Interactions between pretreatment and nutrient concentrations of mussel processing effluents for citric acid production

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Pretreatment of mussel processing effluents for citric acid production by *Aspergillus niger* (CBS 262-65) in submerged culture was studied by means of an orthogonal full factorial experimental design with four independent variables: initial levels of hexacyanoferrate II, methanol, phosphate, and carbon source. The empirical model thus obtained showed the ineffectiveness of the hexacyanoferrate treatment (contrarily that occurs with molasses) and at the same time, a series of complex interactions affecting the acid production not described in previous studies concerned by the separate effects of the variables here considered. Basically, the main effect of the hexacyanoferrate is toxic while the presence of methanol seems to involve morphological changes that increase the superficial area of the mycelial pellets, thereby promoting an unspecific metabolic activation. It therefore enhances the toxic effect of hexacyanoferrate, but also favors citric acid accumulation. The net response depends on the concentrations of the carbon source and phosphorus.

Keywords: Citric acid production; mussel processing effluents; *Aspergillus niger*; experimental design

Introduction

Mussel production by semiculture in the Galician Rias Baixas (North-West Spain) is 250,000 metric tonnes/annum which represents almost half of the world production. More than 60% of it is processed and leads to generation of 300-500 l effluent/metric ton of the raw mollusc.¹ At present, the effluent is dumped in the coastal water, thereby severely polluting the very medium that generates the resources. Its utilization for the recovery of proteins or taurine² and small-scale uses in recovery of glycogen for

cosmetic preparations or as food seasoning are not economical.¹ Recently, its use as substrate, directly and after concentration, with or without saccharification was investigated for various fermentation products such as biomass³⁻⁴ amylases,⁵⁻⁷ gibberellic acid⁸⁻¹⁰ and citric acid.¹¹

Production of citric acid by *Aspergillus niger* from different agro-industrial by-products/wastes of extremely variable composition poses a number of severe problems because of the high sensitivity of the culture to a number of medium ingredients.¹² The yields of citric acid are drastically reduced especially in the presence of metal ions and minerals¹³ and hence, these are binded and inactivated or precipitated by pretreatment of the substrate with potassium hexacyanoferrate II.¹⁴⁻¹⁵ Another important factor is the inclusion of 1-3% methanol or other low molecular weight alcohols which stimulates citric acid production by affecting growth and sporulation through the action on the cell permeability, spatial organization of the membrane, or changes in lipid composition of the cell wall.¹⁶ A relationship between alcohol and tolerance to Mn, Fe, and Zn has also been suggested.¹⁷ Phosphorus is another nutrient whose effect on the production of citric acid has been related to metal content of the media. In media with phosphorus limitation, the effect of trace metals becomes less acute.¹⁸⁻²⁰

All of the above three factors (hexacyanoferrate, methanol, and phosphorus) together with substrate concentration have a critical role in the biosynthesis of citric acid. Obviously, these have complex interactions among themselves and, therefore, assume vital importance especially when a new substrate is employed. A literature survey indicates no work on the use of mussel processing waste (MPW) as substrate for citric acid production except for initiation of the effort by our group.¹¹ Even with other substrates, the studies on these four factors have been done in isolation, thereby ignoring interactive effects among themselves. Consequently, these optimization studies gave values which may not be really optimal; hence, the present studies have been undertaken.

Materials and methods

Microorganism and inoculum preparation

A. niger (CBS 262-65) was used and maintained on malt-yeast extract agar slants at 4°C by subculturing every month. For inoculum preparation, the spores grown on the slant at 30°C for 7 days were scraped in distilled water and the spore density was adjusted to an initial population of 0.5×10^6 spores ml⁻¹ of medium by optical density measurement at 750 nm after calibration between this data and direct hemocytometer counting.

Fermentation medium and culture conditions

The processing of MPW and its concentration were as described elsewhere.⁷ The basic medium (11 M-H) used contains (g l⁻¹): glucose, 112; total nitrogen, 0.675; and total phosphorus, 0.052. The pH was adjusted to 4.0 with 5 N HCl. Sterilization was performed by steaming for 1 h. Cultures were performed in 300-ml Erlenmeyer flasks with 50 ml of medium at 30°C with orbital shaking at 200 rpm. Samples (entire flask) were drawn periodically for analyses at the fermentation times specified in each case.

Experimental plan

Four independent variables (hexacyanoferrate, methanol, phosphorus, and substrate) were considered in order to obtain an orthogonal full factorial design²¹⁻²² with central quadruple replication. Experimental domain and coding criteria are given in Table 1. Taking into consideration the initial concentrations of the substrate and phosphate in the medium 11 M-H, it was diluted with distilled water and supplemented with KH₂PO₄ for obtaining required concentrations of these compounds. The desired concentrations of hexacyanoferrate and methanol were similarly achieved. Potassium hexacyanoferrate II was added to the medium prior to sterilization. Methanol was added after cooling to 30°C.

Analytical methods

Reducing sugars were estimated by the 3,5-dinitrosalicylic acid Reaction²³ as modified by Forouhi and Gunn²⁴ using glucose as standard. Citric acid was measured by the spectrophotometric method of Marier and Boulet²⁵ once its accuracy was confirmed by the chromatographic method of Chapman and Horvat.²⁶ The metal ion contents of the medium were determined by atomic absorption spectrophotometry.²⁷ Mycelial cells

from the fermented medium were separated by paper filtration, washed with distilled water, and dried to constant weight over P_2O_5 under reduced pressure in a dessicator/oven at $55^\circ C$. All the experiments and analyses were performed in duplicate.

Results and discussion

Selection of the ranges of the variables

The results of the earlier work on production of citric acid by *A. niger* CBS 262-65 from MPW and the literature reports¹²⁻¹³ formed the basis for selection of the range of each of the four variables studied. For example, earlier results showed marked reduction in the production and yield of citric acid from MPW when the medium was supplemented with Fe and Mg (unpublished data). Although these results suggested the presence of an excess of these metals in the MPW, it is unlikely, however, that the medium will have higher levels of low molecular weight components since the MPW has undergone ultrafiltration and diafiltration using a cut-off membrane of 100 kD molecular mass.⁷ The estimation of the metals in the medium by atomic absorption spectrophotometry gave interesting results. The levels of Fe, Mn, Zn, Cu, and Mg in the medium 11 M-H were 1.68, 0.22, 1.17, 0.52, and 17.75 mg l⁻¹, respectively. Except for Mg, these values are much lower than those reported by Chanda et al.¹⁵ and Sanchez-Marroquin et al.²⁸ for canesugar molasses.

Effect of potassium hexacyanoferrate

II concentration

There is general agreement to admit a double effect of hexacyanoferrate on citric acid production: positive, due to the formation of complexes with metals that precipitate or are biologically inactive; and negative, by inhibiting growth. It is not clear, however, if the inhibition is due to a direct effect or to the diminution of the content of metals to levels restrictive of growth. Some authors suggest the convenience of an excess of free hexacyanoferrate.²⁹⁻³⁰ In general, the optimum concentration that appears in bibliography shows a great deal of variability even with the same type of substrate and must be established empirically. Experiments on the effect of potassium hexacyanoferrate II treatment of medium 11 M-H, however, at the level of 10, 20, 50,

100, 500, and 1,000 mg l⁻¹ medium were conducted. The medium turned blue when the concentration of potassium hexacyanoferrate II was 20-200 mg l⁻¹ versus milky white with the use of the binding agent at > 200 mg l⁻¹ due to protein precipitation.³¹

Data after a 48-h incubation (Figure 1) indicate that hexacyanoferrate affects the growth of the culture even at as low as 10 mg l⁻¹. In terms of inhibition percentage of biomass formation (0, the effect of hexacyanoferrate dose (D) showed a sigmoidal response characteristic of the toxic effects which can be adjusted to a conventional logistic equation:

$$I = \frac{K}{1 + \text{EXP}(a - bD)} \text{ in witch } a = \ln \left(\frac{K}{I_0} - 1 \right) \text{ and}$$

K is the maximum biomass inhibition, I₀ the intercept, and b the specific inhibition constant by Quasi-Newton minimization method. This gave a value of 315 mg l⁻¹ for ID₅₀. In terms of citric acid production, the chelating agent up to 200 mg l⁻¹ concentration does not significantly affect the titers, but this concentration of potassium hexacyanoferrate II is the one which affects biomass formation significantly. Perhaps it is this effect of hexacyanoferrate on the biomass formation which improves citric acid production by directing the sugars consumed into citric acid biosynthesis. This can be observed in Figure I at intermediate levels of the binding agent.

The only effect of hexacyanoferrate treatment, thus, is a slight increase in the yield of citric acid in the case of MPW. The data, in turn, support the hypothesis that the concentrations of metals in this waste are not at a sufficient level to affect citric acid production; however, this variable (at 20-150 mg l⁻¹) was included in the statistical method of optimization, in order to determine its interactions with other variables.

Effect of methanol on culture morphology

The data on morphological characteristics of the culture showed that in the presence of methanol, the mycelia was in the form of smaller, homogeneous-sized pellets of less than 1 mm diameter in contrast with the pellets of 3-5 mm diameter in the case of the medium without methanol and, consequently, resulted in a higher number of pellets when the medium contained methanol. This observation could be noticed at as early as

24 h of incubation; thus, methanol seems to increase the biomass surface area and thereby promotes increased mass and substrate/product transfers.

Kinetic results

The data on the effects of various variables on biomass formation, citric acid production, residual glucose levels, and pH of the medium at 100, 196, and 288 h incubation periods (Figure 2) show that methanol addition stimulates citric acid production and the rate of glucose consumption even in the presence of hexacyanoferrate. In media without methanol, the addition of hexacyanoferrate shows a minor negative effect on the citric acid production, but a more marked effect on glucose consumption.

It is interesting to point out that the interactions between hexacyanoferrate and methanol are resolved in a different way in terms of biomass formation since the addition of methanol overcame the toxic effect of hexacyanoferrate. In the presence of methanol, the growth of the culture is not affected upon addition of 150 mg l^{-1} hexacyanoferrate; however, in its absence when the level of hexacyanoferrate increases from 20-150 mg l^{-1} the increase in biomass is inhibited by 40-50% as is also evident from the results of the preliminary experiment.

Mathematical model

Based on three different responses (citric acid and glucose consumption at three incubation periods and biomass at two incubation periods), a mathematical model has been developed. The criterion for acceptance of the models is based on contrasting the coefficients by means of the t of the Student test ($\alpha < 0.05$), and Fischer's F (also ($\alpha < 0.05$)) for the global validity of the resulting equation, considering both the experimental error/total error and lack of fitting/experimental error ratios.

At all three incubation periods, the citric acid production was consistently described by means of a linear model with interactions. With respect to biomass and glucose consumption, the significance of the differences between total media and central media provides evidence in all of the cases for different curvatures. The results summarized in

Table 2 for the maximum concentration of citric acid allow the proposal of the following model. (Y is citric acid in g l⁻¹ at 196 h):

$$Y = 10.57 + 3.74S - 2.89F + 8.58M - 1.61SP - 1.40SF + 3.47SM - 2.86FM - 1.42SPM \quad (1)$$

If the exigency of the Student's test are raised to $\alpha < 0.01$, the model is reduced to:

$$Y = 10.57 + 3.74s - 2.89F + 8.58M + 3.47SM - 2.86FM \quad (2)$$

Although Eq. (2) provides less accurate predictions (adjusted $r^2 = 0.879$) than Eq. (1) (adjusted $r^2 = 0.935$), it surpasses both Fisher tests at $\alpha < 0.05$ and, therefore, is acceptable as a "broad" model for the most significant terms of the system.

If, on the contrary, the Student's test exigencies are reduced to $\alpha < 0.1$, the predictions improve slightly, but the model is unnecessarily complicated. This model, however, is interesting since it indicates the fact that phosphorus concentration is a significant first-order variable with a negative sign.

Eq. (1) hence, is selected for the system. Standardized residuals are acceptable and lacking in suspicious regularities, and linear correlation between observed and expected values (Figure 3) only reflects the small second-order component which is not significant according to the criterion of lack of fitting/experimental error ratio.

One of the first considerations is concerned with the high susceptibility of response to the factors studied, i.e., the effect of the selected variables multiplied by a factor of -4 the response in the center of the experimental domain.

Between the first-order terms (in addition to the obvious effect of the substrate upon the response), the system behaviour appears greatly influenced by a strong predominance of the favorable effects of methanol in contrast to the unfavourable effects of hexacyanoferrate; thus (compare Figures 4A and 4B), high responses are absent at high levels of hexacyanoferrate whose role in this medium seems to be of a toxic nature especially in the absence of a high metal concentration. Inversely, high responses are never obtained with low methanol levels.

Comparing left and right sides of Figure 4B, it can be appreciated that the phosphorus (even lacking in first-order effect) becomes essential for promoting the favorable effect of methanol at low levels of substrate. Secondly in (Figs. 4A and 4B), a very slight favorable effect of hexacyanoferrate at any phosphorus concentration is apparent especially at low substrate levels. Finally (Figure 4C), the effect of methanol reveals a complex behavior. At a high substrate level, it overcomes the negative effect of hexacyanoferrate independently of the phosphorous concentration. At a low substrate level, it reverses the effect of hexacyanoferrate which was slightly positive with low methanol; thus, methanol causes enhancement at the same time citric acid accumulates and hexacyanoferrate is toxic. The net effect depends on the concentrations of the carbon source and phosphorus.

It thus seems reasonable to attribute to methanol an unspecific activating role in the microbial metabolic processes. An hypothesis is also supported by the fact that phosphorus, a nutrient of essentially energetic character, contributes to enhancing the effect; however, it is difficult to justify that the low levels of substrate favors such manifestations.

On the basis of the present results, it can be concluded that the pretreatment of MPW medium with hexacyanoferrate is not required, but the addition of methanol to the medium significantly improved the production of citric acid. A literature survey indicates that such enhancement of citric acid productivity is common for methanol, ethanol, and other low molecular weight alcohols.^{13,17} The suitability of MPW for ethanol production was demonstrated in a previous work.⁷ This opens up a new possibility for using either a two-stage process (first ethanol production by *Saccharomyces cerevisiae* and then citric acid production by *A. niger*) or a mixed culture fermentation of MPW for production of citric acid with the goal of reducing the cost of production.

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Table 1 Experimental domain and codification criteria of the independent variables

	Coded values	Natural values			
		Substrate S (g l ⁻¹)	Phosphorus P (mg l ⁻¹)	K ₄ [Fe (CN) ₆] F (mg l ⁻¹)	Methanol M (% v/v)
	-1	62	50	20	0
	0	78	100	85	1.5
	1	94	150	150	3.0
Increments	1	16	50	65	1.5
Codification:		V _c : Coded value			
$V_c = (V_n - V_o)/dV_n$		V _n : Natural value			
Decodification:		V _o : Natural value in the center of the domain			
$V_n = V_o + (dV_n * V_c)$		dV _n : Increment of V _n corresponding to one unit of V _c			

Table 2 Results of the experimental plan and analysis of the significance of the proposed model

S	P	F	M	Y	$\hat{Y}$	Coefficients	T	Model	
1	1	1	1	12.94	16.18	10.57	27.927	10.568	
1	1	1	-1	1.26	0.63	3.74	8.831	3.736	S
1	1	-1	1	28.44	30.47	-1.17	2.771	NS	P
1	1	-1	-1	2.34	3.49	-2.89	6.822	-2.886	F
1	-1	1	1	23.01	22.24	8.58	20.283	8.581	M
1	-1	1	-1	1.75	1.02	-1.61	3.811	-1.613	SP
1	-1	-1	1	39.93	36.53	-1.40	3.318	-1.404	SF
1	-1	-1	-1	2.57	3.88	3.47	8.199	3.469	SM
-1	1	1	1	9.81	10.64	0.26	0.620	NS	PF
-1	1	1	-1	1.98	3.29	-1.19	2.807	NS	PM
-1	1	-1	1	14.87	19.31	-2.86	6.751	-2.856	FM
-1	1	-1	-1	1.33	0.54	-0.12	0.278	NS	SPF
-1	-1	1	1	6.77	4.58	-1.42	3.350	-1.418	SPM
-1	-1	1	-1	1.74	2.90	-0.96	2.266	NS	SFM
-1	-1	-1	1	15.23	13.25	0.34	0.804	NS	PFM
-1	-1	-1	-1	0.73	0.15	-0.13	0.307	NS	SPFM
0	0	0	0	12.83	10.57	Average response = 10.57			

0	0	0	0	10.69	10.57	Average central response = 11.67	
0	0	0	0	9.80	10.57	Var(Ee) = 2.864	
0	0	0	0	13.34	10.57	$t(\alpha < 0.05; v = 3) = 3.182$	
				SS	df	QM	
Model		1963.17	8	245.40	QME/QMEe = 2.454	$F_3^{11}(\alpha = 0.05) = 8.77$	
Error		77.32	11	7.03	QMLF/QMEe = 3.000	$F_3^{18}(\alpha = 0.05) = 8.85$	
Exper. error	8.59	3	2.86			$r^2 = 0.962$	
Lack of fitting	68.73	10	8.60			adj $r^2 = 0.935$	
Total		2,040.49	19	107.39			

$\hat{Y}$ and $\hat{Y}$: observed and expected values of citric acid (g l^{-1}), respectively; NS: not significant coefficient; SS: sum of squares; QM: quadratic means. E: total error; Ee: experimental error; LF: lack of fitting. Variables according to Table 7

Figure 1 Effect of the treatment with potassium hexacyanoferrate II among citric acid production and biomass in medium 11 M-H after an incubating period of 48 h (top). The horizontal lines indicate the levels of the corresponding control culture. Toxic effect in terms of inhibition percentage of biomass formation adjusted (Quasi-Newton) to a conventional logistic equation (bottom). Citric acid, CIT; biomass, B; consumed glucose, Gc; inhibitor dose, ID_{50}

Figure2 Experimental plan results represented as a function of time. Absence of methanol, (O or $\diamond$); presence of methanol, ($\bullet$ or $\blacklozenge$); absence of hexacyanoferrate, (O or $\blacklozenge$); and presence of hexacyanoferrate, (O or $\bullet$). Notes same s Figure 7

Figure 3 Correlation between observed and expected values according to the model proposed

Figure 4 Response surfaces corresponding to citric acid concentration after 196 h. A function of substrate and methanol: with a low level of hexacyanoferrate (A) or a high level of hexacyanoferrate (B). For A and B, left figure has a low level of phosphorus and right figure has a high level of phosphorus. A function of hexacyanoferrate and methanol (C). With a high level of substrate and low level of phosphorus (left). With a low level of substrate and a high level of phosphorus (right)