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REGULAR ARTICLE

Production and characterization of biomass and exopolysaccharides obtained in submerged culture under different initial pHs used in the cultivation of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*

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ABSTRACT

Colletotrichum gloeosporioides (GC003) and *Rhizopus stolonifer* (RS001) were cultivated in two different liquid culture media: LC1 (glucose 40 g L^{-1}, yeast extract 3 g L^{-1} and tryptone peptone 2 g L^{-1}) and LC2 (glucose 40 g L^{-1}, yeast extract 3 g L^{-1} and tryptone peptone 10 g L^{-1}) for the production of mycelial biomass and exopolysaccharides (EPS). By using the liquid culture (LC2) under pH of 4.5 presented the highest biomass content (15.73 g L^{-1}) in the propagation of *Rhizopus stolonifer*. The highest production of exopolysaccharides (1.74 g L^{-1}) was obtained by the liquid culture (LC2) under pH of 4.5 in the cultivation of *Colletotrichum gloeosporioides*. The results presented that the production of biomass and exopolysaccharides (EPS) is directly related with the pHs values and the strain used in the cultivation.

Keywords: *Colletotrichum gloeosporioides;* Biomass; Exopolysaccharides; Liquid culture media; *Rhizopus stolonifer*

INTRODUCTION

Exopolysaccharides (EPS) have showed different applications in the food, pharmaceutical, cosmetic and other industries due to their propierties such as: bioadhesives, probiotics, gelling, thickeners, bioadsorbents, emulsifiers and stabilizers (Xu et al., 2006; Öner et al., 2016; Gil-Ramírez et al., 2018). The most common microbial EPS producers are: bacteria and fungi, among the most important conditions to produce EPS are: pH ranged from 3.0 to 6.5, temperature between 22 °C and 30 °C (Nguyen et al., 2012; Mahapatra and Banerjee, 2013). Submerged liquid is the most common culture used in the production of biomass and exopolysaccharides, this culture medium produces larger amounts of mycelium in restricted spaces (such as industrial bioreactors) and has uniform distribution in the substrate, reducing culture periods without significant problem of contamination (Bae et al., 2001; Kim et al., 2002; García-Cruz et al., 2019).

Studies have presented that the use of phytopatogenic fungi such as: (*Colletotrichum* sp. and *Rhizopus* sp.) to improve the biodegradation of organic wastes like: mangoes, oranges for being used in the composting process without cause biologic hazards or several damage to crops (Mena-Nevarez et al., 2012). Valenzuela-Cobos et al. (2020b) presented the highest biodegradation of plantain rachis with a treatment composed of $1.75x10^6$ spores of *Colletotrichum gloeosporioides* + $1.75x10^6$ spores of *Rhizopus stolonifer* in 1 L of sugar cane sterilized. However, there are not studies about the production of exopolysaccharides in submerged culture under different initial pHs (3.5, 4.5, 5.5, 6.5 and 7.5) of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*.

The aim of this investigation was to evaluate the mycelium growth kinetics on malt extract agar, the biomass and the exopolysaccharides (EPS) production from two submerged culture under different initial pHs (3.5, 4.5, 5.5, 6.5 and 7.5) used in the cultivation of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*.

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MATERIALS AND METHODS

Strains

In this experiment was used the following phytopatogenic fungi: *Colletotrichum gloeosporioides* (GC003) and *Rhizopus stolonifer* (RS001). The strains are maintained on MEA dishes. Stocks of all strains are deposited at the fungal collection of Research and Development Laboratory of Ecuahidrolizados.

Solid culture media

The culture media was prepared by dissolving 18 g of malt extract and 15 g of bacteriological agar in 1 L of distilled water using an Erlenmeyer flask. The flask was sterilized in autoclave at 15 psi (121 °C) for 15 min, subsequent, 10 mL of sterile medium were poured into Petri dishes. The dishes with the medium solidified were put in plastic bags and incubated at 28 °C for 24 h to check the sterility. Then, the Petri dishes without contamination were used for propagation of the mycelium of phytopathogenic fungi (Eger et al., 1976; Coello-Loor et al., 2017).

Growth culture conditions

Liquide Culture 1 (LC1): 1 L of distilled water with glucose (40 g L^{-1}), yeast extract (3 g L^{-1}) and tryptone peptone (TP) 2 g L^{-1}.

Liquide Culture 2 (LC2): 1 L of distilled water with glucose (40 g L^{-1}), yeast extract (3 g L^{-1}) and tryptone peptone (TP) 10 g L^{-1}.

The two liquid culture were adjusted to five different initial pHs: 3.5, 4.5, 5.5, 6.5, 7.5 by addition of either 1N NaOH or 1N HCl (Taskin et al., 2012).

Mycelial growth kinetics with Gompertz model

The mycelial growth rate on Petri dish with MEA at 20 °C was estimated by the diameter of the colony until the total colonization, and the growth velocity was determinate used the following nonlinear regression model (Valenzuela-Cobos et al., 2017; Valenzuela-Cobos et al., 2020a):

$$\log N = A+C*\exp\{-\exp[-B(t-M)]\},$$

where:
A, B, C= parameters of the model
t= days of mycelial colonization
M=day with maximum growth rate
log N= growth kinetics
μ_{max}= M/ (A*C), where: M= day with maximum growth rate

To determinate the maximum growth (μ_{max}) on MEA and the lag time (λ) was calculated by using the μ_{max} value on the Hill model (Gibson et al., 1987; Hills and Wright, 1994):

$$\lambda = [\ln(1+(\mu_{max}/v)]/ \mu_{max}, \text{ where: } v= \mu_{max}$$

Biomass production

Two discs of mycelium (5.5 mm) of *Colletotrichum gloeosporioides* (GC003) and *Rhizopus stolonifer* (RS001) were cut from the edge of MEA dishes, and then were inoculated in 100 mL of the two solutions of liquid culture (LC1 and LC2). All production studies were carried out at 25 °C and 200 rpm in a shaking incubator for 6 days. Cellular biomasses were separated by using a 17000 rpm centrifuge at 4 °C, then was washed from the sieve with distilled water, filtered through Whatman #1 filter paper, and dried to constant weight at 70 °C (Lakzian et al., 2008).

Exopolysaccharides production

The culture broth and the water used to wash the biomass off the sieves were filtered through Whatman #1 filter paper and evaporated to 50 mL at 80 °C using a heating plate. This reduced volume was added to 150 mL of ethanol (98%), in order to precipitate the polysaccharides. Finally, the precipitate exopolysaccharides (EPS) were filtered out, dried to constant weight at 40 °C (Wagner et al., 2004; Rasulov et al., 2013).

Statistical analysis

In all analyzes, a completely randomized design and the results were studied using one-way analysis of variance (ANOVA) to determine the significance of individual differences at $p<0.05$ level, the maximum growth specific speed (μ_{max}) and the lag phase (λ) of the fungi strains cultivated on MEA dishes, the biomass content and the exopolysaccharides produced by the submerged liquid (LC1 and LC2) under different initial pHs (3.5, 4.5, 5.5, 6.5 and 7.5) used in the cultivation of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*, when statistical differences were found, the Duncan Test with $\alpha = 0.05$ was applied. The analyses were carried out using statistical software (Statgraphic ver. 16).

RESULTS AND DISCUSSION

Mycelial growth kinetics of dikaryotic strains on MEA

The Gompertz and Hill models describe the best growth tendencies both in terms of statistical accuracy and simplicity (McDonald and Sun, 1999), by using these models were determinate the maximum growth specific speed (μ_{max}) (Gil et al., 2011) and the lag time (λ) (Hills and Wright, 1994) of the phytopatogenic fungi strains.

Colletotrichum gloeosporioides showed the highest maximum growth specific speed (μ_{max}) of 0.47 mm d^{-1}, while *Rhizopus stolonifer* presented μ_{max} of 0.14 mm d^{-1} (Table 1). Sangeetha and Rawal (2009) obtained μ_{max} values between 4.00 to 9.81 mm d^{-1} for eight strains of *Colletotrichum gloeosporioides*

Table 1: Comparation of μ_{max} and λ values of the phytopathogenic fungi strains on MEA

Strains	μ_{max} (d^{-1})	CV (%)	λ(d)	CV (%)
Colletotrichum gloeosporioides (GC003)	0.47±0.14^{B}	30	1.60±0.48^{a}	30
Rhizopus stolonifer (RS001)	0.15±0.08^{A}	57	7.10±5.59^{b}	78

*Uppercase letters in each column indicated significant difference among the μ_{max} values on MEA, while lowercase letters in each column indicated significant difference among the λ values on MEA at level $p < 0.05$ according to Duncan's test, n=10

cultivated on PDA agar at 15 °C, while (González and Sutton, 2005) determined values of μ_{max} since 11.90 to 12.40 mm d^{-1} for three strains of *Colletotrichum gloeosporioides* cultivated on PDA agar at 26 °C. Ochoa-Velasco et al. (2018) reported μ_{max} value of 7.44 mm d^{-1} for one strain of *Rhizopus stolonifer* cultivated on PDA agar at 28 °C. The specific growth rate (μ_{max}) is the ability of the strain to degrade polysaccharides and lignocellulosic materials in substrates (Liu et al., 2017). Based on the results, the strain of *Colletotrichum gloeosporioides* can absorb the nutrients on MEA in shorter time in comparison with the other strain used in the investigation. The specific growth rate (μ_{max}) is directly related with the medium and temperature used in the cultivation of the fungi strain.

Phytopatogenic fungi strain of *Colletotrichum gloeosporioides* presented the lowest lag phase (λ) being of 1.60 d, whereas *Rhizopus stolonifer* presented lag phase of 7.10 d. Sangeetha and Rawal (2009) obtained lag phase (λ) ranged from 0.07 to 0.17 d for eight strains of *Colletotrichum gloeosporioides* cultivated on PDA agar at 15 °C, while (González and Sutton, 2005) determined values of λ between 0.55 and 0.59 d for three strains of *Colletotrichum gloeosporioides* cultivated on PDA agar at 26 °C. Ochoa-Velasco et al. (2018) reported λ value of 0.42 d for one strain of *Rhizopus stolonifer* cultivated on PDA agar at 28 °C. The lag phase indicates the adaptability of the strain to new conditions (Chatterjee et al., 2015). Based on the results of our study, the fungi strain of *Colletotrichum gloeosporioides* can adapt in less time on MEA in comparison with the other strain in the research.

Biomass and exopolysaccharides production

Table 2 shows the production of biomass from the two submerged liquid under different initial pHs (3.5, 4.5, 5.5, 6.5 and 7.5) used in the cultivation of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*.

The liquid culture (LC1) under different initial pHs (3.5, 4.5, 5.5, 6.5 and 7.5) used in the cultivation of *Colletotrichum gloeosporioides* presented biomass content since 7.50 to 12.77 g L^{-1}, while using the liquid culture (LC2) under different initial pHs presented biomass production ranged

Table 2: Biomass production obtained from *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* cultivated in two different liquid culture media under different initial pHs

Strains	Liquid culture	pH	Biomass (g L^{-1})	CV (%)
Colletotrichum gloeosporioides (GC003)	LC1	3.5	12.17±0.31^{f}	2.51
		4.5	12.77±0.06^{g}	0.45
		5.5	10.80±0.36^{d}	3.34
		6.5	9.47±0.35^{b}	3.71
		7.5	7.50±0.36^{a}	4.81
	LC2	3.5	12.50±0.26^{f}	3.64
		4.5	13.43±0.31^{h}	3.79
		5.5	11.50±0.20^{e}	2.30
		6.5	10.23±0.41^{c}	3.61
		7.5	9.27±0.21^{b}	3.60
Rhizopus stolonifer (RS001)	LC1	3.5	12.37±0.45^{D}	3.64
		4.5	14.70±0.56^{F}	3.79
		5.5	11.50±0.26^{C}	2.30
		6.5	9.73±0.35^{B}	3.61
		7.5	8.47±0.31^{A}	3.60
	LC2	3.5	13.37±0.25^{E}	1.88
		4.5	15.73±0.40^{G}	2.57
		5.5	12.83±0.12^{D}	0.90
		6.5	11.27±0.21^{C}	1.85
		7.5	9.67±0.06^{B}	0.60

*LC1 (glucose 40 g L^{-1}, yeast extract 3 g L^{-1} and tryptone peptone 2 g L^{-1}) and LC2 (glucose 40 g L^{-1}, yeast extract 3 g L^{-1} and tryptone peptone 10 g L^{-1})
* Lowercase letters in each column indicated significant difference among the biomass production obtained from the two different liquid culture media (LC1 and LC2) under different initial pHs used in the cultivation of Colletotrichum gloeosporioides, while uppercase letters in each column indicated significant difference the biomass production obtained from the two different liquid culture media (LC1 and LC2) under different initial pHs used in the cultivation of Rhizopus stolonifer at level $p < 0.05$ according to Duncan's test, n = 3

from 9.27 to 13.43 g L^{-1}. The results obtained using the liquid culture (LC2) under pH of 4.5 presented the highest biomass content (13.43 g L^{-1}) in the cultivation of *Colletotrichum gloeosporioides*, while the lowest content of biomass (7.50 g L^{-1}) was presented by the liquid culture (LC1) under pH of 7.5 in the propagation of this fungi strain. For otherwise, using the liquid culture (LC1) under different initial pHs in the cultivation of *Rhizopus stolonifer* showed biomass content between 8.47 and 14.70 g L^{-1}, whereas using the liquid culture (LC2) under different initial pHs presented biomass production since 9.67 to 15.73 g L^{-1}. The results obtained using the liquid culture (LC2) under pH of 4.5 presented the highest biomass production (15.73 g L^{-1}) in the propagation of *Rhizopus stolonifer*, whereas the lowest content of biomass (8.47 g L^{-1}) was presented by the liquid culture (LC1) under pH of 7.5 in the propagation of this fungi. Diamantopoulou et al. (2014) showed biomass since 10.50 g L^{-1} to 22.50 g L^{-1} per day for different edible fungi cultivated on submerged cultures at pH of 6.2. Papagianni (2004) indicates that the acid pH limits mycelial growth.

Table 3 presents the production of exopolysaccharides from the two liquid culture under different initial pHs

Table 3: Exopolysaccharides obtained from two different liquid culture media under different initial pHs used in the cultivation of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*.

Strains	Liquid culture	pH	Exopolysaccharides (g L^{-1})	CV (%)
Colletotrichum gloeosporioides (GC003)	LC1	3.5	1.20±0.00^{e}	0.31
		4.5	1.51±0.08^{g}	5.40
		5.5	1.03±0.03^{d}	3.24
		6.5	0.87±0.03^{b}	3.00
		7.5	0.81±0.00^{a}	0.88
	LC2	3.5	1.31±0.03^{f}	2.06
		4.5	1.74±0.02^{h}	1.42
		5.5	1.23±0.03^{e}	2.53
		6.5	0.99±0.02^{c}	2.61
		7.5	0.96±0.03^{c}	3.00
Rhizopus stolonifer (RS001)	LC1	3.5	1.08±0.04^{C}	3.33
		4.5	1.31±0.03^{E}	2.03
		5.5	0.98±0.01^{B}	1.79
		6.5	0.92±0.00^{B}	0.65
		7.5	0.84±0.01^{A}	2.14
	LC2	3.5	1.21±0.02^{D}	1.91
		4.5	1.50±0.06^{F}	4.27
		5.5	1.20±0.07^{D}	5.51
		6.5	1.07±0.04^{C}	4.05
		7.5	0.93±0.05^{B}	4.97

*LC1 (glucose 40 g L^{-1}, yeast extract 3 g L^{-1} and tryptone peptone 2 g L^{-1}) and LC2 (glucose 40 g L^{-1}, yeast extract 3 g L^{-1} and tryptone peptone 10 g L^{-1}) *Lowercase letters in each column indicated significant difference among the exopolysaccharides production obtained from the two different liquid culture media (LC1 and L C2) under different initial pHs used in the cultivation of Colletotrichum gloeosporioides, while uppercase letters in each column indicated significant difference the exopolysaccharides production obtained from the two different liquid culture media (LC1 and LC2) under different initial pHs used in the cultivation of Rhizopus stolonifer at level $p < 0.05$ according to Duncan's test, n = 3

(3.5, 4.5, 5.5, 6.5 and 7.5) of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*.

The liquid culture (LC1) under different initial pHs used in the propagation of *Colletotrichum gloeosporioides* showed exopolysaccharides production ranged from 0.81 to 1.51 g L^{-1}, while using the liquid culture (LC2) under different initial pHs presented exopolysaccharides content since 0.96 to 1.74 g L^{-1}. The results obtained using the liquid culture (LC2) under pH of 4.5 presented the highest exopolysaccharides production (1.74 g L^{-1}) in the cultivation of *Colletotrichum gloeosporioides*, while the lowest content of exopolysaccharides (0.81 g L^{-1}) was showed by the liquid culture (LC1) under pH of 7.5 in the propagation of this strain. Otherwise, using the liquid culture (LC1) under different initial pHs in the cultivation of *Rhizopus stolonifer* showed exopolysaccharides content between 0.84 and 1.32 g L^{-1}, whereas using the liquid culture (LC2) under different initial pHs presented production of exopolysaccharides ranged from 0.93 to 1.50 g L^{-1}. The results obtained using the liquid culture (LC2) under pH of 4.5 presented the highest exopolysaccharides content (1.50 g L^{-1}) in the cultivation of *Rhizopus stolonifer*, whereas the lowest content of exopolysaccharides (0.84 g L^{-1}) was showed by the liquid culture (LC1) under pH of 7.5 in the propagation of this phytopathogenic fungi.

The submerged culture represents an alternative form of fast and efficient production of mycelial biomass and exopolysaccharides (Confortin et al., 2008). Medium with nitrogen source tends to be the most expensive. Peptones represent not only a source of organic nitrogen but also a source of amino acids or specific peptides. They are defined as protein hydrolysates that are readily soluble in water and are not precipitable by heat, by alkalis or by saturation with ammonium sulphate. The most common peptones used for microbiological studies are bacto-peptone, tryptone peptone (TP), fish peptone (FP), meat peptone, neopeptone and protease peptone (Parrado et al., 1993; Dufosse et al., 1997; Taskin and Erdal, 2011; Vasileva-Tonkova et al., 2007). The use of source of organic nitrogen such as: tryptone-peptone in the medium is directly related with the high production of biomass and exopolysaccharides.

CONCLUSIONS

The highest production of biomass and exopolysaccharides was obtained by the liquid culture (LC2) under pH of 4.5 in the cultivation of *Rhizopus stolonifer* and *Colletotrichum gloeosporioides* respectively.

The production of biomass and exopolysaccharides is modified with the different values of pH and also the strains.

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Authors' contributions

Juan Diego Valenzuela-Cobos has developed the experimental plan. Onay Adonys Mercader-Camejo performed the analysis. René Oscar Rodríguez-Grimón verified the analytical methods and discussed the results and wrote the final manuscript. Ana Grijalva-Endara and Raúl Marcillo-Vallejo supervised the findings of this work.

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