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Effect of nitrogen source and pH on siderophore production by *Rhodotorula* strains and their application to biocontrol of phytopathogenic moulds

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The production of rhodotorulic acid, a siderophore synthesized by *Rhodotorula* strains, was improved with the objective of achieving the biocontrol of phytopathogenic moulds. Rhodotorulic acid increased up to 60% in the presence of urea as a nitrogen source, pH near to 8 and a C:N ratio of 8:1. The siderophore-containing spent medium showed *in vitro* antifungal activity against important plant pathogens including *Botrytis cinerea*, which causes grey mould on a wide variety of host plants including numerous commercial crops. The antifungal activity was related to siderophore concentration. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 226–229.

Keywords: rhodotorulic acid; biocontrol; phytopathogenic moulds; yeasts

Introduction

Microorganisms elaborate a variety of low-molecular-weight high-affinity chelating agents, which solubilize ferric iron in the environment and transport it into the cell. These compounds are generically known as siderophores [12]. There is substantial evidence to believe that siderophores play some role in the biocontrol of phytopathogenic microorganisms by sequestering iron, and thereby inhibiting either pathogen growth or metabolic activity [15].

Siderophores produced by soil *Pseudomonas* species have been studied as biocontrol agents of soil-borne and aerial plant diseases. Many rhizosphere *Pseudomonas* species promote plant growth and inhibit pathogenic bacteria and fungi by producing siderophores [8]. The effect of a siderophore (Pseudobactin 358) produced by *Pseudomonas putida* on the suppression of fusarium wilt of carnations has been reported [7].

Recently, we demonstrated that rhodotorulic acid, a siderophore produced by *Rhodotorula glutinis* strains, improved the biological control of blue rot caused by *Penicillium expansum* in harvested apples. Moreover, conidial germination and mycelial growth of *P. expansum* were closely related to the siderophore concentration [4]. Because the biosynthesis and secretion of siderophores are strictly regulated by environmental factors [4,5,14], the goal of the present work was to increase production of rhodotorulic acid by *Rhodotorula* strains by manipulating culture medium composition and pH. As this study is a part of a project for developing a biocontrol method of postharvest rots in apples and pears, antifungal activity of the siderophore on different postharvest pathogens was also assayed.

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Materials and methods

Microorganisms

R. glutinis SL 57 and *Rhodotorula rubra* SL 29, isolated from apples and identified in our laboratory [3], were used in the experiments and were maintained on a semisynthetic minimal medium (in grams per liter): glucose 5, $(NH_4)_2SO_4$ 0.5, yeast extract 0.05, $K_2H_2PO_4$ 0.2, $MgCl_2{\cdot}6H_2O$ 0.2 and agar-agar 20, at pH 4.5.

Culture medium and growth conditions for siderophore production

Culture medium for siderophore production consisted of (in grams per liter) sucrose 25, $(NH_4)_2SO_4$ 4, K_2HPO_4 3, citric acid 1, $MgSO_4$ 0.08, and $ZnSO_4$ 0.002, pH 6.8.

Rhodotorula strains were grown in 1000-ml flask cultures with 200 ml of production medium with shaking (200 rpm) for 72 h at 30°C. Inoculum was grown in the same medium for 24 h at 30°C with shaking (200 rpm). The inoculum consisted of 20 ml of this actively growing culture.

Siderophore detection in culture supernatants

Siderophore was detected according to the ferric perchlorate assay [13]. Supernatants obtain from cultures by centrifugation were used. The assay consisted of mixing 0.5 ml of culture supernatant, or a suitable dilution thereof, with 2.5 ml of 5 mM $FeCl_3$–0.1 M $HClO_4$ in a cuvette followed by measurement of the maximum optical density (OD_{max}) versus a blank similarly prepared from sterile medium. The relationship of OD to concentration was assessed using the Lambert–Beer law.

The siderophore was isolated, purified and characterized by RMN spectroscopy [2].

Effect of the siderophore on spore germination and the mycelial growth of phytopathogenic moulds

Liquid (50 ml) was inoculated with 2 ml of a mould spore suspension (6.5×10^6 spores/ml) plus either 10 ml of sterile

culture supernatant (final concentration of siderophore: 0.5, 1 and 1.5 g/l) or 10 ml of sterile distilled water. After 4 h of incubation, samples were withdrawn, fixed on glass slides and stained with lactophenol supplemented with aniline blue (Smith 1963) [16]. These preparations were observed with a light microscope (Olympia) at a magnification of 500×. Conidial germination was assessed by counting at least 100 conidia per slide.

After 48 h, cultures were filtered and the retained mycelium was dried and weighed. Each experimental treatment was replicated in three different Erlenmeyer flasks. Values expressed as percentages were submitted to angular transformation before statistical analysis. Experiments were repeated at least twice.

Statistical analysis

Data were analyzed by an analysis of variance followed by Fisher's least significant difference.

Results

Effect of exogenous amino acids on rhodotorulic acid production

For determining the effect of arginine, ornithine and glutamic acid on rhodotorulic acid production, the production medium was supplemented with the substance to be assayed (10 mM final concentration). Results are shown in Figure 1. Ornithine and glutamic acid stimulated siderophore production by *R. rubra* and *R. glutinis*. In contrast, arginine did not have any significant effect on siderophore production. The amount of siderophore produced by *R. rubra* was 30% higher when the production medium was supplemented with ornithine. Similar results were obtained with *R. glutinis*. The effect of glutamic acid was similar to that of ornithine.

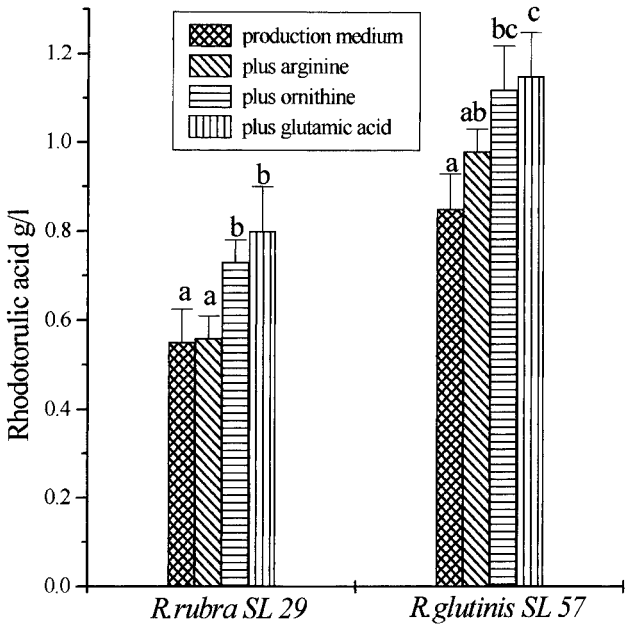

Figure 1 Effect of exogenous amino acids on rhodotorulic acid production by *Rhodotorula* strains. All values are means of triplicates ± SD. Means designated with the same letter are not significantly different ($p = 0.05$).

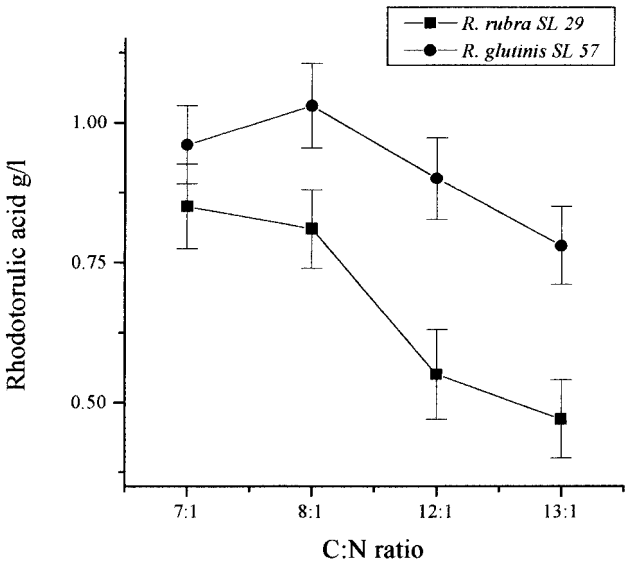

Figure 2 Effect of C:N ratio on rhodotorulic acid production by *Rhodotorula* strains. All values are means of triplicates ± SD.

Nitrogen source, C:N ratio, and siderophore production

Preliminary studies carried out using production medium supplemented with different combinations of sucrose and ammonium sulfate (Figure 2) showed that siderophore accumulation increased with an increment in nitrogen concentration. The combination of 25 g/l of sucrose and 6 g/l of ammonium sulfate represented the optimum C:N ratio (8:1). Subsequently, assays were performed in a medium with a C:N ratio of 8:1 in which the nitrogen source was changed by a mixture of ammonium sulfate and urea (sucrose 25 g/l, ammonium sulfate 4 g/l, urea 1 g/l). In this medium the amount of siderophore produced by either *R. rubra* or *R. glutinis* increased up to 60%.

Further experiments were then performed with the aim of determining whether siderophore production was stimulated by urea or by the increment in the nitrogen concentration.

The production of siderophore was carried out in media with urea and ammonium sulfate as nitrogen sources. These compounds were added separately or together to culture media. Siderophore production increased when the nitrogen source was urea or urea plus ammonium sulfate (Figure 3). The amount of siderophore produced by *R. rubra* was 1.0 g/l in medium 2 (urea plus ammonium sulfate C:N, 8:1) and 1.6 g/l in medium 3 (urea, C:N, 8:1), whereas rhodotorulic acid production in medium 4 (ammonium sulfate, C:N, 8:1) was not significantly different from production in medium 1 (ammonium sulfate, C:N, 12:1). The same effects were observed with *R. glutinis*. Siderophore produced by this strain reached 1.9 g/l in medium 3. Changes of pH during culture were different according to the medium used. At 24 h of growth pH decreased to 2.5–3 in media with ammonium sulfate, whereas in media with urea pH increased to 8. Values of pH at the end of experiments were similar to 24-h values. This phenomenon was observed in both *R. rubra* and *R. glutinis*.

Effect of pH

To clarify the effect of pH on rhodotorulic acid production, two variables, nitrogen concentration and pH, were evaluated. An

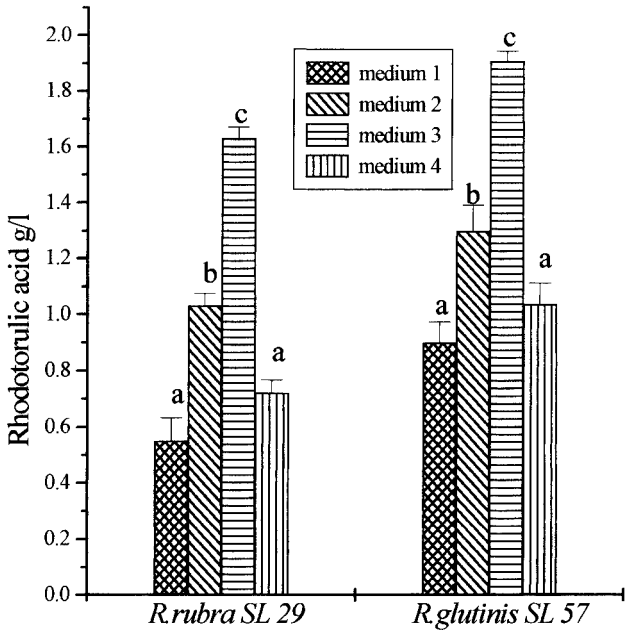

Figure 3 Effect of urea on rhodotorulic acid production by *Rhodotorula* strains. All values are means of triplicates ± SD. Means designated with the same letter are not significantly different ($p = 0.05$). Medium 1: ammonium sulfate 4 g/l. Medium 2: ammonium sulfate 4 g/l plus urea 1 g/l. Medium 3: urea 3 g/l. Medium 4: ammonium sulfate 6.5 g/l.

experimental plan at two levels was designed, therefore 2^2 experiments were carried out. The selected values were nitrogen 0.85 (C:N, 12:1) and 1.35 g/l (C:N, 8:1), pH 3 and 8 and the nitrogen source was ammonium sulfate. The experimental matrix and the results are shown in Table 1. A statistical analysis of these results indicated that nitrogen concentration had the least effect on siderophore production, whereas high pH had a positive influence. The interaction nitrogen–pH showed a highly significative positive effect. ($F=0.01$).

Role of urea in rhodotorulic acid production

To determine whether urea as nitrogen source played some role in the siderophore production, two other experiments were performed. Nitrogen concentration and pH were the same as in the preceding experiments, but in this case, urea was the only nitrogen source used. The experimental matrix and results are shown in Table 1. As

Table 1 Effect of nitrogen concentration and pH on rhodotorulic acid production by *R. rubra* SL 29

Experimental matrix			Rhodotorulic acid production with			
Assay	Nitrogen (g/l)	pH	Ammonium sulfate as N source		Urea as N source	
			g/l	g/g biomass	g/l	g/g biomass
1	0.85	3	0.60	0.07	0.65	0.04
2	0.85	8	1.84	0.14	0.81	0.06
3	1.35	8	1.99	0.16	1.80	0.14
4	1.35	3	0.88	0.07	1.21	0.08

The pH was adjusted with 5 N KOH or 5 N H_2SO_4.

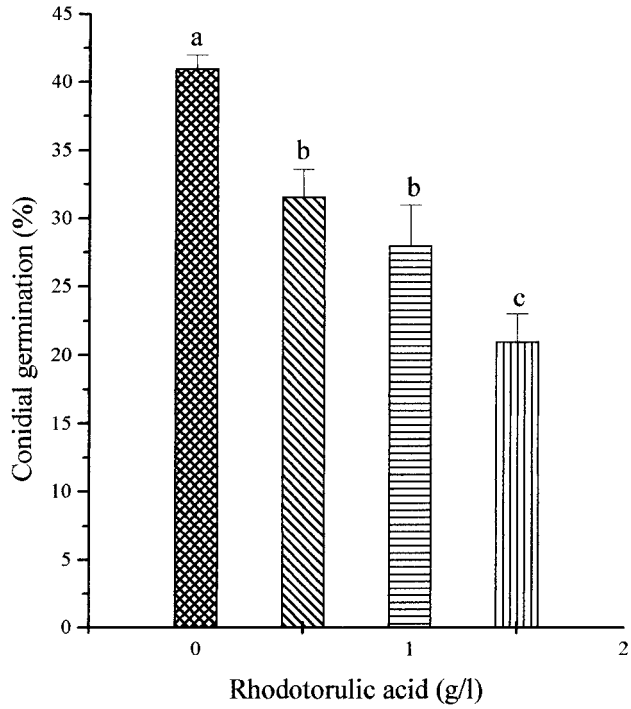

Figure 4 Effect of rhodotorulic acid on germination of *B. cinerea* germination. All values are means of triplicates ± SD. Means designated with the same letter are not significantly different ($p = 0.05$).

in the preceding assay, the interaction between nitrogen–pH showed a positive effect ($F=0.01$). Production of siderophore reached 1.7 and 2 g/l for *R. rubra* and *R. glutinis*, respectively. pH had the least effect on siderophore production whereas a high urea concentration had a positive effect.

Antifungal activity of rhodotorulic acid

Conidial germination and mycelial growth of pathogenic moulds were reduced in the presence of rhodotorulic acid produced by *Rhodotorula* strains. There was a relationship between the siderophore concentration and the inhibition of conidial germination (Figure 4). A reduction of almost 50% in conidial germination of *Botrytis cinerea* with respect to the control was observed when the siderophore concentration was 1.5 g/l. With respect to mycelial growth, siderophore-containing extracts strongly inhibited (50%) the growth of *Cladosporium cladosporoides* and *P. expansum*. *B. cinerea* and *Rhizopus* sp. were also sensitive to spent medium from *Rhodotorula* cultures (Table 2).

Table 2 Antifungal activity of siderophore-containing extract

Phytopathogenic mould	Mycelial growth (g dry wt/l) $\bar{X}$±SD (n)	
	In the presence of siderophore	In the absence of siderophore
P. expansum	0.66±0.15 (3)*	1.4±0.1 (3)
B. cinerea	0.23±0.01 (3)*	0.32±0.02 (3)
C. cladosporoides	0.54±0.1 (3)*	1.07±0.09 (3)
Rhizopus nigricans	1.24±0.08 (3)	1.30±0.15 (3)

*Test results significantly different from control ($p=0.05$).

Discussion

The production of rhodotorulic acid is of interest for biocontrol of postharvest diseases in apples and pears. In a previous work, we demonstrated that it is possible to improve the biological control of blue mold by *R. glutinis* strains when the yeast grows in an iron-deficient medium. Under this condition, the yeast produces rhodotorulic acid, which inhibits conidial germination and mycelial growth of the pathogenic mould. [4].

In the present study, the *P. expansum* controllers *R. glutinis* SL 57 and *R. rubra* SL 29 were used. Each strain produced rhodotorulic acid in iron-deficient medium, but *R. glutinis* was the best producer of the two [3].

Rhodotorulic acid is a hydroxamate-type siderophore and its biosynthetic pathway comes from hydroxylation and acylation of the δ-amino group of the nonproteinogenic amino acid L-ornithine [10].

Biosynthesis of rhodotorulic acid responded to exogenous arginine, ornithine and glutamic acid, as was reported for *Rhodotorula pilimanae* [1]. Rhodotorulic acid biosynthesis was stimulated by ornithine, which is a direct precursor of the siderophore, whereas arginine had no significant effect. The stimulatory effect of urea on siderophore production was higher than that of ornithine. Urea is a metabolite related to the basic amino acids ornithine and arginine; moreover, it was reported that urea was responsible for accumulation of ornithine and glutamic acid when *Candida guilliermondii* was grown on this compound as the sole nitrogen source [9]. Results of experiments carried out with production media containing urea as the sole nitrogen source showed that urea stimulated siderophore production more than an ammonium sulfate–urea mixture, whereas ammonium sulfate in a higher concentration did not have a significant effect.

In brief, three factors seem to be related to enhancement of siderophore production: the presence of urea, the increase in pH and high nitrogen concentrations.

Factorial designs were carried out with the aim of clarifying the role of each factor. As can be inferred from the results, pH near 8 stimulates siderophore production and this effect is more important than the effect of high nitrogen concentration when ammonium sulfate is used as nitrogen source. There is a positive interaction between pH and nitrogen concentration. As the synthesis of glutamic acid, a precursor of rhodotorulic acid, is favored by a pH between 7 and 8 and by an ample supply of a suitable nitrogen source [17], it is possible to infer that the same conditions could stimulate rhodotorulic acid production. Although the stimulatory effect of urea could also be related to an increment in glutamic acid biosynthesis it is not possible to discard the concept that urea *per se* enhances siderophore production. As can be seen in the results of the second factorial experiment, the positive effect of urea was highly significant. Perhaps this compound would favor the accumulation of ornithine as in other yeasts [9].

The siderophore-containing spent medium from *Rhodotorula* strains showed *in vitro* antifungal activity against an important plant pathogen, *B. cinerea*, which causes grey mould on a wide variety of host plants including numerous commercial crops [6]. Experiments testing inhibition of conidial germination was performed with *B. cinerea* because this mould is extremely difficult to control. In addition, the ability of this pathogen to rapidly develop resistance to a wide variety of fungicides [6] has highlighted the increasing need for alternative forms of control including biological control.

Extracts containing siderophore alone or combined with antagonist microorganisms would be an interesting alternative for control of postharvest diseases including the grey mould pathogen *B. cinerea*.

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References

1 Akers H, M Llinás and JB Neilands. 1972. Protonated amino acid precursor studies on rhodotorulic acid biosynthesis in deuterium oxide media. *Biochemistry* 12: 2283–2291.
2 Atkin C, J Neilands and H Phaff. 1970. Rhodotorulic acid from species of *Leucosporidium*, *Rhodosporidium*, *Rhodotorula*, *Sporodiobolus*, and *Sporobolomyces* and a new alanine-containing ferrichrome from *Criptcoccus melibiosum*. *J Bacteriol* 103: 722–733.
3 Calvente V, D Benuzzi, N Obuchowicz, G Hough and MI Sanz de Tosetti. 1999. Changes in surface microflora of apple and pear fruits by application of pesticides and their relation with bicontrol of post-harvest diseases. *Agro-Food-Industry Hi-Tech* 10: 30–33.
4 Calvente V, D Benuzzi and MI Sanz de Tosetti. 1999. Antagonistic action of siderophores from *Rhodotorula glutinis* upon the post-harvest pathogen *Penicillium expansum*. *Int Biodeterior Biodegrad* 43: 167–172.
5 Duffy B and G Défago. 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Appl Environ Microbiol* 65: 2429–2438.
6 Jarvis WR. 1988. Taxonomy. In: Coley-Smith J, K Verhoeff and W Jarvis (Eds), The Biology of *Botrytis*, Academic Press, London, pp. 1–18.
7 Lemanceau P, P Bakker, W Jan de Kogel, C Alabouvette and B Schippers. 1993. Antagonistic effect of nonpathogenic *Fusarium oxysporum* Fo47 and Pseudobactin 358 upon pathogenic *Fusarium oxysporum* f. sp. *Dianthi*. *Appl Environ Microbiol* 59: 74–81.
8 Loper J. 1990. Molecular and biochemical bases for activities of biological control agents: the role of siderophores. In: Liss AR (Ed), New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases. NY, pp. 735–747.
9 Makarova E and D Grigoryan. 1975. Effect of urea and amino acids on the ornithine cycle on the biomass accumulation and amino acids synthesis by *Candida guilliermondii*. *Prikl Biokhim Mikrobiol* 11: 322–325.
10 Neilands JB. 1981. Microbial iron compounds. *Annu Rev Biochem* 50: 715–731.
11 Neilands JB. 1989. Siderophore systems of bacteria and fungi. In: Beveridge TJ and RJ Doyle (Eds), Metals Ions and Bacteria. Wiley, New Jersey, pp. 141–164.
12 Neilands JB. 1993. Siderophores. *Arch Biochem Biophys* 302: 1–3.
13 Payne S. 1994. Detection, isolation and characterization of siderophores. *Methods Enzymol* 235: 329–344.
14 Raaska L and T Mattila-Sandholm. 1995. Effects of iron level on the antagonistic action of siderophores from non-pathogenic *Staphylococcus* spp. *J Ind Microbiol* 15: 480–485.
15 Riquelme M. 1996. Fungal siderophores in plant–microbe interactions. *Microbiol SEM* 12: 537–546.
16 Smith G. 1963. Equipo y Técnicas de laboratorio. In: Introducción a la Micología Industrial (Ed), Acribia, Zaragoza, pp. 303–305.
17 Vandercasteele JP, J Lemal and M Coudert. 1975. Pathways and regulation of glutamate synthesis in *Corynebacterium* sp. overproducing glutamate. *J Gen Microbiol* 90: 178–180.