Revista Brasileira de Biociências
Brazilian Journal of Biosciences

ISSN 1980-4849 (on-line) / 1679-2343 (print)

ARTICLE

Optimization of the cultivation conditions for the production of an antimicrobial compound by *Trichosporon japonicum* QU139

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Received: May 19 2010 Received after revision: December 12 2010 Accepted: December 28 2010
Available online at http://www.ufrgs.br/seerbio/ojs/index.php/rbb/article/view/1596

ABSTRACT: (Optimization of the cultivation conditions for the production of an antimicrobial compound by *Trichosporon japonicum* QU139.) Strain *Trichosporon japonicum* QU139 showed antimicrobial activity against several pathogenic fungi, and has good potential for being used in the development of a new antimicrobial agent. The objective of this work was to optimize the conditions for the production and detection of the antimicrobial compound produced by this strain. The antimicrobial effect was evaluated by the well method against sensitive cells of *Cryptococcus gattii* C20 in GYP and YM media at different pH values. GYP medium at pH 4.5 ± 0.5 and 24h of incubation, at 25ºC and 150 rpm, were defined as the best medium and cultivation conditions. The methodologies employed for the isolation and concentration of the antimicrobial compound were negative for antimicrobial activity, suggesting that it may not have a protein nature. The nature of the compound needs to be fully explored, and other methodologies, especially the ones for purification of glycolipids, must be employed in order to obtain a purified compound for biotechnological purposes.
Key words: *Cryptococcus gattii*, killer yeasts, biotechnological potential, *Trichosporon japonicum*.

RESUMO: (Otimização das condições de cultivo para a produção de um composto antimicrobiano por *Trichosporon japonicum* QU139). A cepa *Trichosporon japonicum* QU139 mostrou atividade antimicrobiana contra vários fungos patogênicos, tendo um bom potencial para o desenvolvimento de um novo agente antimicrobiano. O objetivo do trabalho foi a otimização da produção e detecção do composto antimicrobiano. O efeito antimicrobiano foi avaliado pelo método de poços contra células sensíveis de *Cryptococcus gattii* C20 em meios GYP e YM em diferentes pH. O meio GYP, em pH 4.5 ± 0.5 e 24h de incubação a 25ºC e 150 rpm, foram definidos como o melhor meio e condições de cultivo. As metodologias empregadas para isolamento e concentração do composto antimicrobiano foram negativas para atividade antimicrobiana, sugerindo que ele possa não ter natureza protéica. A natureza do composto precisa ser mais bem explorada e outras metodologias, especialmente as utilizadas em purificação de glicolipídeos, devem ser empregadas para obter um composto purificado visando aplicações biotecnológicas.
Palavras chave: *Cryptococcus gattii*, leveduras *killer*, potencial biotecnológico, *Trichosporon japonicum*.

INTRODUCTION

The occurrence of fungal infections in the human population, caused by pathogenic fungi, has grown in recent times (Ghannoum & Rice 1999, Lupetti *et al.* 2002, Kanafani & Perfect 2008). The antifungal agents used to treat these infections belong to a few groups of drugs, and there are reports of resistance to many of these, while others are highly toxic to the patient (Lunardi *et al.* 2006, Severo *et al.* 2009). These facts emphasize the need for the development of new therapies. *Cryptococcus neoformans* and *C. gattii* cause an illness called cryptococcosis, which is one of the most common mycoses in immunodepressed patients. *Cryptococcus gattii* is usually associated to immunocompetent humans and animals, while *C. neoformans* affects immunocompromised patients (Severo *et al.* 2009).

The production of antimicrobial compounds by yeasts is common and has been described in more than a hundred species and twenty genera. These compounds belong to two main types: glycoproteins and glycolipids (Golubev 2006). Glycoproteins, called mycocins or killer toxins, have been studied for over 40 years and a lot is known about the molecular characteristics of these toxins and their modes of action (Magliani *et al.* 1997, Schmitt & Breining 2002). Antibodies obtained from mycocins are being studied for the development of antifungal vaccines (Polonelli *et al.* 1994, Magliani *et al.* 2004, Kabir *et al.* 2009, Krishnaswamy *et al.* 2010). The fungicidal activity of the extracellular glycolipids was discovered recently and is less known. Besides the clinical area, these antimicrobial compounds have applicability in the food industry, especially in fermentative processes, and in the biocontrol of plant and crustacean diseases (Santos & Marquina 2004, Comitini *et al.* 2004, Wang *et al.* 2007).

In previous experiments performed in our laboratory, 21 yeast strains were tested against several pathogenic fungi, including different species of *Candida*, *C. neoformans*, *C. gattii*, dermatophytes, *Sporothrix schenckii* and *Fonsecaea pedrosoi*. Based on the results obtained, the yeast *Trichosporon japonicum* QU139 was chosen for the development of a new antifungal agent with a broad

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spectrum of action. Fuentefria *et al.* (2008) showed that *T. japonicum* QU139 was capable of inhibiting all of the one hundred clinical and environmental isolates of *C. gattii* and *C. neoformans* tested.

One of the major drawbacks for the industrial use of antimicrobial compounds produced by yeasts is the lack of accurate data on toxin production and activity (Barandica *et al.* 1999). In order to better characterize the production of the antimicrobial compound secreted by *T. japonicum* QU139, the objective of this work was to discover what the optimal cultivation conditions are for the production and detection of the antimicrobial compound produced by this strain, with the intention of purifying the compound in the future.

MATERIALS AND METHODS

Yeast strains

The strain *T. japonicum* QU139, isolated from artisanal cheese in the state of Rio Grande do Sul, Brazil (Landell *et al.* 2006) and deposited in the Yeast Culture Collection/UFRGS, was used as the mycocinogenic yeast. The sensitive strain *Cryptococcus gattii* C20 was a clinical isolate from a HIV positive patient, serotype B, molecular type VGIII, and *mating type* α.

Culture media

The cultures were maintained in GYP agar (2% glucose; 1% peptone; 0.5% yeast extract; 2% agar) that was covered with sterile mineral oil, at 4°C. The medias for toxin production and detection were GYP broth and agar and YM broth and agar (1% glucose; 0.5% peptone; 0.3% yeast extract; 0.3% malt extract; 2% agar), which were both buffered with 0.05 M citrate-phosphate buffer at different pH values (3.0 to 6.0).

Production and detection of the antimicrobial compound

Approximately 10^3 cells of *T. japonicum* QU139 were inoculated in 250 mL Erlenmeyer flasks containing 100 mL of each medium, and then incubated for 48 hours. Modifications in the incubation temperature and agitation were also tested: 25ºC or 30ºC, and 80 rpm or 150 rpm, respectively. 30 µL of each culture supernatant, for each treatment, were inoculated in 9mm diameter wells on a Petri dish containing the respective media and pH, previously inoculated at the surface with 10^3 sensitive cells of *C. gattii* C20. The activity of the antimicrobial compound was evaluated by measuring the diameter (mm) of the inhibition zone around the well after incubation for 48h at 25°C. Data presented refer to the average of two independent experiments with less than 10% standard deviation.

Monitoring of viable cells:

100 µL of each culture supernatant were inoculated in GYP agar and incubated for 48h at 25°C for quantification of viable cells. The number of generations and the specific growth rate were calculated according to the following formulae:

$$N = \frac{\log Nt - \log N_0}{\log 2}$$

$$\mu = \frac{N}{T}$$

Where:

N: number of generations

log Nt: log of cell number at the end of the log phase

log N_0: log of cell number at the beginning of the log phase

μ: specific growth rate

T= duration of the log phase

All the numbers represent the average of two different experiments.

Isolation and concentration of proteins / glycoproteins

The methodologies employed for the isolation and concentration of the antimicrobial compound, so it could be purified at a later date, were performed according to Scopes (1994), Santos & Marquina (2004), Buzzini *et al.* (2004) and Comitini *et al.* (2004). *Trichosporon japonicum* QU139 was grown in GYP pH 4.5 for 24 hours at 25°C.

Precipitation with ammonium sulfate

100 mL of the culture supernatant were precipitated with ammonium sulfate in four saturation levels (0–20%; 20–40%; 40–65% and 65–80%). The experiment was conducted under refrigeration (4°C) and agitation (80 rpm). The salt quantity necessary for attaining each saturation level was calculated based on the volume of culture supernatant subjected to precipitation, according to Scopes (1994). After the addition of the corresponding quantity of salt, the suspension was maintained for 30 minutes under agitation followed by 30 minutes under rest. The suspension was centrifuged (10,000xg for 15 min) and the precipitate was suspended in 2 mL of citrate-phosphate buffer (0.05M, pH 4.5). The sample was dialyzed against the same buffer overnight. After this process, the activity in 30 µL was evaluated by the well method.

Precipitation with ethanol

1 L of the culture supernatant was centrifuged, filter-sterilized (0.45 µm), precipitated with 70% volume of cold ethanol, and kept at 4°C overnight. After centrifugation (25 min at 3,000xg), the pellet was suspended in 50 mL of citrate-phosphate buffer (0.05 M, pH 4.5). Activity was evaluated by the well method.

Ultrafiltration

The protocol was adapted from Comitini *et al.* (2004). 100 mL of the culture supernatant was filter-sterilized

Table 1. Halo diameters (mm) produced by the yeast *T. japonicum* QU139 in GYP and YM at 25ºC and 80 rpm.

pH	GYP				YM			
	16h	24h	40h	48h	16h	24h	40h	48h
pH 3.0	0	0	17	17	15	22	0	0
pH 4.0	18	20	27	24	15	22	27	18
pH 5.0	20	21	25	27	16	22	26	20
pH 6.0	18	20	24	23	15	22	26	20

(0.45 mm), concentrated to a final volume of 2 mL in an Amicon YM10 (10kD cut-off), kept at 4°C and evaluated by the well method.

Freeze-drying

The filter-sterilized culture supernatant was also subjected to freeze-drying for five days. The pellet was suspended in 50 mL of citrate-phosphate buffer, at pH 4.5, and tested by the well method.

Co-culture

10^3 cells of *C. gattii* C20 in 10 mL of GYP broth were inactivated using an autoclave and added to 100 mL of *T. japonicum* QU139 in the same medium (pH 4.5, 150 rpm, for 24h) in order to know if the killer strain needed the contact with a sensitive cell to produce the antimicrobial compound. The supernatant of the co-culture was filter--sterilized and evaluated by the well method.

RESULTS

The size of the inhibition haloes produced by *T. japonicum* QU139 in GYP and YM at 25ºC and 80 rpm can be seen in Table 1. Both media showed the biggest haloes at 40–48 hours of incubation at pH 4.0 to pH 6.0. Incubation at 30ºC resulted in smaller inhibition haloes (data not shown). As the results were similar for both media, all other experiments were performed using GYP. An increase in agitation to 150 rpm resulted in the reduction of the incubation time required for the best toxin production (Table 2). The biggest haloes were produced at pH 4.0 to pH 5.0 in 24 hours. It is possible that higher rpm values would reduce the incubation time.

Growth curves were plotted along the experiments for comparison with the production of the antimicrobial compound. The peak of production of the antimicrobial compound coincided with the late exponential phase for all pH values tested (Tab. 2, Fig. 1). The specific growth rate and the duration of the lag and log phases of each growth curve are shown in Tab. 3. Although the lowest specific growth rate was obtained at pH 5.5, pH 4.0 to 5.0 were the best for antimicrobial compound production (Tab. 2). Therefore GYP medium at pH 4.5 ± 0.5 and incubation at 25ºC with 150 rpm were defined as the best medium and cultivation conditions for production of the antimicrobial compound by strain *T. japonicum* QU139.

Table 2. Halo diameters (mm) produced by the yeast *T. japonicum* QU139 in GYP at 25ºC and 150 rpm.

pH	Halo diameter (mm)				
	24h	30h	36h	42h	48h
pH 4.0	29a	24a	25a	27a	24a
pH 4.5	32a	26a	26a	26a	26a
pH 5.0	32a	24a	22a	22a	23a
pH 5.5	24b	21a	21a	22a	19b

*Values followed by the same letter do not differ statistically by the Tukey Test (P = 0.05).

Table 3. Specific growth rate and duration of the lag and log phases for the strain *T. japonicum* QU139 in GYP medium at 25°C.

pH	μ (h-1)	Duration of the phase (hours)	
		lag	log
pH 4.0	0,87 a*	12	12
pH 4.5	0,66 b	6	18
pH 5.0	0,64 b	6	18
pH 5.5	0,36 c	6	30
CV (%) 1,18			

*Values followed by the same letter do not differ statistically by the Tukey Test (P = 0.05).

The methods for protein isolation were negative for antimicrobial activity, while the controls, with the culture supernatants containing cells, were always capable of producing inhibition haloes against the sensitive strain *C. gattii* C20 (data not shown). Co-cultivation of *T. japonicum* QU139 with dead cells of *C. gattii* C20 also did not produce inhibition haloes when the cell-free culture supernatant was tested.

DISCUSSION

Several factors may influence the activity of antimicrobial compounds produced by yeasts, such as pH, temperature and media composition. Usually, nutritionally rich media containing glucose, peptone and yeast extract and a low pH are used (Golubev 2006, Mimee *et al.* 2009). The peak of toxin production by *T. japonicum* QU139 coincided with the end of the log phase, as observed for *Kluyveromyces phaffii* (Comitini *et al.* 2004). Toxin production lowered along the stationary phase. Barandica *et al.* (1999) proposed a mathematical model to explain toxin production by yeasts. According to these authors,

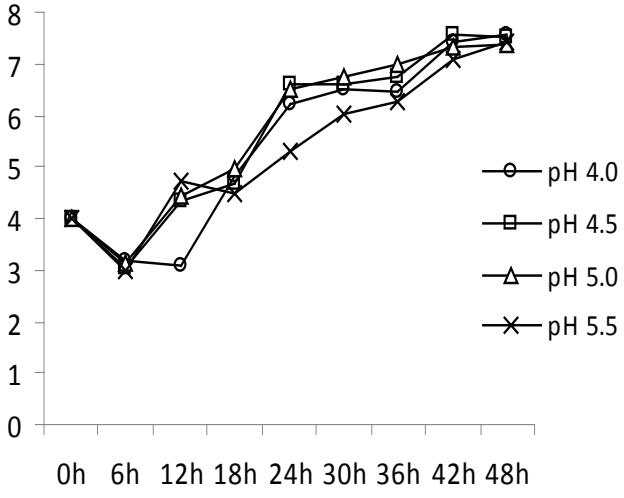

Figure 1. Growth curve of *Trichosporon japonicum* QU139 in GYP, at 150 rpm and 25°C, at pH 4.0 (o), 4.5 (□), 5.0 (Δ), and 5.5 (×).

the process of toxin synthesis is simultaneous with its inactivation during the log phase, but synthesis does not take place along the stationary phase, resulting in lower toxin levels.

The size of the inhibition haloes produced by *T. japonicum* QU139 were higher than the ones obtained by Fuentefria *et al.* (2008) for the same strain using *C. gattii* C20 as the sensitive yeast. This was probably due to the difference in the number of sensitive cells (10^6 cells), pH (5.5), medium (YM) and incubation time (3–5 days) applied by Fuentefria *et al.* (2008). These authors suggested the use of a panel of killer yeasts for biotyping *C. neoformans* / *C. gattii* in cell x cell experiments. The different halo sizes obtained in the present study points to the need of using the purified toxins in biotyping experiments instead of the intact cells as suggested by Buzzini *et al.* (2007).

The methodologies used in this study for purification of the antimicrobial compound are the ones usually applied for the isolation/concentration of glycoproteins (Soares & Sato 2000, Santos & Marquina 2004). But the activity of *T. japonicum* QU139 against the sensitive cell was only evident when there was live cell-cell contact. It is possible that the compound was degraded and lost its activity during the aggressive conditions for precipitation or due to the presence of proteases. Alternatively, the agitation of the culture may have caused the inactivation of the antimicrobial compound, as reported for *Saccharomyces cerevisiae* and *Kluyveromyces lactis* (Kotani *et al.* 1977, Wilson & Whittaker 1989). In this case, the best results obtained in the present study for 150 rpm compared to 80 rpm may be due to a better growth of *T. japonicum* QU139 under higher agitation and oxygenation. Finally, another explanation may be that the antimicrobial compound does not have a protein nature.

The occurrences of protein and non-protein antimicrobial compounds in the genus *Trichosporon* have already been reported. *Trichosporon pullulans* was reported to produce a protein toxin with the molecular weight of 15kDa associated to the presence of dsRNA (Golubev *et al.* 2002). In previous studies, the strain *T. japonicum* QU139 presented dsRNA (Fuentefria 2007). More recently, *Trichosporon porosum* Pa20 was shown to produce a glycolipid (non-protein) compound active against ascomycetous and basidiomycetous fungi (Kulakovskaya *et al.* 2009, 2010). The nature of the compound produced by *T. japonicum* QU139 needs to be fully explored, and other methodologies, especially the ones for purification of glycolipids, must be employed in order to obtain a purified compound for biotechnological purposes.

ACKNOWLEDGEMENTS

The authors thank CAPES and FAPERGS for the financial support, Dr. Marilene Henning Vainstein for the strain *C. gattii* C20, K.P. Kowalski for the technical assistance, and F. Bücker and M. Strochein for the statistical analysis.

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