to be adequate feedstocks for bioethanol production. Productivities and ethanol yields are similar to the obtained with conventional carbon sources, glucose and sucrose and may attain high product yields. The use of agriculture wastes is a valuable contribution for ethanol production, in next future as a 2nd generation bioethanol, promoting a sustainable biofuel production and avoiding the depletion of agriculture resources, a determinant strategy for not causing a negative impact on food production.

Ethanol produced from renewable and cheap agricultural products provides reduction in green house gas emission, carbon monoxide, sulfur, and helps to eliminate smog from the environment. Bioethanol, both renewable and environmentally friendly, is believed to be one of the best biofuels alternatives if supported by national legal and strategic energy orientations.

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