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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Microalgae cultivation for biosurfactant production

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Microalgae can be used as both food and a source of bioactive compounds, such as oils, vitamins and biosurfactants. An important factor contributing to the production costs of bioactive compounds, such as biosurfactants, is the carbon source. One way to decrease production costs is by reducing carbon without a concomitant reduction in productivity. Biosurfactants have a wide range of industrial applications, particularly in the food industry, where they are used as emulsifiers and thickeners. We have investigated the use of cyanobacteria *Arthrospira* sp. LEB 18 and *Synechococcus nidulans* LEB 25 and of chlorophytes *Chlorella minutissima* LEB 108, *Chlorella vulgaris* LEB 106 and *Chlorella homosphaera* for the production of biosurfactants using autotrophic and mixotrophic cultivation. The strains were grown in Erlenmeyer photobioreactors containing appropriate media with $NaHCO_3$ as the autotrophic carbon source and glucose or molasses for mixotrophic growth. The results obtained demonstrate the potential of organic carbon sources to stimulate both the growth of microorganisms and biosurfactant production. Furthermore, the data highlight the potential of using molasse, a low-cost byproduct, as an organic substrate for microalgae cultivation.

Key words: Bicarbonate, biosurfactants, glucose, molasse, superficial tension.

INTRODUCTION

The world production of surfactants exceeds 3 million tons per year, nearly all of which are petroleum derivatives, with 70 to 75% of this production used by industrialized countries (Banat et al., 2000). Biosurfactants are biologically produced and composed by complex molecules and encompass a wide variety of chemical structures, such as glycolipids, lipopeptides, lipoproteins, neutral lipids, fatty acids and phospholipids (Desai and Banat, 1997).

Interest in biosurfactants has increased due to their diversity and potential application in areas such as food processing, environmental protection, pharmaceuticals and the recovery of oily residues. The surfactants produced by microorganisms have the advantage of being biodegradable and possess a great deal of specificity (Sundaram and Thakur, 2015).

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Biossurfactant production uses microalgae because these organisms are major producers of glycolipids, phospholipids and neutral lipids (Rodolfi et al., 2009). Furthermore, these microorganisms can also be a source of biocompounds that have applications in pharmaceutical and food industries, like biopigments and essential fatty acids, direct application of biomass in animal and human feed, biofuel production (H_2, biodiesel, bioethanol and biogas), and carbon dioxide biofixation (Morais et al., 2015; Pandey et al., 2014; Bellou et al., 2014).

A major advantage of cultivating microalgae for biosurfactant production is that many of these microorganisms fall into the Generally Recognized As Safe (GRAS) category. Such certified organisms have no risk of toxicity or pathogenicity and can be used for applications in the food and pharmaceutical industries (Soccol et al., 2013).

Commercial biosurfactant production is limited due to the high costs involved, particularly with respect to culture media. The use of cheaper substrates, such as molasses and glucose, may reduce the cost factor and make production economically viable. Mixotrophic microalgae culture can significantly enhance the growth of microalgae, resulting in cell densities three to ten times higher than those obtained in autotrophic culture (Bhatnagar et al., 2011). For *Arthrospira* microorganisms, the use of molasse as the carbon source is the most influential factor for maximizing biomass concentration and specific growth rate (Andrade and Costa, 2007). Furthermore, an organic substrate in the culture medium can reduce nocturnal loss of biomass because cellular energy demand can be supplied by respiration (Torzillo et al., 1991).

We investigated the cyanobacteria strains *Arthrospira* sp. LEB 18 and *Synechococcus nidulans* LEB 25 and the chlorophyte strains *Chlorella minutissima* LEB 108, *Chlorella vulgaris* LEB 106 and *Chlorella homosphaera* for the production of biosurfactants in autotrophic and mixotrophic cultivation.

MATERIALS AND METHODS

Mixotrophic cultivation of *Arthrospira* sp LEB 18

Maintenance and growth of *Arthrospira*.sp. strain LEB 18 (Morais et al., 2008) was performed over 10 days in Zarrouk medium (Zarrouk, 1966) and was supplemented during the dark period with a total of 1, 3, 5, 7 or 9 $g.L^{-1}$ of glucose or molasses (Indumel, Brazil) added to the cultures at a rate of 10% per day. The cultures were carried out in 2 L Erlenmeyer photobioreactors maintained at 30°C and under a light intensity of 41.6 µmol m^{-2} s^{-1} generated by 40 W fluorescent lamps in a 12 h photoperiod. The cultures were stirred by injecting sterile air with a specific flow rate with volume of air per volume of medium per minute (vvm) of 0.5 (Costa et al. 2000). The initial LEB 18 concentration was 0.15 g L^{-1} (Radmann et al. 2007). The glucose concentration was analyzed using the glucose-oxidase enzymatic method (Laborlab, Campinas, Brazil). The culture analysis were performed in duplicate.

Selection of microalgae for biosurfactant production

The organisms used in this study were the cyanobacteria *Arthrospira* sp. LEB 18 and *S. nidulans* LEB 25 and the chlorophytes *C. minutissima* LEB 108, *C. vulgaris* LEB 106 and *C. homosphaera*.

The cyanobacteria were maintained and grown in Zarrouk medium (Zarrouk, 1966), and the chlorophytes were maintained and grown in BG-11 medium (Rippka et al., 1979). Autotrophic growth used a total of 16.8 g L^{-1} sodium bicarbonate as the carbon source (Chen et al., 1996). Mixotrophic growth was conducted over 20 days using glucose as total carbon source of 5 $g.L^{-1}$ (selected as described in the previous section), which was added to the cultures during the dark period at a rate of 5% per day. Glucose was added incrementally because adding the total amount at the beginning of the experiment caused precipitation and, thus, reduced nutrient availability.

Before glucose addition the residual glucose concentration (see below) was measured in the culture medium to determine whether the previous aliquot had been utilized during the light phase.

Cultures were carried out in 2 L Erlenmeyer photobioreactors maintained at 30ºC and were exposed to a light intensity of 41.6 µmol m^{-2} s^{-1} generated by 40 W fluorescent lamps with 12 h photoperiod.

The cultures were stirred by injecting sterile air with a specific flow rate of 0.5 vvm (Costa et al., 2000). The initial biomass concentration was 0.15 $g.L^{-1}$ (Radmann et al. 2007). The glucose concentration was analyzed using the glucose-oxidase enzymatic method (Laborlab, Campinas/Brazil). All tests and analysis were performed in duplicate.

Analytical determinations

Microalgal growth

The increase in biomass was monitored daily by measuring the optical density of the cultures at 670 nm using a spectrophotometer (FEMTO 700 Plus) and a previously constructed standard curve relating dry weight and optical density. The following parameters were evaluated: maximum biomass concentration ($X_{max,}$ g L^{-1}); maximum yield, (P_{max}, g L^{-1} d^{-1}), obtained from $P = (X_t - X_0)/(t - t_0)$, where X_t is the biomass concentration (g L^{-1}) at time t (d) and X_0 the biomass concentration (g L^{-1}) at time t_0 (d) (Schmidell et al., 2001); and the maximum specific growth rate ($\mu_{max,}$ d^{-1}) by exponential regression of the logarithmic growth phase (Bailey and Ollis, 1996).

Biosurfactant activity

Culture samples were taken every two days and sonicated for 15 min in an ultrasonic bath to break the cell walls, and the surface tension of the sonicate was immediately evaluated using the ring method using a digital tensiometer (Kruss Processor Tensiometer K-6, Germany) and the sample in contact with air (Rodrigues et al., 2006). The results were expressed as minimum surface tension over time (TS_{min}, mN m^{-1}).

Statistical analysis

All reported values represent the average value of the analysis of three replicates. An ANOVA was performed followed by Tukey's

test ($p<0.05$) for mean comparison using the Statistica 8.0 software for Windows (Statsoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Arthrospira sp LEB 18 mixotrophic cultivation

All cultures showed cell growth over 10 days except for assays with added molasses concentrations of 7 and 9 g L^{-1}, which showed cell death in 3 days. In experiments measuring cell growth curves with glucose, adaptation was not observed, while the cultures with molasses spent approximately three days from adapt to the conditions subjected to the microorganism. In the dark phase of photosynthesis, the microorganisms consume their own energy source, reducing growth rate and, consequently, cell concentration, and recovering again during the light period phase (Nelson and Cox, 2011). However, comparison of the spectrophotometric readings in the dark and light periods revealed that there was no decrease in cell growth in the dark phase because of the addition of organic carbon sources that maintained the growth rate (Figure 1).

Chen and Zhang (1997) have reported that the cell growth of mixotrophic cultures is limited by low or high concentrations of organic carbon. High concentrations of carbon can induce cellular stress as a result of excess nutrients in the culture medium. At low concentrations, there was growth restriction due to the shortage of an organic carbon source. This phenomenon was observed in the tests performed with the addition of glucose and molasse.

There was an increase in biomass that was directly proportional to the concentration of the carbon source to 5 g L^{-1}, thereafter, a decrease in cell specific growth rate and productivity was observed using glucose. In cultures with molasse, the largest cell concentration achieved was 3 g L^{-1} of organic source. In trials where higher cell concentrations were obtained for both molasse and glucose, there was also maximum productivity (0.27 and 0.26 g L^{-1} d^{-1}, respectively). Compared with autotrophic culture (0.10 g $L^{-1}d^{-1}$), it was observed that the addition of organic source to crops increased significantly ($p <0.001$) maximum productivity.

Glucose addition increased the maximum specific growth rate 1.9 times during mixotrophic LEB 18 cultivation compared with the autotrophic. In assays using molasses, the maximum growth rate reached in the mixotrophic assay was lower than autotrophic because, in general, this parameter was attained in the exponential growth phase. The growth curves in Figure 1 show that in cultures with molasses, the exponential cell growth phase was not achieved at the end of the experiment for any of the carbon source concentrations. The pH of crops remained between 9.5 and 10.5 which, according to Pelizer et al. (2003), is the optimal range for *Arthrospira* growth.

Microalgae selection for biosurfactants production

According Torzillo et al. (1991), during the dark phase, biomass is reduced because of cellular energy demand, which is supplied by the endogenous microalgal cell reserves formed during the light phase. In cultures where an organic carbon source was added nightly, biomass loss can be minimized during the dark phase, generating higher cell density compared with the autotrophic culture, as observed in the growth curves in Figure 2.

The increase in cell concentration in the mixotrophic condition was observed in the LEB 106 culture, which showed a stationary phase from 5 days of cultivation with glucose addition. In this growth step, the microalgae reached the maximum cell concentration in the culture with organic substrate (1.02 g L^{-1}) (Table 1). The stationary phase of cell growth, which stabilizes the cell concentration, can occur because of a lack of nutrients in the culture medium or due to consumption by microorganisms during development. At this stage, the production of metabolites occurs, such as carbohydrates and lipids; these metabolites are used by the end of this phase as a form to obtain energy until the beginning of cell decline (Schimidell, 2001). In this experiment, the addition of glucose caused an increase in the maximum specific growth rate (0.13 d^{-1}) compared with the autotrophic culture (0.11 d^{-1}), quickly consuming the carbon provided in the culture medium and affecting the stability of cell concentration more rapidly.

The microalgae studied showed better kinetic parameters of growth in crops that were mixotrophically cultured. Chojnacka and Noworyta (2004) observed a similar pattern with higher specific growth speeds for mixotrophic cultivation compared with autotrophic. Due to the different cellular and genetic characteristics of each of the strains studied, the addition of glucose influenced growth kinetic parameters differently. The largest maximum cell concentration during glucose cultivation was obtained for *C. homosphaera* (3.19 g L^{-1}); this experiment achieved the highest maximum mobile productivity (0.32 g L^{-1} d^{-1}) (Table 2). However, increasing cell concentration did not have a proportional relationship to biosurfactant production. In general, the improvement of a target compound is produced by the addition of substrates that create stress conditions for the cells. This stress results in a microalgal metabolism deviation, leading to the production of specific compounds, usually energetic compounds such as lipids and carbohydrates that compose the biosurfactants.

Compared with experiments performed earlier (Figure 1), the LEB 18 exhibited lower growth with the same concentration of glucose (5.0 g L^{-1}) because of the

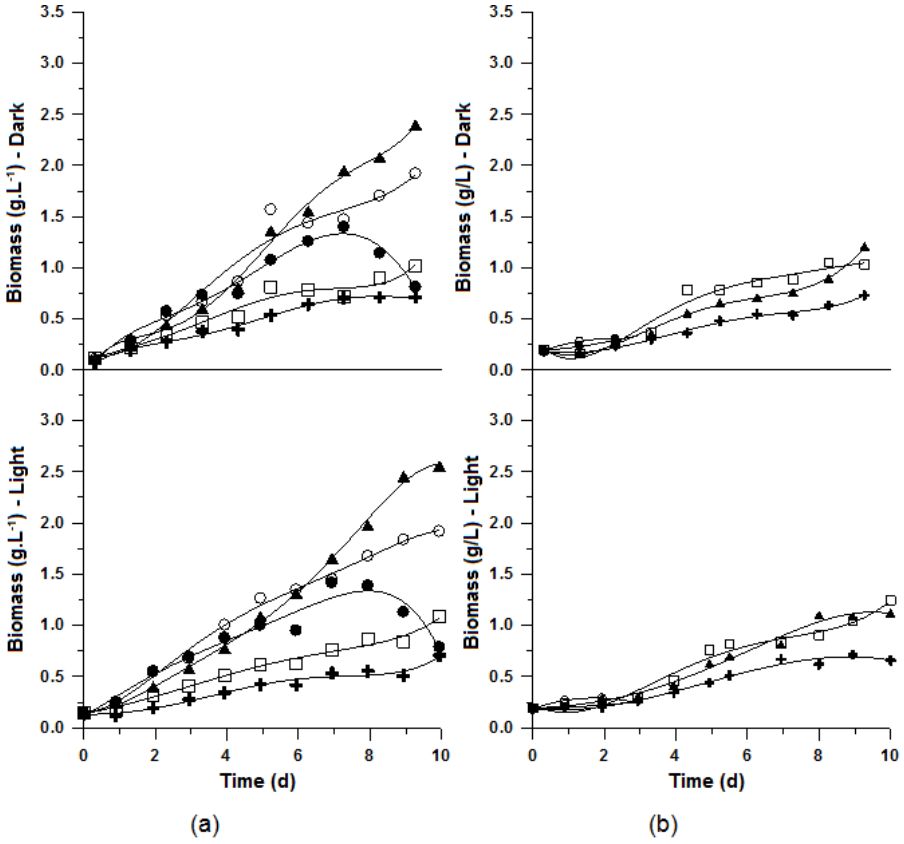

Figure 1. Production of biomass by *Arthrospira* sp. LEB 18 under different concentrations of glucose (a) and molasses (b): (+) E1 (1.0 g.L^{-1}); (≤) E2 (3.0 g.L^{-1}); (▲) E3 (5.0 g.L^{-1}); ○) E4 (7.0 g.L^{-1}); (●) E5 (9.0 g.L^{-1}).

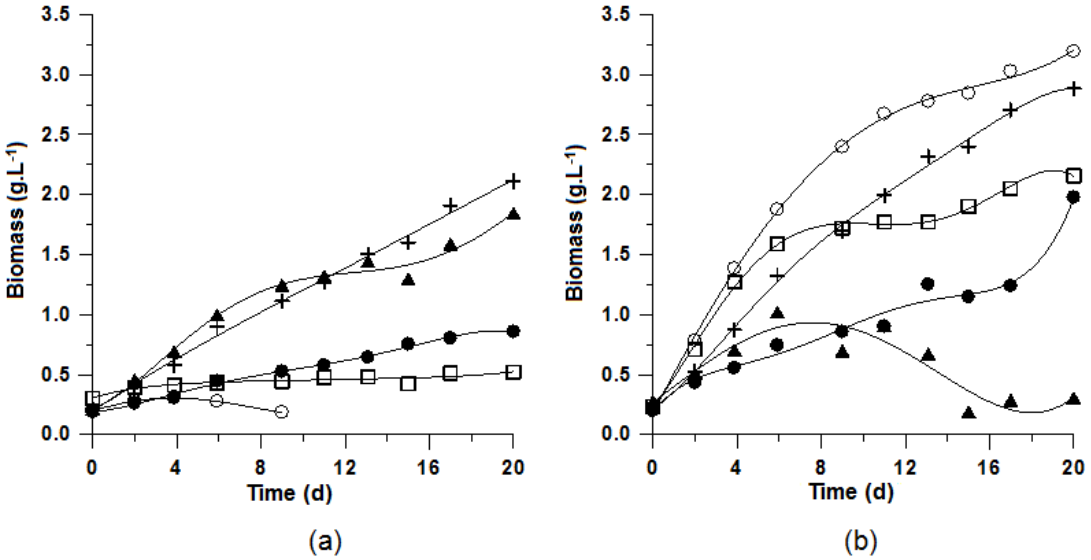

Figure 2. Growth curve for microalgae *Synechococcus nidulans* (+), *Chorella minutissima* LEB 108 (≤), *Chlorella vulgaris* LEB 106 (▲), *Chlorella homosphaera* (○), *Arthrospira* sp. LEB 18 (●). **(a)** Cultures with sodium bicarbonate (16.8 g.L^{-1}). **(b)** Glucose (5.0 g.L^{-1}).

Table 1. Maximum cell concentration (X_{max}, $g.L^{-1}$), productivity (P_{max}, $g.L^{-1}.d^{-1}$) and specific growth rate (μ_{max}, d^{-1}) for the experiments indicated.

Assays	X_{FOC} ($g.L^{-1}$)	X_{max}		P_{max}		μ_{max}	
		Glucose	Molasse	Glucose	Molasse	Glucose	Molasse
1	1.0	0.71±0.10	0.73±0.12	0.08±0.01	0.18±0.01	0.17±0.01	0.08±0.00
2	3.0	1.09±0.09	1.24±0.16	0.13±0.00	0.26±0.01	0.21±0.01	0.11±0.01
3	5.0	2.55±0.21	1.21±0.07	0.27±0.01	0.23±0.00	0.38±0.01	0.12±0.01
4	7.0	1.92±0.15	0.31±0.20	0.27±0.00	0.12±0.01	0.12±0.01	0.03±0.01
5	9.0	1.53±0.05	0.29±0.15	0.25±0.00	0.10±0.01	0.24±0.00	0.02±0.01
Autotrophic	0.0	0.98±0.00		0.10±0.00		0.20±0.00	

X_{FOC}, Concentration of organic carbon source added.

Table 2. Maximum cell concentration (X_{max}, $g.L^{-1}$) productivity (P_{max}, $g.L^{-1}.d^{-1}$), cell growth rate (μ_{max}, d^{-1}) and minimum surface tension (TS_{min}, $mN.m^{-1}$) for *Synechococcus nidulans* (SY), *C. minutissima* (CM), *C. vulgaris* (CV), *C. homosphaera* (CH) e *Arthrospira* sp. LEB 18 (SP) microalgae.

Microalgae	X_{max}		P_{max}		μ_{max}	
	$NaHCO_3$	Glucose	$NaHCO_3$	glucose	$NaHCO_3$	Glucose
SY	2.11±0.52	2.88±0.12	0.16±0.06	0.22±0.01	0.08±0.03	0.14±0.01
CM	0.52±0.00	2.16±0.15	0.04±0.02	0.30±0.05	0.01±0.01	0.22±0.03
CV	1.84±0.29	1.02±0.23	0.15±0.02	0.15±0.08	0.11±0.01	0.13±0.08
CH	0.31±0.00	3.19±0.08	0.06±0.02	0.32±0.00	0.03±0.00	0.03±0.02
SP	1.01±0.07	1.97±0.14	0.06±0.03	0.25±0.18	0.03±0.00	0.16±0.00

different conditions when substrate was added. In the previous experiment lasting 10 days, glucose was added daily at a higher concentration, while in the 20 days experiment, the daily concentration were lower for same added amount of glucose in the same experiments. The addition of substrate occurred daily because adding the total concentration (5.0 g L^{-1}) at the beginning of the cultivation, glucose precipitation occurred, reducing nutrient availability for microalgae. Every day, before the addition of glucose to the cultures, a concentration analysis was performed in the same medium, verifying that it was totally consumed by the microalgae.

Biosurfactant quality is inverse to its surface tension: the smaller the surfactant, the more efficient the surfactant. Carbon source plays an important role in biosurfactant production, influencing the synthesis of induction or repression. According to Cameotra and Makkar (1998), the carbon sources used for the production of biosurfactants can be divided into carbohydrates, hydrocarbons, and vegetable oils and may determine the specificity of the product. According to Cavalero and Cooper (2003) and Hommel et al. (1994), water soluble substrates such as glucose are primarily used in cell metabolism for synthesis of the hydrophilic portion of the biosurfactant molecule, while the lipophilic substrates are used for the production of the lipophilic portion.

Surface tension reduction was found for all microalgae studied, occurring during biosurfactant production in mixotrophic and autotrophic experiment, with an increasing concentration of this product during cultivation time. Among the cultures with added glucose, LEB 18 had the highest reduction in surface tension: 38% from the initial surface tension, reaching 43 $mN.m^{-1}$ (Figure 3). *S. nidulans* presented an end surface tension value that was similar to *Arthrospira* (44.0 $mN.m^{-1}$), with a reduction of 35% compared to baseline.

Conclusion

In the study of organic carbon sources for the mixotrophic cultivation of *Arthrospira* sp. LEB 18, the best growth was observed in culture using glucose as an organic carbon source at a concentration of 5 g L^{-1}. Under these conditions, the maximum cell concentration was 2.6 times higher compared with autotrophic cultivation. When evaluating the culture of different microalgae species for biosurfactant production, a reduction in surface tension was observed in all microalgae studied, with an increased concentration of this product during cultivation time. LEB 18 had the highest reduction in surface tension: 38%

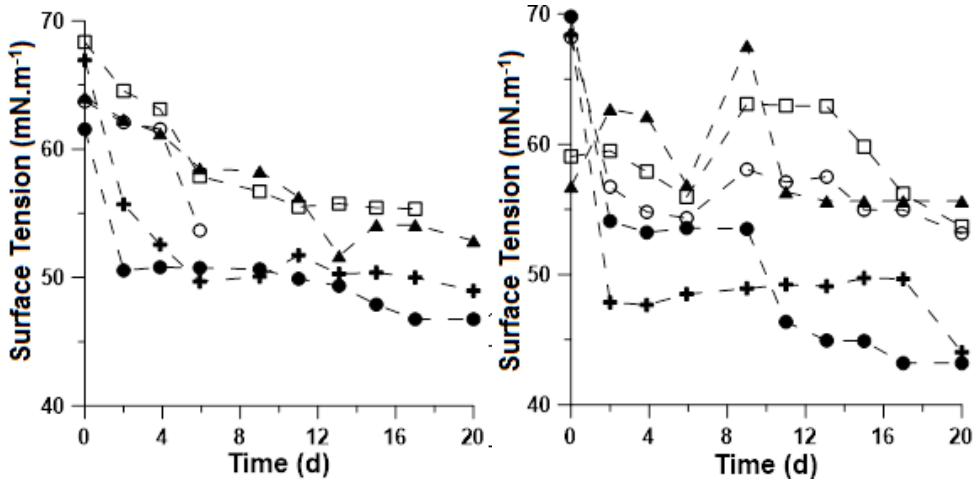

Figure 3. Surface tension over time for microalgae *S. nidulans* (✚), *C. minutissima* (≤), *C. vulgaris* (▲), *C. homosphaera* (○), *Arthrospira* sp. LEB 18 (●). **(a)** Cultures with sodium bicarbonate (16,8 $g.L^{-1}$); **(b)** Glucose (5 $g.L^{-1}$).

from the initial, reaching 43 $mN.m^{-1}$. These results demonstrate the potential of organic carbon sources to stimulate both the growth of microorganisms and biosurfactant production. Furthermore, the data highlight the potential of molasses, a low-cost byproduct, as an organic substrate for microalgae cultivation.

Conflict of Interests

The authors have not declared any conflict of interests.

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Carbon and nitrogen sources differently influence *Penicillium* sp. HC1 conidiation in solid and liquid culture

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This work evaluates the effect of different carbon and nitrogen sources on conidiophore and conidia formation in *Penicillium* sp. HC1, a cellulolytic and xylanolytic fungi arousing industrial interest. A factorial design was used having two variables: A carbon source (glucose, sucrose, cassava starch, wheat bran, and rice flour) and a nitrogen source (tryptose, yeast extract, $(NH_4)_2HPO_4$, and KNO_3). The resulting 20 combinations were evaluated in both solid and liquid medium. Different C:N ratios (5:1, 10:1, 20:1, and 40:1) were also evaluated for one of the combinations. The results revealed the influence of both carbon and nitrogen sources on conidiophore and conidia morphology and the amount of conidia produced; however, this depended on culture condition. A particular culture's condition could also influence conidia tolerance to stressful conditions; conidia having close to 100% tolerance were obtained in liquid media having complex carbon sources and inorganic nitrogen sources. Regarding the C:N ratio, it was found out that nitrogen limitation increased conidia tolerance for both conditions (solid, liquid), the effect being more noticeable in submerged conditions. Understanding the effects of nutrition on conidia production and quality in fungi having industrial interest is a key issue when developing large-scale production.

Key words: Complex carbon source, conidia, conidiophore, inorganic nitrogen source, medium conditions.

INTRODUCTION

Fungi can reproduce themselves sexually or asexually; they thus produce a variety of structures which have evolved and become adapted to their habitat and, in some cases, to their hosts (Steyaert et al., 2010). Conidia

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formation (asexual propagules) is very important for reproduction and rapid dissemination and can lead to producing mycelia rapidly in favourable environmental conditions. Such property, and the fact that they are structures having greater tolerance to different types of stress than vegetative cells, has led to conidia suspensions being widely used in biotechnological-based industry for producing seed cultures or obtaining a formulated final product (Feofilova et al., 2011).

Solid state fermentation (SSF) or submerged fermentation (SmF) is used for large-scale fungi culture. SSF has been used since ancestral times, offering several advantages over SmF, the most important being that it can reproduce the natural process of fungal growth, thereby leading to higher yields of metabolites, growth or asexual propagule formation. Nevertheless, SSF has numerous disadvantages concerning SmF; these would include low mixture efficiency, difficulty in scaling-up, difficulty in controlling different culture parameters, such as pH, temperature, aeration, oxygen transfer, and the great impurity of the products so obtained, thereby increasing recovery costs (Couto and Sanromán, 2006). The foregoing means that SmF continues to be used more in large-scale industrial processes. However, the greatest problem regarding SmF is the culture system *per se* (Grimm et al., 2005), because fungi may have different structural forms throughout their lifecycles influencing the culture's rheological properties and fungal metabolism and thus metabolite production (Grimm et al., 2005; Znidarsic and Pavko, 2001; Papagianni, 2004). In addition, SmF conditions are not ideal for conidia formation (Znidarsic and Pavko, 2001; Hadley and Harrold, 1958; Morton, 1961; Thomas et al., 1987; Boualem et al., 2008) and conidiogenesis is not easily achieved in SmF, due to the relatively good availability of nutrients. Mechanisms controlling asexual propagule formation differ between species (Roncal and Ugalde, 2003; Znidarsic and Pavko, 2001) and most still remain unknown.

Specifically, concerning the genus *Penicillium*, inducing conidiogenesis in SmF has been studied for many years, given the commercial interest shown regarding some species from this genus. Foster et al. (1945) showed that *Penicillium notatum* conidia could be produced in SmF, having morphology and activity similar to that obtained in surface cultures. However, conidia formation only occurred if the culture medium contained a high calcium concentration (0.5 to 5%) (Foster et al., 1945). Such finding has been proven for several *Penicillium* species, such as *Penicillium cyclopium*, *Penicillium griseofulvum*, *Penicillium paxilli*, *Penicillium bilaii* and *Penicillium oxalicum* (Roncal and Ugalde, 2003). Nutrient limitation is another factor determining the induction of conidiogenesis. Contrary to submerged hyphae, aerial hyphae grow outside basal medium separated from the nutrients, leading to aerial hyphal detecting nutrient limitation which could thereby induce the start of conidiogenesis (Roncal and Ugalde, 2003). Hadley and Harrold (1958) found that conidiogenesis in *P. notatum* was connected to nitrogen metabolism, since reduced nitrate levels in the medium increased the ability to produce conidia and reduced calcium requirement (Hadley and Harrold, 1958). Nitrogen limitation provokes conidiogenesis in most *Penicillium* sp. (Roncal and Ugalde, 2003). However, conidiogenesis induction due to carbon limitation has also been reported. For example, low glucose concentration in *P. chrysogenum* restricts vegetative growth, thereby inducing conidia formation (Righelato et al., 1968). Other nutritional conditions could induce conidiogenesis; regarding *P. griseofulvum*, neither conidiophores nor conidia are formed in submerged culture in culture medium containing glucose and nitrate, even with nitrogen limitation, but may be induced in the presence of very high glucose concentrations or by adding defined concentrations of calcium or copper (Morton, 1961).

It has been reported that culture conditions, such as pH, oxygen, and exposure to visible light during mycelial growth affect conidia formation in terms of their amount and their morphological and physiological characteristics, as tolerance to thermal and oxidative stress by ultraviolet (UV) radiation. This pattern has been studied in entomopathogenic fungi, such as *Beauveria bassiana* (Chong-Rodríguez et al., 2011), *Metarhizium anisopliae* (Hallsworth and Magan, 1994), *Metarhizium robertsii* (Rangel et al., 2011), *Paecilomyces farinosus* (Hallsworth and Magan, 1994) and *Paecilomyces fumosoroseus* (De la Torre and Cárdenas-Cota, 1996; Vidal et al., 1998), and phytopathogens, such as *Colletotrichum acutatum* (de Menezes et al., 2015) and *Colletotrichum truncatum* (Jackson and Schisler, 1992). Few reports regarding the genus *Penicillium* have dealt with the relationship between culture conditions and the characteristics of the conidia so obtained. Pascual et al. (2000), found that *P. oxalicum* conidia viability, hydrophobicity, and efficiency (in terms of biocontrol) differed when produced in liquid culture or in solid culture, those produced in solid medium being more efficient (Pascual et al., 2000).

The present work studies how carbon and nitrogen sources and culture condition (solid or liquid) affect conidiophore and conidia formation and also their tolerance to different types of stress in *Penicillium* sp. HC1, a fungus of industrial interest given its ability to degrade lignocellulose residues.

MATERIALS AND METHODS

Organisms and inoculation

Penicillium sp. HC1 was selected from a screening study of cellulolytic microorganisms isolated from rhizosphere soils of rice crops located at Tolima and Meta, Colombia (Gutiérrez-Rojas et al., 2012). This isolate has been deposited in the Centraalbureau voor Schimmelcultures Fungal Biodiversity Center (CBS-KNAW) as CBS 136205. The inoculum for all experiments consisted of a suspension having 10^8 conidia.ml^{-1} which was prepared from a

Table 1. Combinations of carbon and nitrogen sources to evaluate their influence on growth and conidia production in *Penicillium* sp. HC1, on solid and liquid media.

Culture media number	Carbon source ($g.L^{-1}$)	Nitrogen source ($g.L^{-1}$)	Culture media number	Carbon source ($g.L^{-1}$)	Nitrogen source ($g.L^{-1}$)
1	Sucrose (20.00)	Tryptose (7.98)	11	Cassava starch (21.59)	$(NH_4)_2HPO_4$ (4.57)
2	Sucrose (20.00)	Yeast extract (9.18)	12	Cassava starch (21.59)	KNO_3 (7.00)
3	Sucrose (20.00)	$(NH_4)_2HPO_4$ (4.63)	13	Wheat bran (27.00)	Tryptose (7.42)
4	Sucrose (20.00)	KNO_3 (7.08)	14	Wheat bran (27.00)	Yeast extract (9.04)
5	Glucose (20.00)	Tryptose (7.58)	15	Wheat bran (27.00)	$(NH_4)_2HPO_4$ (4.30)
6	Glucose (20.00)	Yeast extract (8.72)	16	Wheat bran (27.00)	KNO_3 (6.58)
7	Glucose (20.00)	$(NH_4)_2HPO_4$ (4.40)	17	Rice flour (21.31)	Tryptose (5.67)
8	Glucose (20.00)	KNO_3 (6.73)	18	Rice flour (21.31)	Yeast extract (6.90)
9	Cassava starch (21.59)	Tryptose (7.88)	19	Rice flour (21.31)	$(NH_4)_2HPO_4$ (3.28)
10	Cassava starch (21.59)	Yeast extract (9.60)	20	Rice flour (21.31)	KNO_3 (5.03)

potato dextrose agar (PDA) culture, incubated at 28°C for seven days.

Effect of different carbon and nitrogen sources on conidia formation

Different carbon sources, simple or chemically defined (sucrose and glucose) and complex (cassava starch, wheat bran, and rice flour) and different nitrogen sources, organic (yeast extract and tryptose) and inorganic ($(NH_4)_2HPO_4$ and KNO_3) (Table 1), were used in solid and liquid media (in L^{-1}: 0.5 g $MgSO_4 \cdot 7H_20$, 0.5 g KCl, 1 g K_2HPO_4, 0.2 mg $FeSO_4$, 0.2 mg $CaCl_2$, 0.02 mg $CoCl_2 \cdot 6H_20$, 0.001 mg $CuCl_2 \cdot 2H_20$, 0.02 mg $NiCl_3 \cdot 6H_20$, 0.003 mg $MnCl_2 \cdot 4H_20$, 0.01 mg $ZnSO_4 \cdot 7H_20$, 0.3 mg H_3BO_3, and 0.003 mg $NaMoO_4 \cdot 2H_20$ at pH 6.0), adding a fixed amount of carbon and nitrogen source to obtain a C:N ratio (10:1). The organic carbon concentration was determined by the Walkley-Black method and total nitrogen concentration by Kjeldahl method. Solid media were prepared with 10 $g.L^{-1}$ agar. Conidia suspension (50 µl) was inoculated in a well at the centre of a Petri dish. Cultures were incubated at 28°C. Liquid medium was prepared in 100 ml Erlenmeyer flasks with 20 ml working volume and inoculated with 2 ml of the conidia suspension, incubated at 28°C and shaken at 100 rpm on an orbital shaker. After 4 days' incubation, a sample was taken from both solid and liquid media for morphological characterisation by image analysis on an optical microscope (Leica DM1000) with a digital camera (Leica, ICC50 HD). Conidia suspensions were obtained after 8 days' incubation in which the amount of conidia was determined by haemocytometer as well as their viability and tolerance to stress. All experiments were performed in triplicate.

Effect of the carbon:nitrogen ratio

Medium 8 (glucose: KNO_3) was selected and the amount of nitrogen source added varied, keeping the amount of carbon (20 $g.L^{-1}$) constant, so that different C:N ratios were obtained (5:1, 10:1, 20:1 and 40:1; 13.48, 6.73, 3.37, and 1.68 gL^{-1} KNO_3, respectively) and then evaluated in solid and liquid medium. All experiments were done in triplicate.

Germination percentage

Germination percentage was evaluated for determining conidia viability. Three 5 µl aliquots of a 10^8 conidia.ml^{-1} suspension were inoculated on Petri dishes containing water agar and incubated at 28°C for 18 to 20 h. Conidia germination percentage was calculated by counting under a microscope, a minimum of 100 conidia (germinated and non-germinated) per each 5 µl aliquot. All evaluations were carried on in triplicate.

Tolerance to thermal stress

Aliquots containing 1 ml of 10^7 conidia.ml^{-1} suspension were heated at 45 and 50°C for 1 h; control was kept at room temperature (RT) (García-Rico et al., 2011). Germination percentage was calculated after treatment time had elapsed. All experiments were done in triplicate.

Tolerance to oxidative stress

A suspension of 10^8 conidia.ml^{-1} was mixed with hydrogen peroxide to reach a final concentration of 0, 100, 110, 120, 130, 140, and 150 mM, and then incubated for 30 min at RT (García-Rico et al., 2011). After this incubation time, germination percentage was calculated. All experiments were done in triplicate.

Tolerance to UV radiation

A 10 ml of 10^8 conidia.ml^{-1} suspension was submitted to UV radiation in a laminar flow chamber (Streamline laboratory products EN 1822.1) at 20 cm constant distance from UV lamp. A 1 ml sample was taken at different intervals of time: 0, 0.5, 1, 2, 3, 5, and 10 min according to Rangel et al. (2011), with some modifications. Germination percentage was calculated. All experiments were done in triplicate.

Statistical analysis

Differences between conidiophore and conidia morphology

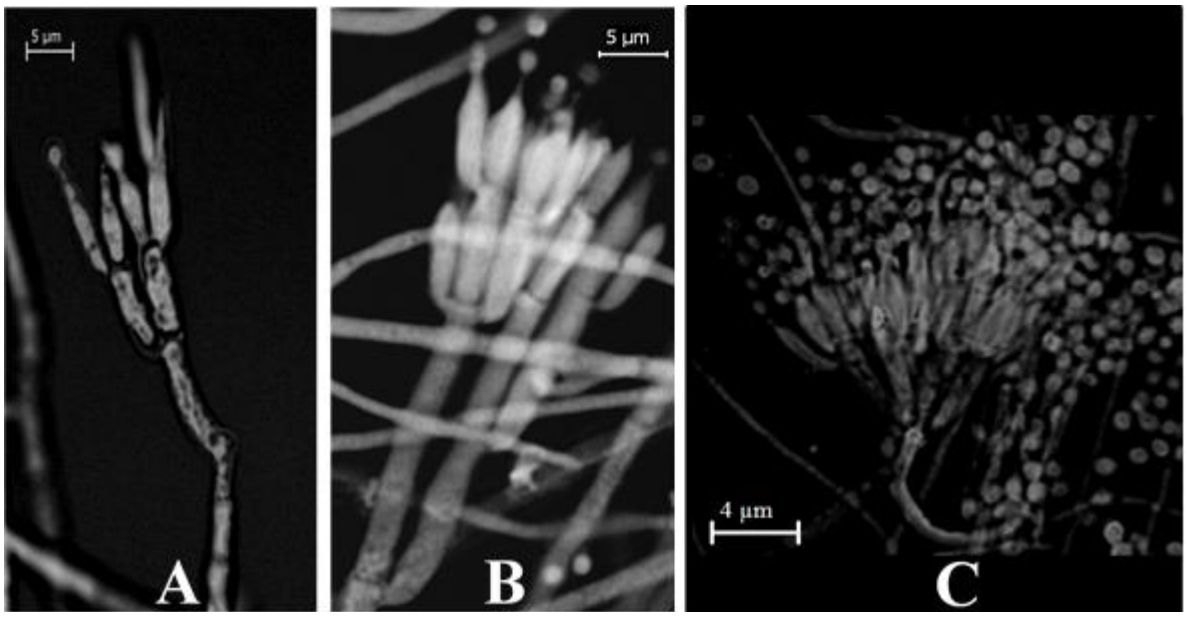

Figure 1. Conidiophores and conidia of *Penicillium* sp. HC1 after 4 days incubation at 28°C on different culture media. A: Liquid medium (ME11), B: Solid medium - simple carbon source (ME5), C: Solid medium- complex carbon source (ME17). 100x.

as well as the amount of conidia produced under all conditions (in solid and liquid) and tolerance to stress were evaluated by analysing one-way variance (ANOVA) and Tukey test having 0.05 significance level. Two-way analysis of variance (ANOVA) was also used for determining the influence of the variables separately and the interaction between them on the amount of conidia produced. Pearson correlation was used for measuring the correlation between conidiophore morphology and the tolerance of the conidia produced. SPSS version 21 and Design Expert version 7 statistical software was used for all the analysis.

RESULTS

Effect of the type of carbon and nitrogen source on conidia formation

Figures 1 and 2 show the results of microscopic characterisation. Differences were observed between the conidiophores obtained using simple carbon sources (SS) and complex sources (CS) in solid medium. The conidiophores were wider in the latter (CS: 14.73 ± 4.48 µm; SS: 9.41 ± 3.58 µm), having more phialides (CS: 9 ± 2; SS: 5 ± 2) and the phialides, in turn, were much bigger (CS: 8.67 ± 1.76 µm; SS: 8.21 ± 1.57 µm) and wider (CS: 2.18 ± 0.4 µm; SS: 1.92 ± 0.33 µm). Reproductive structures were not observed in all liquid media, for that reason it is not possible to stablish differences between types of sources. However, the data did show that conidiophores obtained in submerged condition were shorter (12.98 ± 2.62 µm), narrower (7.37 ± 1.92 µm) and had less phialides (2 ± 1) than those obtained in solid media with any of the sources evaluated here. Differences were only found regarding the conidia size obtained in solid medium with complex carbon sources, being larger (2.50 ± 0.49 µm × 2.16 ± 0.46 µm) than those obtained in solid medium with simple sources (2.04 ± 0.41 µm × 1.77 ± 0.39 µm) or in liquid medium (1.87 ± 0.42 µm × 1.67 ± 0.39 µm).

The highest conidia production in solid medium was obtained in culture medium 9 (5.82 ± 0.032 Log_{10} (conidia).$mm^{2})^{-1}$) (cassava starch + tryptose) and 14 (5.77 ± 0.003 Log_{10} (conidia).$mm^{2})^{-1}$) (wheat bran + yeast extract) and the lowest (4.49 ± 0.041 Log_{10} (conidia).$mm^{2})^{-1}$) in culture medium 7 (glucose + $(NH_4)_2HPO4$) (Figure 3A and B). The two-way analysis of variance (ANOVA) (Table 2) showed that both carbon and nitrogen sources and the interaction between them had a significant effect ($p<0.0001$), being the highest with carbon source ($F=37.80$). Complex carbon sources in liquid medium seemed to favour conidia production (Figure 3D); the highest values were obtained with medium 13 (wheat bran + tryptose) and 19 (rice flour + $(NH_4)_2HPO_4$) (6.84 ± 0.70 and 6.64 ± 0.88 Log_{10} (conidia.ml^{-1}), respectively. The lowest values were obtained with simple carbon sources (Figure 3C), the lowest being 4.95 ± 0.52 Log_{10} (conidia.ml^{-1}), obtained in medium 6 (glucose + yeast extract). Unlike the solid media, nitrogen source in liquid media did not show a significant influence on conidia production ($p=0.9686$), carbon source having greater influence ($p<0.0001$, $F=22.42$).

Effect of the type of carbon and nitrogen source on conidia tolerance

Figure 4 shows the tolerance to thermal stress (50°C) of

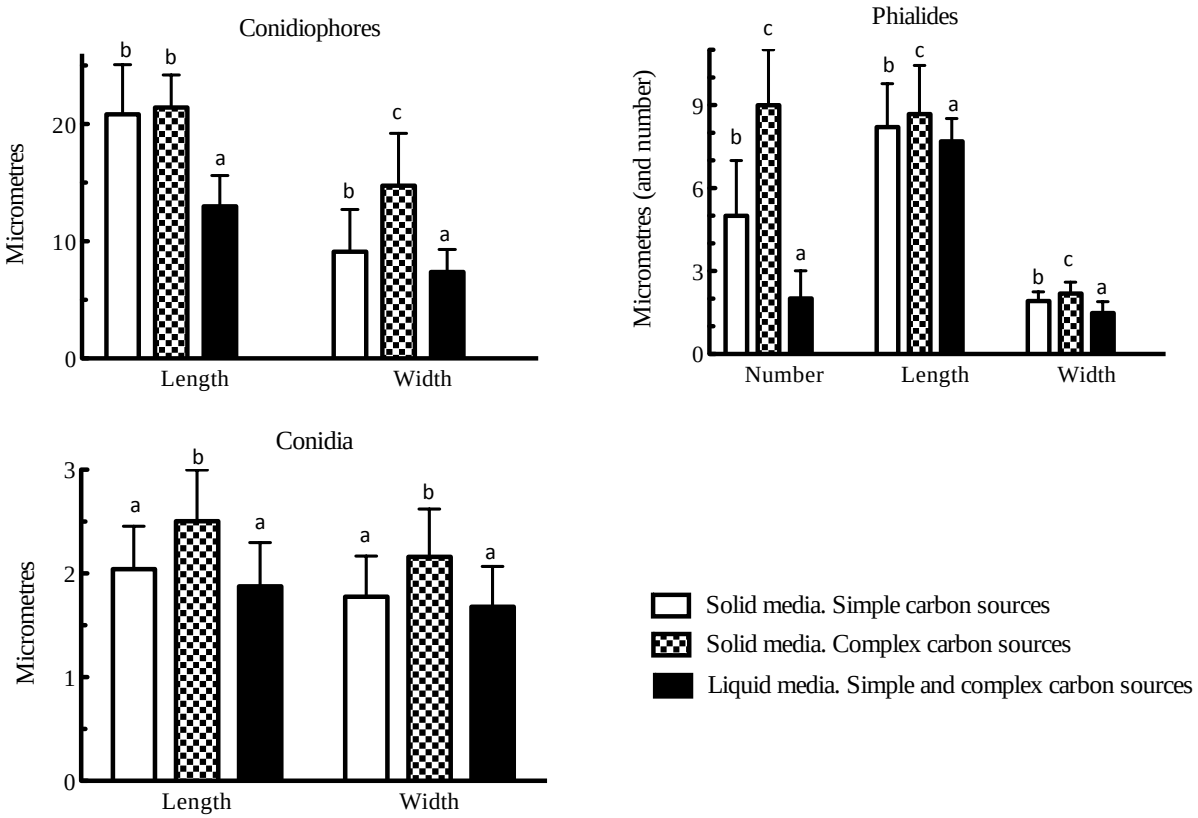

Figure 2. Morphological characteristics of *Penicillium* sp. HC1 conidiophores and conidia obtained from different solid and liquid culture media. The same letters indicate no significant difference according to Tukey test (95% significance).

Table 2. Effect of carbon and nitrogen sources on conidia production, on solid and liquid media.

Factor	Conidia production			
	Solid media conidia. $(mm^2)^{-1}$		Liquid media $(conidia.ml^{-1})$	
	F value	P value	F value	P value
A	37.80	< 0.0001	22.42	< 0.0001
B	16.67	< 0.0001	0.084	0.9686
A × *B*	10.11	< 0.0001	4.47	< 0.0001
Model		< 0.0001	7.55	< 0.0001
R^2	0.8897		0.7052	
Adjusted R^2	0.8372		0.6119	
Adequate precision	16.836		8.814	

A: Carbon source; B: Nitrogen source.

conidia obtained in all solid and liquid media. The relative germination percentage of conidia obtained from solid media was 16% on average, with 56.7% coefficient of variation, whilst this was 37.02%, with 99.1% coefficient of variation, from liquid media. The incidence of complex or simple carbon and organic or inorganic nitrogen sources in solid media was not evident.

Conidia obtained from liquid media had a clear tendency towards high tolerance (close to 100%) in media having complex carbon sources (cassava starch, wheat bran, and rice flour) combined with inorganic nitrogen sources ($(NH_4)_2HPO_4$ and KNO_3). The conidia obtained from liquid media 11 (cassava starch + $(NH_4)_2HPO_4$) and 19 (rice flour + $(NH_4)_2HPO_4$) showed tolerance at 45°C, oxidative stress and UV radiation. Conidia obtained from PDA (8 days culture) were used as the standard for comparison (Figure 5). These results showed that the conidia obtained from in these two media

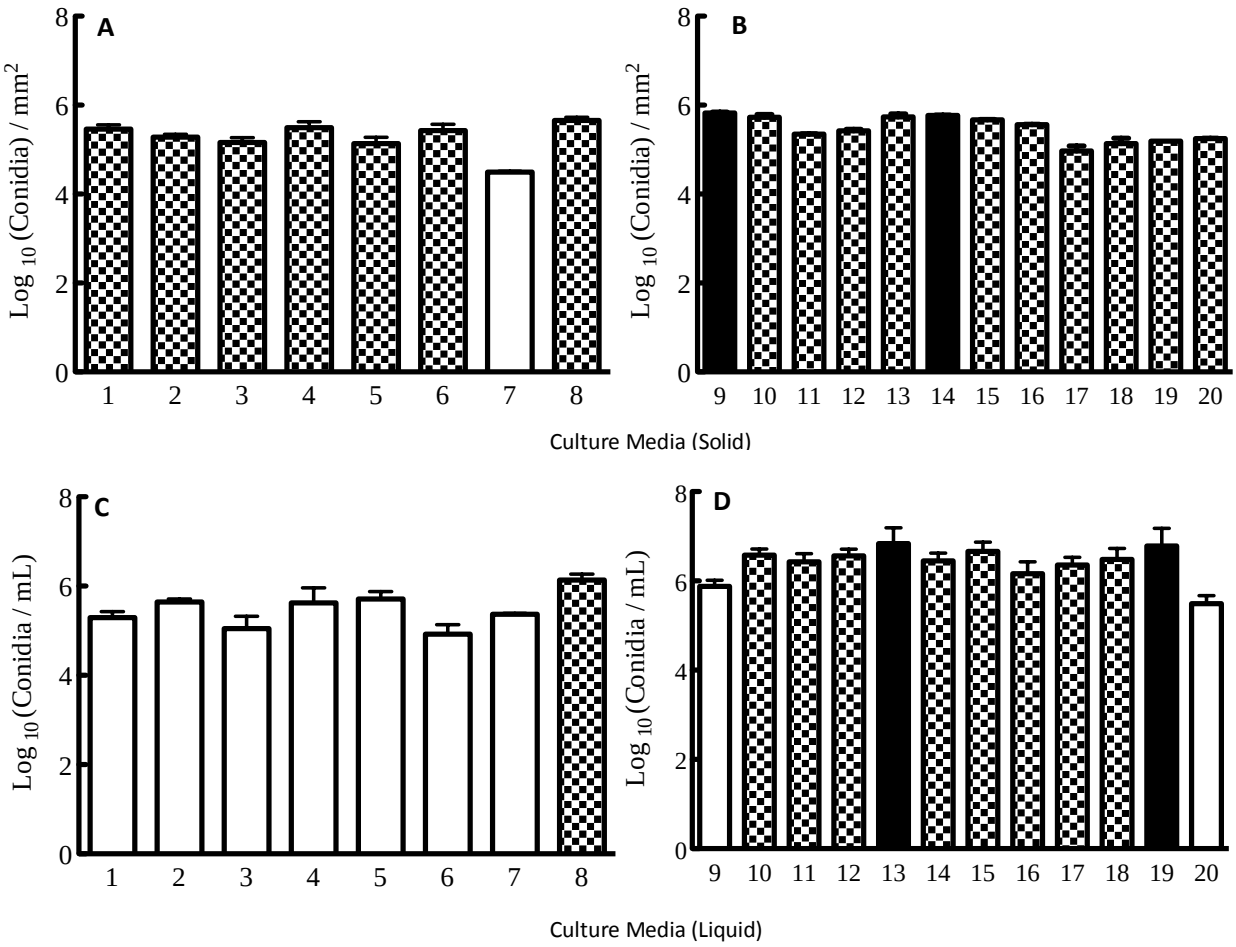

Figure 3. Production of conidia in different culture media. (A) Solid media - simple carbon sources. (B) Solid media - complex carbon sources. (C) Liquid media - simple carbon sources. (D) Liquid media - complex carbon sources. Black bars represent the higher responses, white bars represent the lower responses and squared bars represent the intermediate responses, according to Tukey test ($p<0.05$).

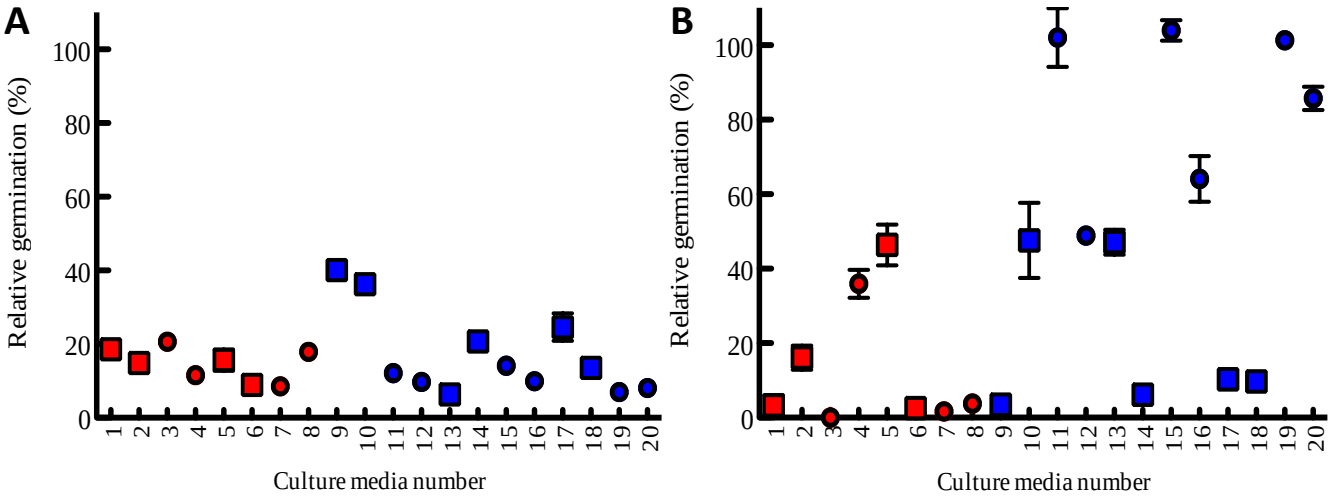

Figure 4. Survival of *Penicillium* sp. HC1 conidia obtained on different culture media exposed to thermal stress (50°C for one hour). Solid media (A), liquid media (B). Red symbols represent simple carbon sources and blue symbols represent complex carbon sources. Square symbols represent organic nitrogen source and round symbols represent inorganic nitrogen source.

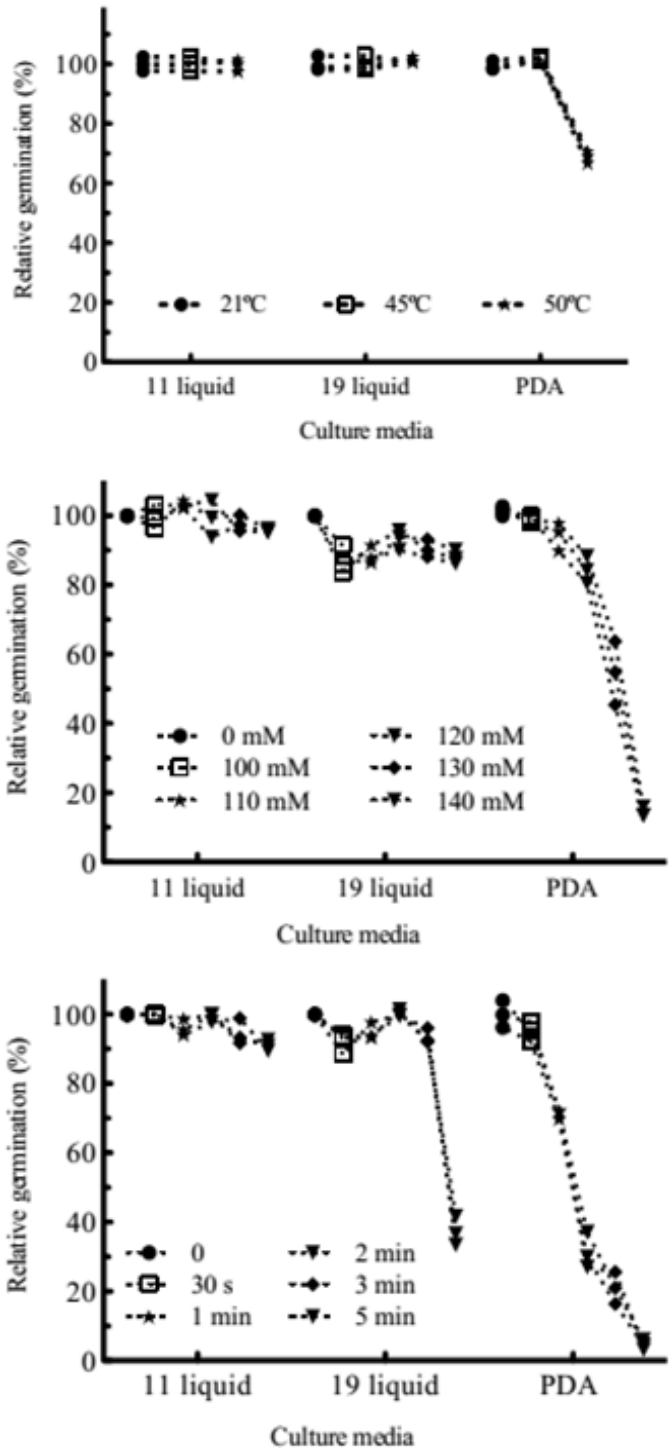

Figure 5. Tolerance to thermal stress (A), oxidative stress (B) and UV radiation (C) of *Penicillium* sp. HC1 conidia in liquid (11 and 19) and PDA media.

were not only more tolerant to temperature (Figure 5A) but also to oxidative and stress caused by UV radiation (Figure 5B and C).

Correlation between conidiophores' morphological characters and conidia's tolerance to thermal stress

Pearson correlation between the conidiophores' morphological characteristics and the results of the 50°C tolerance test, regarding conidia produced in different culture conditions, was analysed. Positive and statistically significant correlations were obtained in all cases (Table 3). The results suggested that the conidia obtained from larger structures tended to be more tolerant; this was evident regarding solid media where the most tolerant conidia were obtained from cassava medium starch as carbon source. The structures having most phialides were also obtained with such media. However, lower tolerance values were not obtained in all liquid media; in fact, the most tolerant conidia were obtained in liquid media with complex carbon sources and inorganic nitrogen sources (media 11, 15, and 19). This result suggests that this ratio is not always direct and does not just depend on one condition, such as solid or liquid medium or simple or complex carbon source, but rather on the interaction of many factors.

Effect of the carbon:nitrogen ratio on conidia tolerance

Conidia were obtained in two conditions (solid and liquid), in culture medium with simple carbon source (glucose) and inorganic nitrogen source (KNO_3), and then submitted to thermal stress (50°C) for establishing the effect of C:N ratio on conidia tolerance. It was seen that nitrogen limitation for both conditions increased conidia tolerance (Figure 6). In a non limiting nitrogen ratio (5:1), conidia obtained in solid medium had 1.41 ± 0.87 germination percentage and those in liquid medium 25.66 ± 0.87; whilst a limiting nitrogen ratio (40:1) increased such percentage to 5.34 ± 0.33 in solid media and to 53.66 ± 2.70 in liquid media. This effect was more noticeable in submerged ($p<0.000$, $F = 55.322$) than in solid condition ($p=0.003$, $F=10.805$). Two-way analysis of variance (ANOVA) showed that this culture condition had greater influence on response ($p<0.0001$, $F=80.13$) than C:N ratio ($p<0.0001$, $F=11.72$).

DISCUSSION

Studying culture conditions' influence on conidia production has been limited to entomopathogenic fungi of industrial interest (Jackson and Schisler, 1992; Hallsworth and Magan, 1994; De la Torre and Cárdenas-Cota, 1996; Vidal et al., 1998; Chong-Rodríguez et al., 2011; Rangel et al., 2011; de Menezes et al., 2015), but has been little studied in the genus *Penicillium*. The present work aimed at evaluating the influence of carbon source, nitrogen source, and their interaction on solid and

Table 3. Pearson correlation coefficients of survival percentage at 50°C of conidia obtained from different culture conditions and morphological characteristics of conidiophores

Morphological parameter	Relative germination percentage after heat treatment at 50°C
Conidiophore length	0.374*
Conidiophore width	0.370*
Number phialides	0.445*
Phialide length	0.264*
Phialide width	0.214*

*Correlation is significant at the 0.01 level (2-tailed)

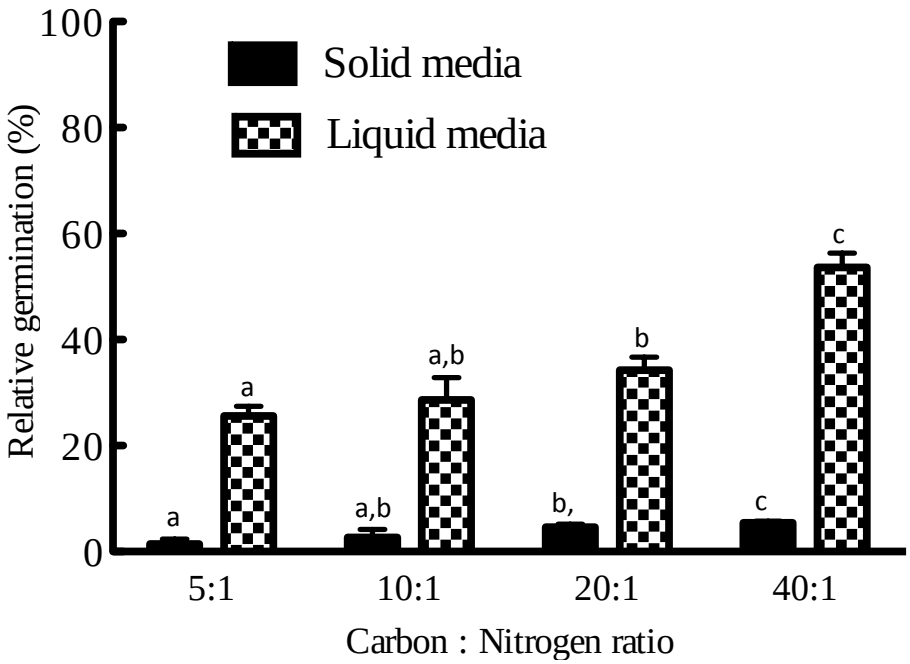

Figure 6. Effect of carbon:nitrogen ratio on *Penicillium* sp. HC1 conidia tolerance to thermal stress (50°C) from liquid (8) and solid (PDA) media.

submerged culture, regarding conidiophore and conidia morphology, the amount of conidia produced and their tolerance to conditions of stress in the cellulolytic fungi *Penicillium* sp. HC1. Krasniewski et al. (2006) studied the effect of culture medium composition in solid culture on conidia production in *Penicillium camemberti* and found that not just concentration, but also the type of nitrogen source influenced conidiogenesis in this fungi; KNO_3 stimulated conidia production whilst $(NH_4)_2SO_4$ was inhibitory, using glucose as carbon source. Whether such clear tendency regarding the type of nitrogen source favouring conidiation (or not) could not be established in our work, probably due to the effect not just being caused by the nitrogen source, but also interaction with the carbon source.

The conidiation pattern was different in liquid media where a clear tendency for obtaining greater conidia production with complex carbon sources emerged. Given the nature of complex sources, some other component could have been exercising an influence on conidiogenesis, which could only be observed in the submerged condition. Mycelium air contact was the dominant stimulus for *Penicillium* sp. conidiophore formation in solid medium (Morton, 1961; Roncal and Ugalde, 2003); such situation did not occur when *Penicillium* sp. HC1 grew submerged, meaning that other inducing factors would have been playing a dominant role. Such factors might have been ions; it has been shown that calcium is fundamental for conidiophore formation in submerged culture in differing *Penicillium* sp. (Roncal and Ugalde, 2003). Roncal et al. (2002) identified a diterpenoide in *P. cyclopium*, they named it conidiogenone, which could act as a hormone at very low concentrations (10^{-7} to 10^{-8} mol.L^{-1}), thereby inducing

conidiogenesis at some calcium concentration. According to their results, conidiogenone and conidiogenol (conidiogenone precursor) were produced from very early growth phases onwards and were continuously released to culture medium, where they became accumulated until reaching a concentration which induced conidiogenesis. It seems that calcium reduces the threshold concentration required in liquid medium for such induction in a yet-to-be-understood way, but which is probably related to this cation's binding to the hyphae external surface (Roncal et al., 2002). The inducing role of other ions, such as Mg, K, Cu, and PO_4 in liquid media has also been reported in *P. griseofulvum*, *P. chrysogenum* (Morton, 1961), and *P. camemberti* (Bockelmann et al., 1999).

Regarding tolerance to temperature (50°C), a clear tendency for obtaining conidia having high tolerance (close to 100%) was obtained in liquid culture with complex carbon sources and inorganic nitrogen sources. Such result was confirmed for conidia obtained in liquid media 11 (cassava starch and $(NH_4)_2HPO_4$) and 19 (rice flour and $(NH_4)_2HPO_4$) where high tolerance to oxidative stress and stress caused by UV radiation was also obtained. The influence of culture medium composition on conidia tolerance has been reported for other genera. Hallsworth and Magan (1994) found differences in polyhydroxy alcohol and trehalose content in conidia from three entomopathogenic fungi (*Beauveria bassiana, Metarhizium anisopliae*, and *Paecylomices farinosus*) when they were cultured in different carbon sources and concentrations (Hallsworth and Magan, 1994). The accumulation of polyols, such as mannitol, and trehalose is a mechanism, which cells use for protecting themselves from stress. Trehalose, for example, can replace water at low water activity and stabilise proteins during desiccation, thereby preserving membrane integrity (Hallsworth and Magan, 1996; Rangel et al., 2008). Cells accumulate these compounds in response to thermal shock, freezing, dehydration, osmotic stress, and carbon limitation and also to stress caused by other agents like UV radiation (Rangel et al., 2008). The interaction of the three factors (submerged culture condition, complex carbon source, and inorganic nitrogen source) may have caused a stressful environment for *Penicillium* sp. HC1, leading it to accumulating compounds, such as those reported in other species, therefore conidia produced in these conditions increased tolerance to the stressing conditions evaluated here.

However, not just the type of carbon and nitrogen source affect conidia tolerance to stress; the C:N ratio also influences such characteristic. Different C:N ratios were evaluated using a simple carbon source; this led to low tolerance (compared to that obtained with complex sources) and these conidia's tolerance we observed to be increased by increasing nitrogen restriction, even though to the detriment of the amount of conidia produced in very limiting ratios (data not shown). The influence of C:N ratio on fungal conidia activity and characteristics has been studied in fungi, such as *Talaromyces flavus* (Engelkes et al., 1997), *B. bassiana*, and *Pochonia chlamydosporia* (Gao and Liu, 2010a), *Paecilomyces lilacinus*, and *M. anisopliae* (Gao and Liu, 2010b), *Lecanicillium lecanii* and *Trichoderma viride* (Gao and Liu, 2009). The relationship between carbon concentration and C:N ratio with conidia production and quality has been clear in all cases; however, this relationship was different for each species studied and depended on factors such as type of carbon source, type of nitrogen source and culture system. The latter was evident in our results as fungal response to nitrogen limitation in solid medium was very different to that obtained in the liquid media where the effect was much clearer.

It was clear that culture medium composition and culture system (solid or liquid) were the critical factors determining the amount and tolerance of conidia, therefore, these factors must be carefully defined for guaranteeing conidia survival in field conditions. However, further studies are needed for establishing which mechanisms are involved regarding the differences in tolerance observed in the culture conditions evaluated here. This work is the first report in which all these parameters (carbon source, nitrogen source, C:N ratio, culture system, amount, and tolerance of conidia) have been evaluated for *Penicillium* sp.

Conflict of Interests

The authors have not declared any conflict of interests.

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