Bioemulsifier production by Aspergillus niger MYA 135: presumptive role of iron and phosphate on emulsifying ability

World Journal of Microbiology and Biotechnology

ISSN 0959-3993
Volume 26
Number 12

World J Microbiol Biotechnol
(2010) 26:2291-2295
DOI 10.1007/
s11274-010-0409-4

World J Microbiol Biotechnol (2010) 26:2291–2295
DOI 10.1007/s11274-010-0409-4

SHORT COMMUNICATION

Bioemulsifier production by *Aspergillus niger* MYA 135: presumptive role of iron and phosphate on emulsifying ability

Verónica Leticia Colin · Mario Domingo Baigorí · Licia María Pera

Received: 18 November 2009 / Accepted: 2 April 2010 / Published online: 17 April 2010

Abstract Microbial emulsifiers are compounds employed in primary mechanisms for bioremediation of petroleum and other hydrocarbon pollutants from the environment. Although emulsifiers of biological origin are produced by microorganisms generally in response to growth on hydrocarbons, *Aspergillus niger* MYA 135 produced a bioemulsifier during fermentation in a sucrose-based culture medium at an initial pH of 5.0 and at 30°C. The production of bioemulsifiers can be strongly influenced by environmental factors. In this connection, a study of the effect of initial pH, the incubation temperature and presence of $CaCl_2$ or $FeCl_3$ in the culture medium was conducted. Emulsification index was increased by 112 and 206% at an initial pH 2.0 or in medium supplemented with $FeCl_3$, respectively. On the other hand, emulsifying ability of *Aspergillus niger* supernatants was detected during the exponential phase, suggesting that bioemulsifiers accumulation and microbial growth would be related. Interestingly, this study suggests that iron and/or phosphate ions would play a key role in maintaining the emulsifying ability. Finally, factorial design was also employed to study the effects of the initial pH, the presence of $FeCl_3$ and the concentration of KH_2PO_4 on the emulsification index.

Keywords *Aspergillus niger* · Bioemulsifier accumulation · Emulsification index · Factorial design

Introduction

Research on bioemulsifiers has expanded considerably in recent years due to their potential use in different biotechnology areas. Emulsifiers derived from natural sources are of particular interest since they exhibit lower levels of toxicity, higher biodegradability and they are under increasing consumers' demand as natural alternatives to their synthetically produced counterparts (Gutierrez et al. 2008).

Microbial emulsifiers are employed in primary mechanisms for bioremediation of petroleum and other hydrocarbon pollutants from the environment (Calvo et al. 2009), since these compounds are able to emulsify hydrocarbons, enhance their water solubility and increase the displacement of oily substances from soil particles. There is a wide diversity of bioemulsifiers due to the large variety of producer microorganisms. However, so far there are only few reports concerning bioemulsifier production by filamentous fungi (Luna-Velasco et al. 2007).

Although emulsifiers of biological origin are produced by microorganisms in response to growth on hydrocarbons (Martínez-Checa et al. 2007), this represents a challenge for subsequent separation of the produced bioemulsifier. Interestingly, there have been a few examples of bioemulsifier production after growth on carbohydrates. This study reports on the production of bioemulsifiers by *Aspergillus niger* MYA 135 in a sucrose-based culture medium. Secondly, it describes the presumptive role of certain medium components in the production and activity of the bioemulsifier.

Materials and methods

Microorganism and culture conditions

Aspergillus niger ATCC MYA 135, from the culture collection at PROIMI (Pilot Plant of Microbiological Industrial Processes, Tucumán, Argentina), was used throughout this study. The basic culture medium (BM) contained (g/l):

V. L. Colin (✉) · M. D. Baigorí · L. M. Pera
PROIMI-CONICET, Av. Belgrano y Pasaje Caseros, T4001 MVB, Tucumán, Argentina
e-mail: veronicacollin@yahoo.com.ar

sucrose, 10.0; KH_2PO_4, 1.0; NH_4NO_3, 2.0; $MgSO_4$, 0.2 and $CuSO_4$, 0.06. Production of the bioemulsifier in BM at initial pH 5.0 and 30°C was used as reference. The effect of modifications in culture conditions on bioemulsifier production was assayed by changing the initial pH of the medium (2.0 to 8.0), the incubation temperature (25 to 37°C) or by the addition of 1.0 g/l of $CaCl_2$ or $FeCl_3$ to the BM. It is important to remark that addition of chlorides did not modify the initial pH of the culture medium.

Culture flasks were inoculated at a final concentration of 10^5 conidia/ml. Fermentations were carried out on an orbital shaker (200 rpm) in 500 ml conical flasks containing 100 ml of culture medium.

Emulsification index and emulsion stability

Bioemulsifiers production was monitored during 7 days. Primarily, the emulsification index (EI-24) of the cultures was determined by mixing equal volumes of a hydrocarbon (kerosene) and culture broth free of cells; the mixture was vortexed for 2 min and left to settle for 24 h. The EI-24 was calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm) (Cooper and Goldenberg 1987). An emulsion was defined as stable when the EI was 50% or higher (Bosch et al. 1988). In addition, the specific EI-24 was also expressed as EI-24%/$g_{biomass}$. Secondly, the EI-24 of the supernatant was also assayed after centrifugation and separation of the precipitate formed in BM suplemented with $FeCl_3$. After centrifugation the supernatant was mixed again with the precipitate or supplemented with each of the BM compounds and emulsification index was measured once more. Culture media without inoculum as well as the BM compounds solutions were used as negative controls.

Emulsifier nature

Culture samples obtaining after 5 days of incubation in BM supplemeted with 1.0 g/l $FeCl_3$ were centrifuged at 2,000×*g* for 15 min at room temperature and then filtered through a 0.45 μm membrane. One volume of acetone was added to 1 volume of cell-free filtrate and kept at 4°C overnight. Dried emulsifier precipitate was dissolved in destilled water and then used as emulsifier source. To investigate the role of peptides in emulsification activity, 990 μl of extract was treated with 10 μl of proteinase K (30 U/mg) at 37°C for 4 h. To investigate the role of lipids, 900 μl of extract was treated with 100 μl of a lipase activity (9.16 U/mg) from *Brevibacillus agri* MIR-E12 at 37°C for 3 h. The partial nucleotide sequence of the 16S rRNA from *B. agri* MIR-E12 was deposited in GeneBank database under accession number EF635412. The presence of reducing sugar was determined after HCl hydrolysis at 100°C by using the dinitrosalicylic acid reagent (Miller 1969). The thermal resistance of the emulsifier was tested by measuring emulsification index after incubation at 100°C for 10 min.

Determination of the biomass

Fungal biomass was determined by taking samples (aliquots) of the culture every 24 h. Mycelium was washed and dried at 105°C until constant weight.

Statistical analysis

Statistical analysis was performed using Infostat (version 2004) software for Windows. Results are presented as the mean ± standard desviation. Statistical significance values of the means were evaluated using one-way analysis of variance. Differences were accepted as significant when $P < 0.05$.

In order to identify main effects and interactions a 2^3 full factorial design was performed. The independent variables and their levels are presented in Table 1. The "+ and −" notation was used to represent the high and low levels of each factor.

Results and discussion

Growth kinetics and bioemulsifier production by *A. niger*

Both EI-24 and specific EI-24 of *A. niger* MYA 135 supernatants as well as biomass production in BM are presented in Fig. 1.

Many researchers have found that the inclusion of hydrocarbons in the growth medium markedly increased bioemulsifiers production. However, this represents a challenge for subsequent separation of the produced bioemulsifier. Interestingly, certain microorganisms are able to produce emulsifying agents when they are grown in water-soluble substrates or carbohydrates (Amaral et al. 2006).

In the present work, production of bioemulsifiers by *A. niger* MYA 135 was observed in the absence of any

Table 1 Independent variables: factors and their levels for a 2^3 full factorial design

Factors	Process parameters	Level (−)	Level (+)
A	pH	2.0	5.0
B	$FeCl_3$ (g/l)	0.0	1.0
C	KH_2PO_4 (g/l)	1.0	1.5

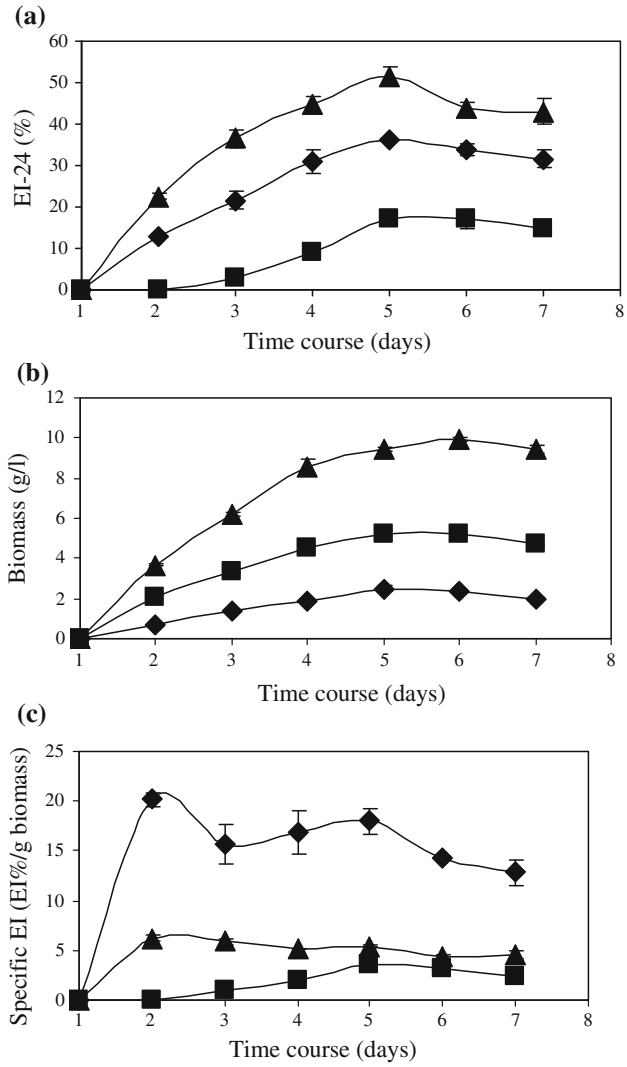

Fig. 1 Time course of bioemulsifier production and growth kinetics of *Aspergillus niger* MYA 135 in BM at 30°C; (*filled square*) Under reference culture conditions, (*filled diamond*) BM at initial pH 2.0, (*filled triangle*) BM supplemented with $FeCl_3$. **a** EI-24 (%), **b** Biomass (g/l) and **c** Specific EI-24 (EI-24%/$g_{biomass}$). *Error bars* represent the standard deviation calculated from at least three independent experiments

water-immiscible substrate. Under reference culture conditions bioemulsifier ability of supernatant was low and unstable (Fig. 1a). However, since the production, chemical compositions and functional properties of the bioemulsifiers can be strongly influenced by environmental factors, in this study the effect of culture conditions on the emulsifier production by *Aspergillus niger* MYA 135 was tested. In previous studies performed on this strain, it was found that the fungal growth and metabolites production such as citric acid (Pera and Callieri 1997) and extracellular lipases (Colin et al. 2010) were strongly affected by the initial pH of the culture medium, the incubation temperature and by the presence of $CaCl_2$ or $FeCl_3$ in the medium. In this study, only at an initial pH 2.0 or in the BM supplemented with $FeCl_3$ the production of bioemulsifiers was significantly increased compared to the reference conditions ($P < 0.05$) (Fig. 1a). Thus, the EI-24 at 5th day of cultivation increased by 112 and 206%, respectively. Under the other culture conditions assayed, no significant bioemulsifier production could be detected.

Kinetics are important in order to determine the relationship between growth and bioemulsifiers production. Although excretion of emulsifiers by most microorganisms only occurs during the stationary growth phase (Zinjarde and Pant 2002), *A. niger* produced bioemulsifiers during the exponential phase, suggesting that their accumulation and microbial growth would be related (Fig. 1a, b). In this connection, the highest EI-24 was detected at 5th day of cultivation, after which it showed a decline. On the other hand, the highest specific EI-24 was observed after 2 days of incubation in BM at initial pH 2.0 (Fig. 1c). However, it is important to mention that the biomass concentration under this environmental condition was lower than 1.0 g/l (Fig. 1b).

Presumptive role of iron and phosphate in bioemulsifier ability

Figure 2a shows the characteristics of an emulsion of kerosene and supernatant of *A. niger* in BM supplemented

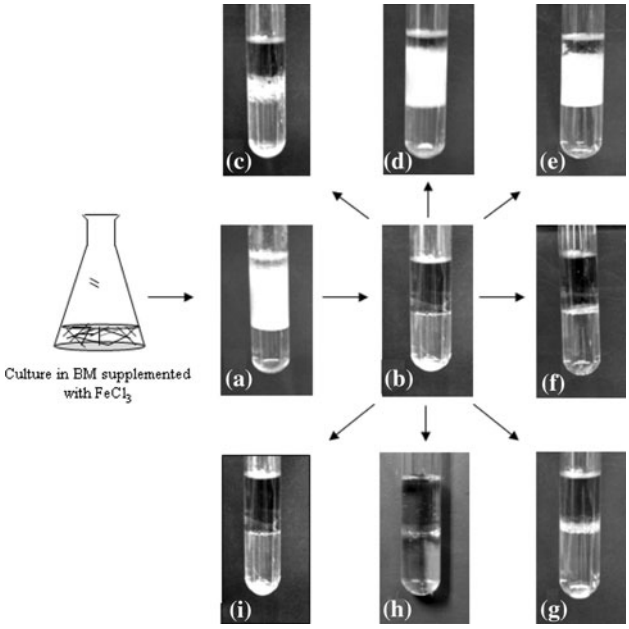

Fig. 2 Characteristics of emulsions of kerosene and supernatants of *A. niger* MYA 135 at 5th day of cultivation: supernatant after filtration of fungal growth in BM supplemented with $FeCl_3$ (**a**); supernatant after centrifugation and separation of the precipitate formed in medium supplemented with $FeCl_3$ (**b**); supernatant mixed again with the precipitate (**c**); supernatant after centrifugation and subsequent addition of KH_2PO_4 (**d**), $FeCl_3$ (**e**), KCl (**f**), NH_4NO_3 (**g**), $MgSO_4$ (**h**) or $CuSO_4$ (**i**)

with $FeCl_3$. The EI-24 at 5th day of cultivation was 52%; the emulsion formed was stable and compact retaining practically 100% of the initial height.

Because the supernatant of the culture supplemented with $FeCl_3$ showed turbidity, probably due to precipitation of ferric phosphate, emulsification index was checked again after centrifugation and separation of the precipitate formed. Interestingly, a complete loss of emulsifying ability of the supernatant was observed (Fig. 2b). Resuspension of the precipitate in the supernatant only recovered 22% of the initial emulsifying ability (Fig. 2c). However, after addition of each of the BM compounds to the precipitate-free supernatant, bioemulsification was only restored in the case of KH_2PO_4 (Fig. 2d) and $FeCl_3$ (Fig. 2e) with 91 and 89%, respectively. It is important to remark that addition of KCl did not restore the emulsion (Fig. 2f), suggesting that only iron and/or phosphate ions could have a key role in maintaining the emulsifying ability. Note the absence of bioemulsification after supplementation of NH_4NO_3 (Fig. 2g), $MgSO_4$ (Fig. 2h) or $CuSO_4$ (Fig. 2i) to precipitate-free supernatant. Finally, addition of iron and/or phosphate to the supernatants of cultures grown under the other culture conditions assayed did not cause emulsifying activity.

Factorial design

In order to identify main effects and interactions a 2^3 full factorial design was performed. According to the results previously described, the effects of initial pH, $FeCl_3$ and KH_2PO_4 on emulsification indices were analyzed after 5 days of cultivation. After this time, there were no significant increases in the studied responses (data not shown). The levels of independent variables are presented in Table 1. Maximum EI-24 was obtained in BM supplemented with $FeCl_3$ at initial pH 2.0, while maximum specific EI-24 was detected in BM at initial pH 2.0 (Table 2). Based on the *P*-values shown in Table 3, the factor effect estimates indicate that both EI-24 and specific EI-24 decrease as the initial pH value and the KH_2PO_4 concentration increase. It was also detected a positive and significant interaction between the initial pH and the presence of $FeCl_3$. Concerning the biomass concentration, it can be

Table 2 Matrix for a 2^3 full factorial design and experimental results measured after 5 days of cultivation

Run	Factors			EI-24 (%)	Biomass (g/l)	Specific EI-24 (EI-24%/$g_{biomass}$)
	pH	$FeCl_3$	KH_2PO_4			
1	+	–	–	17.0 ± 0.5	4.70 ± 0.13	3.6 ± 0.2
2	+	–	+	3.8 ± 0	4.82 ± 0.03	0.8 ± 0
3	–	–	–	32.3 ± 1.5	2.01 ± 0.15	16.1 ± 0.5
4	–	–	+	17.6 ± 1.3	1.76 ± 0.08	10.0 ± 0.3
5	+	+	–	52.1 ± 2.9	9.45 ± 0.11	5.5 ± 0.4
6	+	+	+	19.0 ± 1.4	10.23 ± 0.38	1.9 ± 0.1
7	–	+	–	55.2 ± 4.4	7.57 ± 0.08	7.3 ± 0.5
8	–	+	+	19.5 ± 1.9	7.89 ± 0.06	2.5 ± 0.2

Table 3 Estimated effects analysis for EI-24, biomass and specific EI-24 determined after 5 day of cultivation

Term	EI-24		Biomass		Specific EI-24	
	Effects	*T*-values (*P*-values)	Effects	*T*-values (*P*-values)	Effects	*T*-values (*P*-values)
pH (A)	−8.16	−7.56 ($P < 0.001$)	2.4963	30.46 ($P < 0.001$)	−6.012	−36.78 ($P < 0.001$)
$FeCl_3$ (B)	18.76	17.38 ($P < 0.001$)	5.4613	66.63 ($P < 0.001$)	−3.363	−20.57 ($P < 0.001$)
KH_2PO_4 (C)	−24.19	−22.41 ($P < 0.001$)	0.2438	2.97 ($P = 0.018$)	−4.363	−26.69 ($P < 0.001$)
A B	6.36	5.89 ($P < 0.001$)	–0.3813	−4.65 ($P = 0.002$)	4.813	29.44 ($P < 0.001$)
A C	1.01	0.94 ($P = 0.376$)	0.2062	2.52 ($P = 0.036$)	1.113	6.81 ($P < 0.001$)
B C	−10.21	−9.46 ($P < 0.001$)	0.3063	3.74 ($P = 0.006$)	0.112	0.69 ($P = 0.511$)
A B C	0.29	0.27 ($P = 0.797$)	0.0238	0.29 ($P = 0.779$)	−0.512	−3.14 ($P = 0.014$)

seen that the most relevant variables are by far the initial pH and the $FeCl_3$. Both factors had positive effects. In summary, all factors studied seem to have played a critical role in the bioemulsifier production.

Emulsifier nature

In order to investigate the emulsifier nature, acetone-treated extracts was exposed to different treatments. After proteinase K digestion the extract retain 100% of its emulsification ability. On the contrary, the acid hydrolysis at 100°C completely destroyed this ability. However, the emulsification ability remain constant after heating at 100°C for 10 min. Concerning the presence of reducing sugar, a concentration of 0.067 g/l was detected. Finally, the lipase treatment decreases the EI-24 by 61%. These results suggest us that the emulsifier compound could be a glycolipid.

Concluding remarks

Under the current assay conditions emulsifier production by *A. niger* MYA 135 was reported in sucrose-based culture medium, suggesting that synthesis of a bioemulsifier is not simply a response to the presence of an extracellular hydrocarbon. This production was significantly increased at a highly acidic pH or by addition of $FeCl_3$ to the basic medium. On the other hand, a preliminary study suggests that certain medium compounds would not only play a key role in the production but also in continuation of the ability of the bioemulsifier. Finally, to continue the structural characterization of the bioemulsifiers produced is our next objective, since the potential applications of nonionic emulsifiers in bioremediation could be highly promising.

Acknowledgments This work was partially supported by grants Resolution N° 693/04 from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and CIUNT 26/D 409.

References

Amaral PFF, da Silva JM, Lehocky M, Barros-Timmons AMV, Coelho MAZ, Marrucho LM, Coutinho JAO (2006) Production and characterization of a bioemulsifier from *Yarrowia lipolytica*. Process Biochem 41:1894–1898

Bosch MP, Robert M, Mercade ME, Espuny MJ, Parra JL, Guinea J (1988) Surface-active compounds on microbial cultures. Tenside Surfactants Deterg 25:208–211

Calvo C, Manzanera M, Silva-Castro GA, Uad I, González-López J (2009) Application of bioemulsifiers in soil oil bioremediation processes. Future prospects. Sci Total Environ 407:3634–3640

Colin VL, Baigori MD, Pera LM (2010) Effect of environmental conditions on extracellular lipases production and fungal morphology from *Aspergillus niger* MYA 135. J Basic Microbiol 50:52–58

Cooper DG, Goldenberg BG (1987) Surface-active agents from two *Bacillus* species. Appl Environ Microbiol 53:224–229

Gutierrez T, Shimmield T, Haidon C, Black K, Green DH (2008) Emulsifying and metal ion binding activity of a glycoprotein exopolimer produced by Pseudoalteromonas sp. strain TG12. Appl Environ Microbiol 15:4867–4876

Luna-Velasco MA, Esparza-García F, Cañizares-Villanueva RO, Rodríguez-Vázquez R (2007) Production and properties of a bioemulsifier synthesized by phenanthrene-degrading *Penicillium* sp. Process Biochem 42:310–314

Martínez-Checa F, Toledo FL, El Mabrouki K, Quesada E, Calvo C (2007) Characteristics of Bioemulsifier V2–7 synthesized in culture media added of hydrocarbons: chemical composition, emulsifying activity and rheological properties. Biotechnol Resour 98:3130–3135

Miller GL (1969) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Pera LM, Callieri DA (1997) Influence of calcium on fungal growth, hyphal morphology and citric acid production in *Aspergillus niger*. Folia Microbiol 42:551–556

Zinjarde SS, Pant A (2002) Emulsifier from a tropical marine yeast, *Yarrowia lipolytica* NCIM 3589. J Basic Microbiol 42:67–73