ARTÍCULO CORTO

Study of itaconic acid production by *Aspergillus terrus* MJL05 strain with different variable

Estudio de la producción de ácido itacónico con Aspergillus terreus de la cepa MJL05 con diferentes variables

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Abstract

Itaconic acid (IA) production by *Aspergillus terreus* MJL05 strain was investigated in submerged batch fermentation in a stirred bioreactor to determine the effect of varying the nitrogen, phosphorous and carbon sources concentrations in the production medium. Glycerol, a biodiesel by-product was reported as an efficient substrate to achieve high itaconic acid productivities. This was used as the sole carbon source. The resulting C:N 18, N:P 10.8 and C:P 195 ratios were selected as the best and allowed to improve IA concentration from 11.0 to 27.6 g/l with a volumetric productivity of 0.192 g IA/l.h and a specific productivity 0.013 g IA/g biomass.h. Bioprocess yields, Yx:s 0.27 g d.w. biomass/g substrate; Yp:x 1.63 g IA/g d.w. biomass and Yp:s 0.44 g IA/g substrate, allowed to assume the feasibility of using this strain for IA production.

Key words: Itaconic acid, *Aspergillus terreus*, glycerol.

Resumen

La producción de ácido itacónico (AI) con *Aspergillus terreus* MJL05 se realizó en fermentación sumergida en lote en un biorreactor agitado para estudiar el efecto de la variación de las concentraciones de nitrógeno, fósforo y carbono en el medio de producción. El glicerol, subproducto del biodiesel, fue reportado como un sustrato eficiente para obtener altas productividades de AI. Este fue utilizado como única fuente de carbono. Las relaciones entre nutrientes, C:N 18, N:P 10,8 y C:P 195 fueron seleccionadas como las mejores para aumentar la concentración de AI de 11,0 a 27,6 g/l con un a productividad volumétrica de 0,192 g IA/l.h, y una productividad específica de 0,013 g IA/g biomasa.h. Los rendimientos del bioproceso obtenidos fueron de Yx:s 0,27 g p.s. biomasa/g sustrato; Yp:x 1,63 g IA/g p.s. biomasa y Yp:s 0,44 g IA/g sustrato, lo que permite asumir la factibilidad de usar esta cepa para la producción de AI.

Palabras clave: ácido itacónico, *Aspergillus terreus*, glicerol.

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Introduction

Itaconic acid (AI) is a promising organic acid. It is a white crystalline unsaturated dicarbonic acid in which one carboxyl group is conjugated to the methylene group. IA is used worldwide in the industrial synthesis of resins such as polyesters, plastics, and artificial glass and in the preparation of bioactive compounds in the agriculture, pharmacy, and medicine sectors. There is continued interest in developing biological methods to produce compounds with double bonds that are suitable for the manufacture of various polymers. IA also provides possibilities for selective enzymatic transformations to create useful polyfunctional building blocks (Okabe *et al*., 2009).

In general, itaconic acid production by *A. terreus* depends on temperature (30-40 ºC), continuous aeration, low starting pH (3-5), lower operating pH (2.2-3.8), high glucose concentrations (10-20%), sufficient nitrogen, high magnesium sulfate concentration (0.5%), low phosphate to limit mycelial growth, and adequate levels of trace metals, zinc, copper, and iron (Nubel and Ratajak, 1964; Gyamerah, 1995; Willke and Vorlop, 2001). *Aspergillus terreus* NRRL 1960 has been so far used as a target microorganism for optimizing the fermentation process. The mycelial growth is sensitive to phosphate limitation and to carbon and nitrogen availability (van der Werf *et al*., 2009). Glucose and glycerol were reported as suitable for IA production (Jarry and Seraudie, 1995; Jarry and Seraudie, 1997) although the observed yields and productivities of the process were strictly depend on the selected strain, ambient conditions and media formulation (Meyer, 2008; Okabe *et al*., 2009).

The main aim of this work was to clarify the effect of variable C:N, C:P and N:P concentrations ratios present in the production media onto the yields and productivities of *A. terreus* MJL05 batch fermentation in submerged culture with glycerol as the sole carbon substrate.

Materials and methods

Microorganism. *Aspergillus terreus* MJL05 strain was isolated from vendee's lands. The culture was maintained on malt agar slants at 5 ºC and sub-cultured every month.

Inoculum. *A. terreus* MJL05 was inoculated in malt extract agar plate and incubated at 30ºC until sporulation was obtained. A suspension of spores was obtained by washing the Petri dish cultures with 10 ml sterile saline solution with Tween 80. Erlenmeyer flasks containing 100 ml *M1* medium were inoculated with an aliquot of the concentrated spore's suspension in order to obtain $5x10^7$ spores.ml^{-1} in the production medium. Flasks were shaken at 200 rpm at 30 ºC for 24 h in an orbital shaker. The resulting "pellets" were used as inoculums for the batch fermentation assays.

M1 culture medium in (g/l): glucose 100.0, $MgSO_4.7H_2O$ 2.0, $CuSO_4.5H_2O$ 0.01, KH_2PO_4 0.2, NH_4NO_3 5.0, Cl_2Ca 2.0, pH 5.5. The resulting C, N and P concentration ratios were C:N 23, C:P 888 and N:P 38.3.

All drugs used were from Cicarelli Lab.

Batch stirred tank fermentations with constant glucose and variable glycerol concentrations: Influence of C:N , C:P and N:P ratios on the IA production

Batch fermentation was scaled-up with a 3-l fermentor (Applikon) with a vessel internal diameter of 0.13 m. The fermentor was equipped for the control of agitation (Rushton impellers), stirred speed (1.25 m.s^{-1}), dissolved oxygen (Cole-Parmer electrode) and air flow (measured with a mass flow controller AALBORG GFM317) and adjusted in 0.69 l.min^{-1} (0.4 vvm).

Fermentor was inoculated with 170 ml of "pellets" previously obtained in *M1* Erlenmeyer flasks. Fermentations assays were done with 1.7l working volume at temperature

(30 ºC), pH (3.4 at inoculation time and then was maintained at 2.4 with 4N KOH and 1N H_2SO_4) and each sample was obtained after 12 h during 192h. Biomass (g dry weight/l), residual glucose and IA concentrations (g/l) were determined.

Glucose (*M1* medium) was replaced by glycerol (Cicarelli Lab) (*M2* medium) in the same concentration (100 g/l). Different carbon, nitrogen and phosphorous sources concentrations in order to obtain variable C:N, C:P y N:P ratios were established. KH_2PO_4 (0.2, 0.90 and 1.25 g/l); NH_4NO_3 (1.0, 3.0, 5.0 and 6.5 g/l) and glycerol (90, 100, 105 and 120 g/l) concentrations were varied to obtain *M2a, M2b, M2c, M2d, M2e* media formulations (table 1 a,b). The selected nutrient concentrations were chosen because of the experimental data reported by several authors for IA production with *A. terreus*. Glycerol, ammonium nitrate (1 g/l) and potassium phosphate (0.05 g/l) were used by Jarry and Seraudie (1995, 1997). Kautola *et al.* (1991) and Reddy and Singh (2002) used higher ammonium nitrate concentrations (4 g/l) and Casas López *et al.* (2003) used higher potassium phosphate concentrations (1.51 g/l).

Biomass (g dry weight/l), residual glucose (g/l), residual glycerol (g/l) and IA (g/l) concentrations were determined. The resulting C:N, C:P and N:P ratios of the media culture prepared can be observed in Table 2. Yx:s (g d.w. biomass/g substrate); Yp:x (g IA/g d.w. biomass), Yp:s (g IA/g substrate) , volumetric productivity Pd_v (g IA/ l.h) and specific productivity Pd_s (g IA/g biomass.h) of the bioprocess were determined.

Analytical methods

IA quantification (g/l) was based in bromide method (1mol bromide equal to 1mol IA), (Tsai *et al.*, 2001). Biomass concentration (g. dry weight/l) was determined periodically filtering aliquots of the culture broth on no. 2 filter paper (Whatman); the wet mycelium was washed twice with saline water and with distilled water and dried at 105 ºC. Glucose (g/l) was determined as reducing sugars by

Table 1a. Media formulations with different carbon, nitrogen and phosphorous sources concentrations

(g/l)	M1	M2	M2a	M2b	M2c	M2d	M2e
NH_4NO_3	5.0	5.0	1.0	3.0	3.0	5.0	6.5
KH_2PO_4	0.20	0.20	1.25	1.25	0.90	0.90	0.90
Glucose	100	-	-	-	-	-	-
Glycerol		100	90	90	90	120	105

Table 1b. Carbon, phosphorous and nitrogen concentrations of different production media cultures

(g/l)	M1	M2	M2a	M2b	M2c	M2d	M2e
Nitrogen	1.75	1.75	0.35	1.05	1.05	1.75	2.27
Phosphorous	0.045	0.045	0.28	0.28	0.21	0.21	0.21
Carbon	40	39	35.2	35.2	35.2	47	41

DNS method, (Miller, 1959). Glycerol (g/l) was quantified by enzymatic method for triglycerides TG Color GPO: PAP AA (Wiener lab.) at 505 nm. The results are the average of three independent fermentations in each group of experiments and the standard deviation of the obtained results was always less than 10%.

Results and discussion

When glucose (100 g/l) was used as the sole carbon source, glucose was not exhausted in the culture medium and only 30 g/l were consumed. The yield of IA referred to consumed glucose (Yp:s) was 0.433 g IA/g, final IA concentration was relatively low (13 g/l) and biomass concentration 3.3 g /l (figure 1). The resulting Pd_s (0.027 g IA/g biomass.h) showed an efficient IA synthesis by the fungus but Pd_v was only 0.090 g IA/l.h. Phosphorous was limiting the culture as can be seen in the resulting C:P (888) and N:P (38.8) ratios.

As it can be seen in Table 2, C:N, C:P and N:P affected the final product concentration achieved which was varied between 11.0 to 27.6 g IA/l. Consumed carbon source was also affected (from 30 to 70%). Whenever glycerol was used as substrate, the increase in N:P ratio resulted in higher IA concentration because of the influence of phosphate limitation as it was also reported by several authors (Riscaldati *et al.*, 2000, Willke and Volop, 2001). This observation is not valid for the *M2* medium with glycerol with the highest N:P (33.8) used because the culture was extremely limited by phosphorous and IA yield was affected.

IA concentration was significantly increased with *M2e* medium respect to the observed one corresponding to *M1* and *M2* media. The maximal IA yield as a function of the amount of the carbon source consumed was 0.43 g IA/g with glucose (*M1*) and 0.48 g IA/g with glycerol (*M2c*). When phosphate limitation was accompanied by a N:P ratio between 5-10, there was a better carbon assimilation and a higher IA concentration was obtained. Respect of the IA concentration achieved when glucose (*M1*) and glycerol (*M2*) were fermented by maintaining the same C:N, C:P and N:P concentration ratios in both media, 13.00 and 17.83 g IA/l were obtained respectively. Glycerol seemed to be a better carbon source for this strain for IA production. The other media formulations with glycerol and variable nitrogen and phosphorus concentrations showed that the following ratios, C:N 18.0, N:P 10.8 and C:P 195 (*M2e*) allowed to improve the efficiency of the fermentation. Another technological as-

Table 2. Influence of C:N, C:P y N:P ratios on the IA production

Culture media	C:N	C:P	N:P	% consumed carbon source	IA (g/l)	Biomass (g/l)
M1	23	888	38.8	30	13.00	3.47
M2	22	866	38.8	41	17.83	8.50
M2a	101	126	1.25	45	11.00	6.29
M2b	33	126	3.8	50	19.14	15.19
M2c	33	168	5	51	26.11	8.63
M2d	27	223	8.3	70	25.50	15.53
M2e	18	195	10.8	61	27.60	16.76

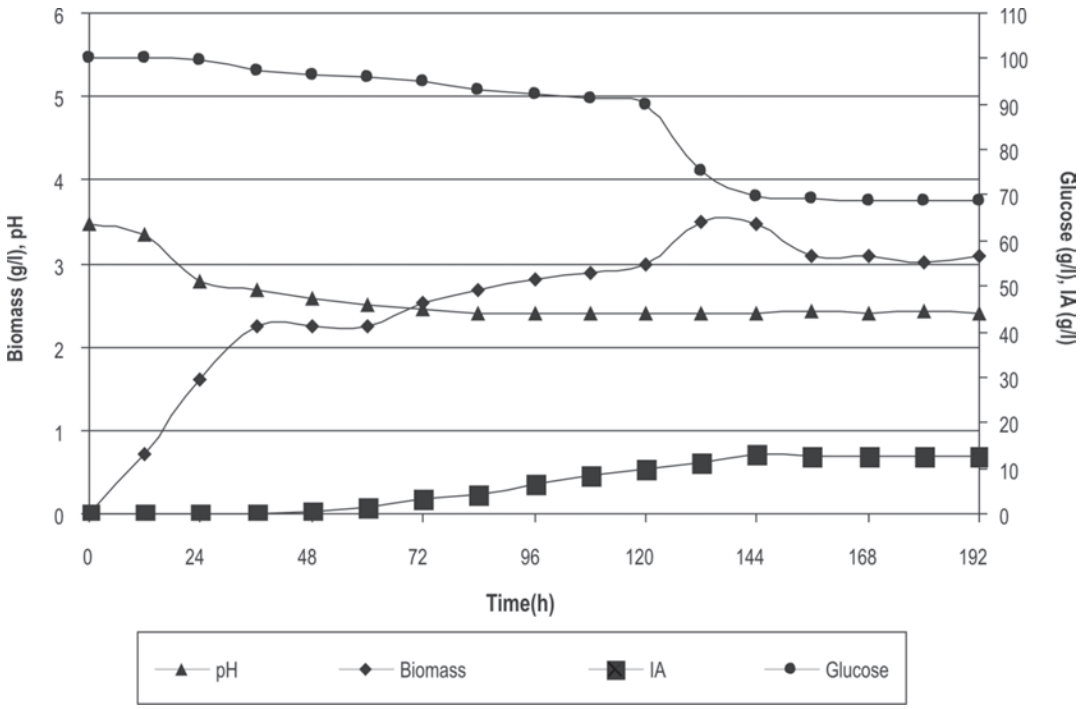

Figure 1. Biomass, glucose and IA concentrations (g/l) and pH in *As. terreus* MJL05 batch fermentation with *M1* medium.

pect to be considered is the resulting yields and productivities of the bioprocess (table 3). The highest values obtained (*M2e*) were Yx:s 0.27 (g d.w. biomass/g glycerol), Yp:x 1.63 (g AI/g d.w. biomass) and Yp:s 0.44 (g AI/g glycerol). Although maximal IA concentration (27.60 g/l) produced by *A. terreus* MJL05 was rather lower that the reported one by other *Aspergillus* strains like TN-484, 51.5 g/l (Gyamerah, 1995); NRRL 1960, 52 g/l (Yahiro *et al*., 1997) and RC4', 67 g/l (Bonnarme *et al*., 1995). MuralidharaRao *et al*. (2007) reported the IA production with Jatropa seed cake and they obtained 24.46 g/l in 120 h with a Pd_v 0.20 g/l.h, similar to the corresponding one to *M2e* medium (this work). Maximal Pd_v (0.192g AI/ l.h) and Pd_s (0.013g AI/g biomasa.h) were obtained with *M2e* medium.

It must be observed that maximum IA production with *M2* glycerol production medium (figure 2) was obtained at 144 h and this experimental data was also observed with all the glycerol media used (*M2a*, *b, c, d* and *e*). This result allowed to improve the volumetric productivity and to lower the costs of the bioprocess.

Conclusions

IA production with *As. terreus* MJL05 were strictly depend on ambient conditions and media formulation. The itaconic acid fermentation process works best under phosphate-limited growth conditions but it is also necessary to maintain N:P between 5 to 10 to improve the IA production. The use of glycerol instead of glucose for IA production by *A. terreus* MJL05 is technological relevant as the increasing biodiesel industry nowadays. Volumetric productivities achieved in this work can be improved by changing the culture system for fed batch and/ or continuous culture, but these results can be considered relevant respect of production media formulations (C:N, C:P and N:P concentrations ratios) in order to increase not only itaconic acid concentration but the yields and productivities of the process as well.

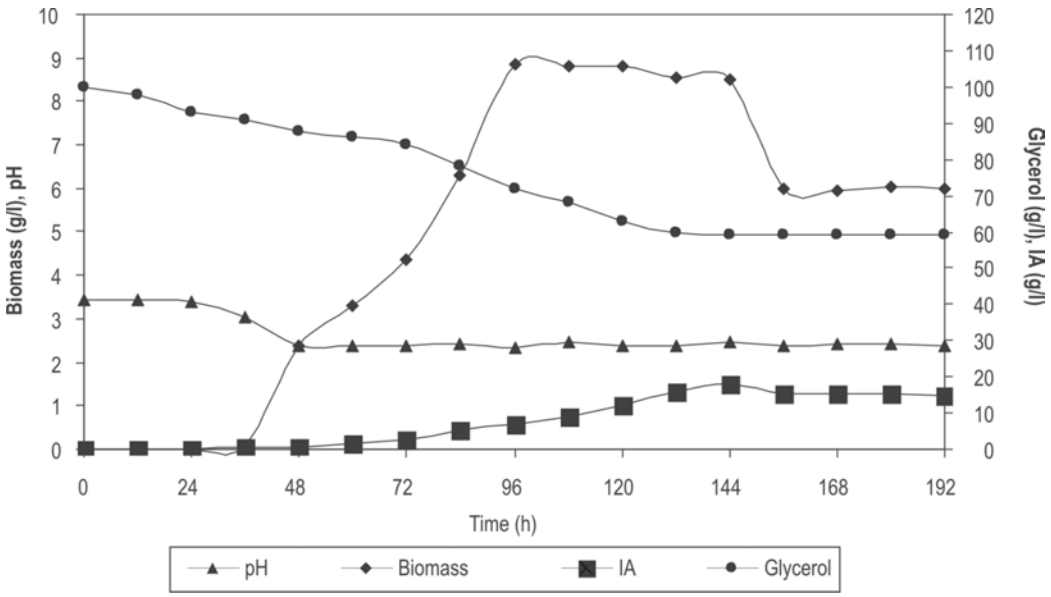

Figure 2. Biomass, glycerol and IA concentrations (g/l) and pH in *As. terreus* MJL05 batch fermentation with *M2* medium.

Table 3. Influence of C:N, C:P and N:P concentration ratios onto bioprocess yields referred to consumed substrates (Yx:s , Yp:s, Yp:x) and volumetric and specific productivities (Pd_v and Pd_s)

Culture media	Yx:s	Yp:s	Yp:x	Pd_v g IA/l.h	Pd_s g IA/l.h
M1	0.11	0.43	3.93	0.090	0.026
M2	0.21	0.44	2.10	0.124	0.010
M2a	0.15	0.23	1.82	0.076	0.013
M2b	0.23	0.43	1.88	0.133	0.013
M2c	0.22	0.48	2.22	0.181	0.015
M2d	0.23	0.37	1.63	0.177	0.013
M2e	0.27	0.44	1.63	0.192	0.013

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