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SHORT COMMUNICATION

Use of cheese whey to enhance *Geotrichum candidum* biomass production in olive mill wastewater

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Abstract *Geotrichum candidum* is a yeast-like filamentous fungus that has attracted industrial interest. The present work investigated *G. candidum* biomass production in agro-industrial wastewaters (olive mill wastewater (OMW) and cheese whey (CW)) as the only substrate. Different solid media (Sabouraud dextrose agar (SDA), CW, OMW, and OMW/CW mixtures in different proportions) were tested. OMW/CW mixtures proved to be suitable for optimal mycelia growth of *G. candidum* with a very high hyphae density. The highest fungal and expansion rate growth of 83 ± 1 mm and 12.4 day^{-1}, respectively, were obtained on a 20:80 mixture of OMW/CW, which was incubated for 7 days. This optimal mixture was used to study the biomass production and the OMW decolorization ability of *G. candidum* in the presence of CW in liquid medium. Liquid cultures were also conducted in OMW and CW separately. After 5 days of incubation, fungal biomass reached 9.26 g l^{-1} in the OMW/CW mixture and only 2.83 g l^{-1} in CW, while no biomass production was observed in OMW alone. OMW decolorization and dephenolization by *G. candidum* also improved in the presence of CW with a decolorization efficiency of 54.5% and a total phenolic reduction of 55.3%, compared with the control which yielded values of about 10% and 15%, respectively. These results suggested that OMW/CW—as the only substrate—could be used as a cost-effective medium to produce *G. candidum* biomass, without the need for water dilution or supplementation with other nutriments.

Keywords Olive mill wastewater (OMW) · Cheese whey (CW) · *Geotrichum candidum* biomass production · Decolorization · Valorization

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Introduction

Food processing industries generate large amounts of liquids by-products, such as olive mill wastewaters (OMW), cheese whey (CW), molasses, and abattoir wastewaters. Only small amounts of these by-products are valorized and recycled into useful products such as food ingredients and animal food. Because of the high cost of some valorization processes, liquid by-products of agro-industries are generally rejected as effluents. Indeed, they cause a great environmental problem because of their large volumes and high organic load values [12, 14]. Furthermore, they are characterized by the presence of biodegradable as well as recalcitrant and biostatic compounds, such as phytotoxic and antibacterial phenolic substances present in OMW [20].

Several methods have been proposed to reduce the ecological impact of these wastes. A better approach would be to view the waste as a possible source to be revalorized by using low-cost processes. Agro-industrial waters have been considered as a potential basis for fermentative production processes [9–11, 14, 22, 24], thanks to the presence of organic matter, such as proteins and simple and complex sugars. Moreover, they could be used as a cheaper water source in fermentation processes instead of pure water [21] that is scarce in the majority of countries.

The imperfect fungus *G. candidum* has long been used as a "starter culture" in cheese manufacturing, due to its

involvement in cheese ripening [8]. Currently, this fungus is used to pretreat industrial wastewaters, such as in the decolorization of crude molasses [16], various dyes [17], and OMW [4, 5, 12]. Decolorization ability of *G. candidum* is essentially attributed to ligninolytic enzymes, including lignin peroxidase (Lip) and manganese-dependent peroxidase (Mnp) [5, 17]. *G. candidum* is also stable in a continuous bioreactor, in comparison with other fungi. Indeed, it is not very sensitive to shear stress because it simultaneously produces mycelium and arthroconidia during growth [4, 5].

Production of fungal biomass that could be used as a starter for biotechnological applications is a promising approach for valorization and bioremediation of various wastes [9, 10]. In the present study and for the first time, to the best of our knowledge, *G. candidum* biomass production was investigated in agro-industrial wastewaters (OMW, CW) as the only substrate. In addition, OMW decolorization ability of *G. candidum* was tested in the presence of CW.

Table 1 Main characteristics of OMW and CW used

Parameters	Fresh OMW	CW
pH[a]	5.2	6.3
Conductivity (ms cm^{-2})	14.3	7.9
TS (g l^{-1})	103.9 ± 1.2	57.5 ± 2.5
TSS (g l^{-1})	3.2 ± 0.5	0.5 ± 0.1
Total ash (g l^{-1})	49.9 ± 1.5	5.0 ± 1.7
Soluble COD (g l^{-1})	148 ± 19.5	60.7 ± 12.0
TKN (g l^{-1})	0.75 ± 0.09	1.61 ± 0.15
P (g l^{-1})	4.78	10.27
P×100/TS (%)	4.60	17.86
Soluble COD/TKN	50/0.25	50/1.32
Reducing sugars (g l^{-1})	51 ± 3.1	1.8 ± 0.51
TP (mg eq gallic acid l^{-1})	1,170 ± 21	ND

Data are reported as mean ± standard deviation of results carried out in triplicate

TS total solids, i.e., dry matter; *TSS* total suspended solids; *Soluble COD* soluble chemical oxygen demand; *TKN* total kjeldahl nitrogen; *P* protein; *TP* total phenolic; *ND* not determined

[a] pH values measured before adjustment and testing

Materials and methods

OMW and CW samples

Two agro-industrial wastewaters (OMW and CW) were used in the present study. OMW, a liquid by-product generated by the olive oil extraction industry, is composed of vegetation water of olives plus washing and processing waters and oil in the form of a very stable emulsion. CW, a liquid by-product of cheese production, retains 55% of milk nutrient (lactose, protein, organic acids, minerals, and vitamins).

OMW and CW were obtained from local factories and stored at −20°C. They were centrifuged at 4,000*g* for 15 min to eliminate suspended solids prior to use. The main characteristics of both wastewaters are given in Table 1.

Geotrichum candidum strain

The fungus *G. candidum* used in this work was isolated from an aerated pilot-scale bubble column fed continuously with OMW [4]. The culture was maintained on SDA at 4°C.

Culture conditions of *G. candidum* on solid media

Solid culture experiments were conducted by using petri dishes (90 mm in diameter, 25 ml medium/petri dish) to examine the capability of *G. candidum* to grow on agro-industrial wastewaters (OMW, CW) as the only substrate. Tested media were adjusted to pH 6 by using NaOH or HCl, added with agar (20 g l^{-1}), and sterilized by autoclaving at 120°C for 20 min. The growth of the fungus on a standard mycological medium SDA was used as a positive control for comparison purposes.

A culture on SDA was prepared for 1 week at 30°C. Then, spores from this culture were suspended in sterile distilled water (containing agar 5 g l^{-1}) at a concentration of 10^6 arthroconidia/ml.

The experimental plates were run in triplicate and they were inoculated at the center with 10 µl of the arthroconidia suspension, and then incubated at 30°C for 7 days.

The fungal growth was monitored by daily measurement of the diameters of the colonies (in mm). Mycelia growth was expressed as expansion rate of colony (mm day^{-1}), i.e., the slope of growth curve (colony diameter = f (time)). Hyphal densities on different solid cultures were observed with the naked eye at the 7th day of the experiment.

Liquid cultures of *G. candidum*

Liquid cultures were conducted to examine more parameters, such as pH, color (optical density at 390 nm, $OD_{390\ nm}$), total phenolic content, Lip and Mnp activities in addition to biomass production, during the growth of *G. candidum* in three different media (CW, OMW, and a 20:80 mixture of OMW/CW). All media were adjusted to pH 6 by using NaOH or HCl and sterilized by autoclaving

at 120°C for 10 min. Experiments were carried out in 250-ml Erlenmeyer flasks containing 100 ml of the appropriate medium. After inoculation with *G. candidum* arthroconidia suspension at an initial concentration of 10^5 arthroconidia/ml, cultures were placed in a rotary shaker at 200 rpm and 30°C for 5 days. Experiments were performed in triplicate and a control culture, without fungi, was also conducted. Measurements were taken daily.

Analytical methods

The biomass content of culture samples was separated from the liquid phase by centrifugation at 6,000*g* for 30 min, dried at 105°C, and weighed by using a highly precise balance. Chemical analyses were carried out in triplicate according to standard methods [1].

Determination of color and total phenolic content

Supernatants obtained by centrifugation of culture broth media at 6,000*g* for 30 min were used to determine in triplicate the color (OD at λ_{max} 390 nm after adjusting pH to 6) and total phenolic content as described previously [23].

Analyses of enzymes

Supernatants obtained by centrifugation of culture broth media at 6,000*g* for 30 min were also analyzed to determine lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) activities according to a published method [15].

Statistical analysis

To evaluate the impact of media composition on *G. candidum*'s growth on a solid culture, ANOVA analyses were conducted by using Statistica software version 6.0 (StatSoft) for Windows. *P* values were determined for two parameters (colony diameter at the 7th day and expansion rate of colony), for all data, for each proportion and different proportions. Effects were considered significant when the *P* value was less than 0.05.

Results

Geotrichum candidum growth on solid media

In a preliminary experiment, solid cultures were conducted to investigate the ability of *G. candidum* to grow on an undiluted fresh raw OMW without any addition of nutriments or dilution. The starting radius of the mycelium was 8 mm. The growth of the fungus using a standard mycological medium SDA was meant to serve as a control for comparison. Results on OMW showed an inhibition of the mycelium growth, which may be due to the elevated phenolic content of 1,170 mg eq gallic acid l^{-1} (Table 1). This probably exerted an inhibitory effect on the fungi. However, *G. candidum* was able to grow on a diluted OMW (Table 2). Moreover, the mycelium growth (colony diameter at the 7th day and the expansion rate of colony) was extensively improved with dilution (significant difference at $P < 0.05$ proved by ANOVA analysis). At the 7th day of growth, the colony diameter and expansion rate for OMW/W 10:90 had doubled to values of 63 mm and

Table 2 Effect of CW addition on the *G. candidum* growth for 7 days on solid medium containing fresh OMW

Media growth	Colony diameter at the 7th day (mm)	Expansion rate of colony (mm day^{-1})	Hyphae density[a] at the 7th day
Sabouraud dextrose agar	85 ± 3	13.9 ± 0.5	Medium
CW	85 ± 1	9.9 ± 0.7	Low
OMW	8 ± 1	0	No hyphae growth
OMW/W 50:50	28 ± 2	4.7 ± 0.4	Low
OMW/W 20:80	40 ± 3	5.8 ± 0.5	Low
OMW/W 10:90	63 ± 2	10.2 ± 1.0	Low
OMW/CW 50:50	57 ± 0	7.8 ± 0.3	Very high
OMW/CW 20:80	83 ± 1	12.4 ± 0.0	Very high
OMW/CW 10:90	80 ± 2	11.8 ± 0.2	Very high

Initial pH = 6; the starting radius of the mycelium was 8 mm. The colony diameters and the expansion rates of colonies are reported as the mean ± standard deviation of results carried out on three samples

CW cheese whey, *OMW* olive mill wastewater, *W* water

[a] Hyphal density is the extent of hyphal branching, i.e., the density of a fungal colony (number of hyphal branches formed per unit area). It is directly related to the concentration of nutrients in growth medium: a sparsely branched colony (low hyphal density) will develop on a nutritionally weak growth medium; a densely branched colony will develop on a nutritionally rich growth medium

10.2 mm day^{-1}, compared to values for OMW/W 50:50, which were just 28 mm and 4.7 mm day^{-1}, respectively. However, the hyphae density remained low.

As shown in Table 2, the growth of the fungus on the optimal diluted OMW or on CW as the only substrate was lower than that on the SDA.

Solid cultures of *G. candidum* on OMW/CW mixtures were carried out for three proportions (50:50, 20:80, 10:90). Results in Table 2 showed an improvement of fungal growth on all OMW/CW mixtures compared with those on all media previously investigated in this study including the synthetic control. ANOVA analysis proved a significant difference (at $P < 0.05$) for the two quantitative parameters (colony diameter at the 7th day and the expansion rate of colony) between all media. A remarkable enhancement was observed for the density of hyphae. The growing mycelium had a very high rate of hyphae density, with minor differences for the various proportions.

SDA showed the largest colony diameter (85 mm) and colony expansion rate (13.9 day^{-1}). However, when hyphae density was taken into consideration, OMW/CW mixtures showed the highest level of improvement in fungal growth (even when compared to the SDA). The highest fungal diameter and expansion rate of 83 mm and 12.4 day^{-1}, respectively, were obtained on OMW/CW 20:80 after 7 days of growth.

OMW decolorization ability of *G. candidum* in the presence of CW

The optimal OMW/CW proportion of 20:80 was used to study the biomass production and the OMW decolorization ability of *G. candidum* in the presence of CW in liquid medium under stirred conditions. The fermentation of diluted OMW alone was taken as control. The monitored biomass, pH, color (residual $OD_{390\ nm}$), residual total phenolic, and LiP and MnP activities are shown in Figs. 1 and 2.

After 5 days of incubation, fungal biomass reached 9.26 g l^{-1} in OMW/CW and just 2.83 g l^{-1} in CW, while no biomass production was observed in OMW (Fig. 1). The highest rate of biomass in CW was observed at the 4th day, although it is still low in comparison with the produced biomass in the OMW/CW mixture.

These results confirm the findings shown previously on solid cultures. A minor difference (about 8%) between the maximum specific growth rates in the OMW/CW mixture ($\mu max = 2.88$ day^{-1}) and in CW ($\mu max = 3.12$ day^{-1}) was noted. It has been observed that the growth of *G. candidum* in media containing CW was accompanied by an increase of pH up to 9, probably due to the release of ammonia during the deamination of amino acids. The latter can be the result of casein degradation in CW [8].

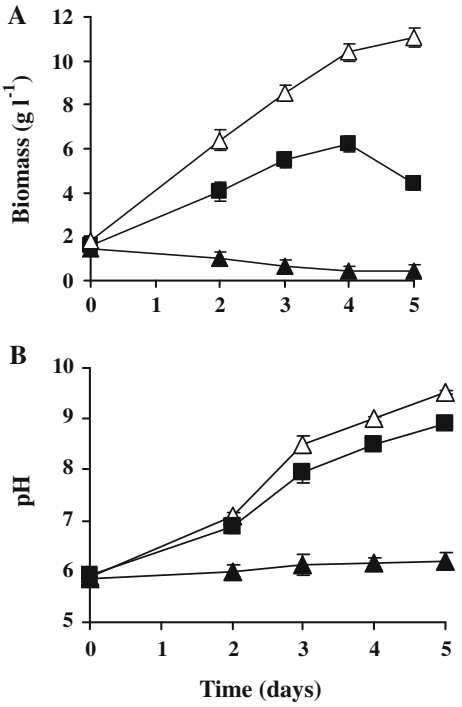

Fig. 1 Time course of biomass (**a**) and pH (**b**) during the growth of *G. candidum* under stirred conditions in CW (*filled squares*), in fresh diluted OMW at a proportion of OMW/W 20:80 (*filled triangles*) and in a 20:80 mixture of OMW/CW (*open triangles*). *CW* cheese whey, *OMW* olive mill wastewater, *W* water. Data are reported as the averages of results carried out in triplicate and *error bars* indicate standard deviations

Mycelium and ramification allow the hydrolysis and adsorption of polyphenols, and then decolorization of OMW. Since the OMW/CW mixture was suitable for mycelium growth, the decolorization of OMW was enhanced to 54.5%, compared with the control which yielded a corresponding value of about 10% after 5 days of culture. The same result was observed with phenolic reduction which is improved from 15 to 55.3% in the presence of CW (Fig. 2).

Ligninolytic enzymes may be responsible for the decolorization and the total phenolic removal of OMW. Their activities were detected after 2 days of culture. In the presence of CW, lignin peroxidase (LiP) activity and manganese-dependent peroxidase (MnP) activity reached about 104 and 97 IU l^{-1}, respectively.

Discussion

According to the present study, preliminary experiments on solid cultures demonstrated that *G. candidum* is unable to grow on an undiluted fresh raw OMW without any addition of nutriments or dilution. This is probably owing to the elevated phenolic content, which can exert an

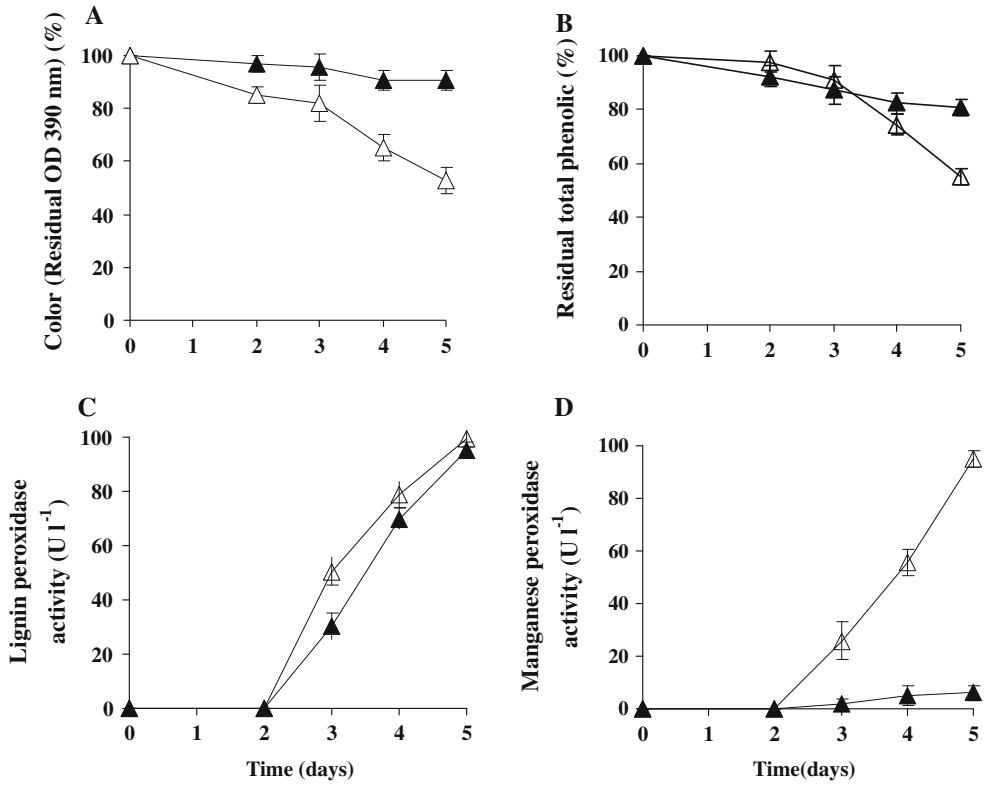

Fig. 2 Time course of color (residual $OD_{390\ nm}$) (**a**), residual total phenolic (**b**), lignin peroxidase (**c**), and manganese-dependent peroxidase (**d**) activities during the growth of *G. candidum* under stirred conditions in fresh diluted OMW at a proportion of OMW/W 20:80 (*filled squares*) and in a 20:80 mixture of OMW/CW (*open squares*). *CW* cheese whey, *OMW* olive mill wastewater, *W* water. Data are reported as the averages of results carried out in triplicate and *error bars* indicate standard deviations

inhibitory effect on the fungi [12, 19]. Indeed, the *G. candidum* growth and the decolorization proved possible in diluted OMW supplemented with nitrogen source (such as ammonium sulfate, ammonium nitrate) as investigated by Asses et al. [5, 6] and by Fadil et al. [12]. Many other fermentative processes [9, 10, 21] are carried out in diluted OMW supplemented with nutriments. Nevertheless, the conventional dilution with water required in some processes, including the present case, leads to a high cost, especially as it is incompatible with the scarce water resources in most Mediterranean countries that produce OMW.

In the present work, CW was chosen as an inexpensive raw material that can dilute OMW and, at the same time, supply missing nutriments. In fact, CW contains a high content of protein (about 17.86% of dry matter) which counterbalanced the low levels of nitrogen in OMW (4.60%) and therefore helped to optimize the C/N ratio. Another reason for choosing this raw material is the overlapping of its overproduction period with OMW's (winter–spring).

Various recent studies investigated biomass production in CW or OMW for use as starter cultures. Aguirre-Ezkauriatza et al. [3] examined the technical feasibility of producing high added value probiotic *Lactobacillus casei* biomass from deproteinized and non-supplemented milk whey. They recommended high cell density fed-batch strategies for commercial production of *L. casei* biomass. Similarly, the production of novel dairy starter cultures (specifically, *Kluyveromyces marxianus, Lactobacillus bulgaricus*, and kefir yeasts) was carried out in CW as a raw material [18]. Furthermore, Altieri et al. [2] evaluated the feasibility of using OMW as an ingredient for commercial cultivation of a value-added mushroom *Agaricus bisporus* (Lange) Sing.

The cofermentation of OMW with raw agro-industrial materials was investigated in previous works. In a recent study, it was shown that decolorization and the total phenolic reduction of OMW by lactic bacteria *Lactobacillus paracasei* was improved by CW addition [7]. Martinez-Garcia et al. [19] also used CW as a co-substrate for the aerobic pretreatment of OMW by the yeast *Candida tropicalis* followed by anaerobic co-digestion. Anaerobic co-digestion of OMW has also been investigated with abattoir wastewaters [13] leading to a simultaneous enhancement of biogas production and bioremediation of effluents.

Findings in the present work have highlighted a remarkable stimulating effect of OMW on the production

of *G. candidum* biomass in CW. In fact, biomass level produced in CW increased when OMW was added. In a similar vein, OMW-based media have been proved to exert a noteworthy stimulating effect on the production of citric acid by *Yarrowia lipolytica*. Both final citric acid concentration and the conversion yield of citric acid produced per unit of sugar consumed were higher when compared with the respective parameters obtained from trials without added OMW [21].

Conclusion

Results in the present work suggest that OMW/CW—as the only substrate—can be used as an optimal cost-effective medium to produce *G. candidum* biomass, without the need for water dilution or supplementation with other nutriments. OMW decolorization and dephenolization are associated with the biomass production.

This process leads to a pretreated effluent in addition to biomass. Further experiments will follow in order to test the possibility of using the biomass produced in OMW/CW as an adapted starter for treatment of raw-fresh and stored OMW. Anaerobic co-digestion of the pretreated effluent also should be examined for studying the opportunity of improving bioremediation and biogas production.

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