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Isolation of Carvacrol Assimilating Microorganisms

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Dedicated to the memory of Professor Vera Johanides

Summary

Several bacteria and fungi capable of assimilating carvacrol were isolated from the herbs oregano, thyme and savory, and pine tree (resin, bark and needles). When cultivated in a liquid medium with carvacrol, as a sole carbon source, the bacterial isolates from savory and pine consumed the carvacrol in the range of 19–22 % within five days of cultivation. The fungal isolates grew much slower and after 13 days of cultivation consumed 7.1–11.4 % carvacrol.

Pure strains belonging to the bacterial genera of *Bacterium*, *Bacillus* and *Pseudomonas*, as well as fungal strain from *Aspergillus*, *Botrytis* and *Geotrichum* genera were also tested for their ability to grow in medium containing carvacrol. Among them, only in *Bacterium* sp. and *Pseudomonas* sp. carvacrol uptake was monitored. Both *Pseudomonas* sp. 104 and 107 consumed the substrate in the amount of 19 %. These two strains also exhibited the highest cell mass yield and the highest productivity (1.1 and 1.2 g/L per day).

Key words: selection, carvacrol, bacterial isolates, fungal isolates

Introduction

Carvacrol (2-*p*-cymenol or 5-isopropyl-2-methylphenol) is one of the main components of the essential oils of some *Labiatae (Laminaceae)* members like oregano, thyme and savory, the content of which can reach up to 86 % (*1*–*3*). The volatile oils of some conifers also contain carvacrol (*4*). It has been indicated that the antioxidant activity of the essential oils of the above-mentioned herbs is due to the carvacrol, its isomer thymol and some other phenols (*1*,*5*). They possess antibacterial activity and therefore find application in treating oral diseases (*6*). Their antifungal activity is used against phytopathogenic fungi (*7*). Additionally, these essential oils exhibit analgesic activity which is also related to the carvacrol content (*8*).

Despite the powerful antimicrobial characteristics of the oils, Chamberlain and Dagley (*9*) found a *Pseudomonas* strain able to degrade thymol completely and carvacrol only partially. The authors proposed a metabolic pathway of thymol that involves *meta*-ring opening of a trihydric phenol, 3-hydroxythymo-1,4-quinol to 3,7-di-methyl-2,4,6-trioxo-octanoate. The hydrolysis of the latter, catalyzed by β-ketolase, yields acetate, 2-ketobutyrate and isobutyrate. β-ketolases encompass ten known enzymes that hydrolyze carbon-carbon bonds of β-diketo compounds. They are frequently encountered in microbial, plant and animal cells (*10*). Although the difference between thymol and carvacrol is only in the position of the OH-group (Fig. 1), no description of microbial degradation pathways of carvacrol has been published so far.

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Here we report on the isolation of microorganisms capable of assimilating carvacrol as a sole carbon source. In addition, we tested a few microorganisms that already grew on phenolic compounds and some other microorganisms, for which we expected to exhibit ability of growing on carvacrol. This report should be looked upon as a first step of our long-term study on microbial metabolism of carvacrol with emphasis on β-ketolases.

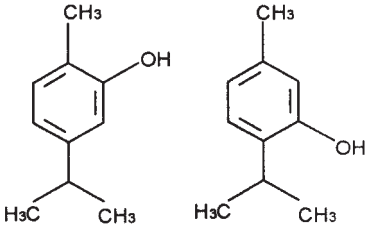

Fig. 1. The molecules of carvacrol (a) and thymol (b)

Material and Methods

Microorganisms

Pure bacterial cultures of *Pseudomonas* sp. 104, *Pseudomonas* sp. 107, *Bacterium* sp. 101 and *Bacillus* sp. 102 that grow on phenolic compounds were kindly supplied by Professor J. Ziberovski from the Faculty of Agriculture in Skopje. The cultures were maintained on peptone agar slants containing carvacrol, at 4 °C.

The fungi used in this study, *Aspergillus niger* ATCC 60363, *A. niger* MK-15, *A. niger* DSM 821, *Botrytis cinerea* Be-4 and *Geotrichum candidum* M-2, are taken from the Culture collection of the Faculty of Technology and Metallurgy in Skopje. They were maintained on PDA slants at 4 °C.

Materials

For isolation of bacteria capable of assimilating carvacrol, the following plant materials were used: savory, thyme and oregano together with the soil on which they grew, then, resin, needles and a bark from a pine tree and the soil under the tree. Leaves of savory, thyme and oregano, as well as pine needles, were taken for the isolation of fungi.

Isolation of microorganisms

The bacteria were isolated using enrichment culture technique in a mineral medium, suggested by Prof. D.W. Ribbons, with the following composition (g/L): KH_2PO_4 6.8, NaOH 1.4, $(NH_4)_2SO_4$ 0.26, $MgSO_4 \cdot 7H_2O$ 0.24, $FeSO_4 \cdot 7H_2O$ 0.08, and carvacrol 0.3, as a sole carbon source. Since the carvacrol is volatile, its stock solution was filter sterilized and added to the cooled bulk solution. The isolation of cultures was performed on agar plates with the above-mentioned medium, and on rich medium plates containing (g/L): peptone 10, yeast extract 5, NaCl 5, agar 18 and carvacrol 0.3. All cultures were maintained on the latter medium supplemented with carvacrol.

The fungi were isolated on a selective PDA-medium containing (g/L): potatoes 300, glucose 10, carvacrol 0.3 and agar 15. Further isolation and selection was performed on the same medium without glucose and on a peptone-agar medium, containing (g/L): peptone 10, agar 15 and carvacrol 0.3. The isolation of the cultures, in all experiments, was performed at 30 °C. The isolates were maintained on PDA supplemented with carvacrol.

Cultivation conditions

The ability of the isolated bacteria to assimilate carvacrol was tested in liquid culture in 100-mL shake flasks with 20 mL of the liquid mineral medium. The medium was inoculated with 24-h old culture grown on agar and extracted with 5 mL sterile water. Temperature of cultivation was 30 °C.

The pure bacterial cultures were grown under the same conditions as the bacterial isolates in this study.

The isolated fungi were cultivated in 500-mL shake flasks with 100 mL of liquid peptone medium and in mineral Czapek-medium containing (g/L): $NaNO_3$ 6, K_2HPO_4 2, $MgSO_4 \cdot 7H_2O$ 1, KCl 1, $FeSO_4 \cdot 7H_2O$ 0.02 and carvacrol 0.3.

The pure fungi were cultivated under the same conditions as the fungi isolated in this study. The medium was inoculated with spores at 5 % (by volume). Temperature of the cultivation was 30 °C.

Analytical methods

Cell growth was determined gravimetrically by drying the separated cell mass at 105 °C. Reversed phase HPLC with UV detection (270 nm) was used for measuring carvacrol concentration on a LiChrospher RP-18 column (Merck), with acetonitrile/water (volume ratio 60/40) as a mobile phase and a flow rate of 1 mL/min at room temperature.

Results and Discussion

From numerous bacterial isolates derived from plant and soil materials six isolates exhibited better growth on the selective media. Of the fungal isolates we present only two, originating from oregano and pine needles. No fungal isolates could be obtained from thyme and savory. Perhaps this is due to the higher carvacrol content in these herbs (*11*), and the strong fungicidal properties of the carvacrol (2,7).

Morphological characteristics of the isolated cultures are given in Table 1. All bacterial isolates grew in a similar way forming white and yellow smooth colonies. The isolates from pine parts and oregano were distinguished with ellipsoidal cells, while those isolated from thyme and savory had spherical shape. The fungal isolates showed different growth pattern; the oregano isolate, after being adapted to the medium, covered the whole surface of the agar plate with sporangia springing from the surface of the mycelium. The pine needles isolate formed white cotton-like round colonies with sporangia stemming from the centre of the colonies. The colonies had different size with up to 3 cm in diameter.

The isolation of the bacterial cultures, as well as their further selection, was mostly performed in the mineral

Table 1. Morphological characteristics of the isolated microorganisms

Name of the isolate	Plant source	Microscopic characteristics	Visual characteristics
Bacteria			
$1B_{CA}$	Thyme	Spherical cells	White
$2\ B_{CA}$	Oregano	Ellipsoidal cells	White
$3\ B_{CA}$	Resin from pine	Ellipsoidal cells	White
$5\ B_{CA}$	Pine needles	Ellipsoidal cells	White
$7\ B_{CA}$	Pine bark	Ellipsoidal cells	White
$8\ B_{CA}$	Savory	Spherical cells	Yellow
Fungi			
$1F_{CA}$	Oregano	Ascospores	White mycelium, black spores
$2F_{CA}$	Pine needles	Conidiospores	White mycelium, brown spores

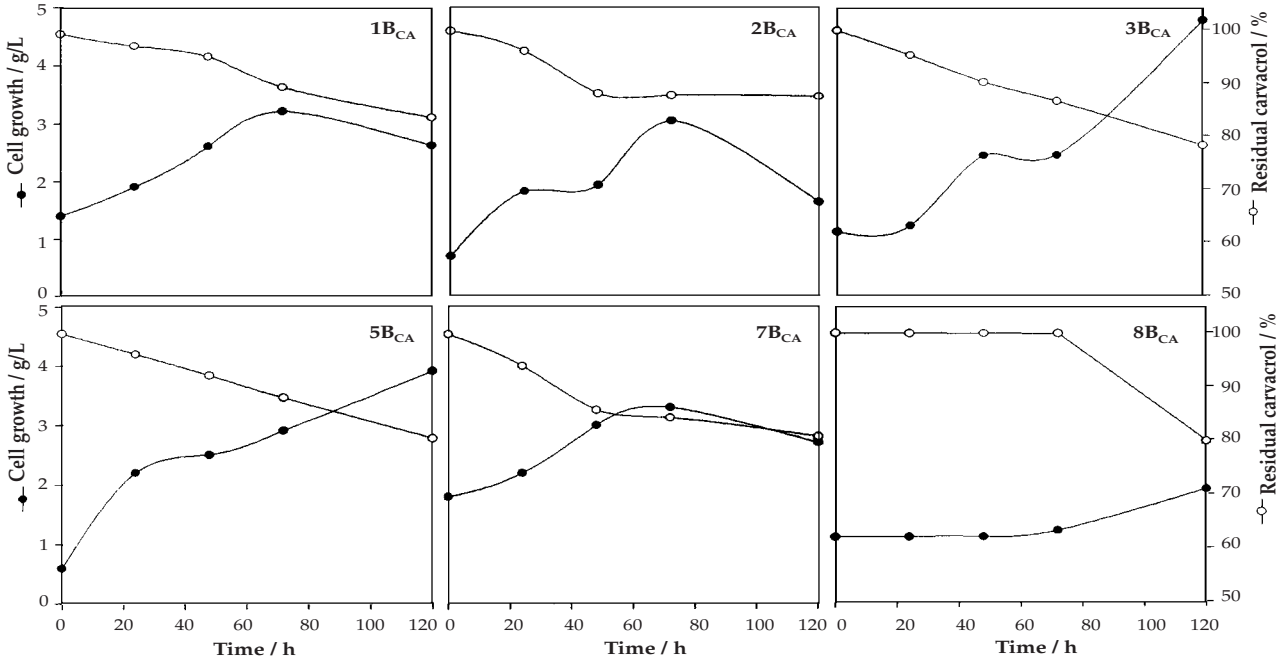

Fig. 2. Dynamics of growth (●–●) and substrate consumption (○–○) of the isolated bacteria cultivated in a liquid mineral medium The names of the isolates correspond to those listed in Table 1

(minimal) medium. The idea behind it was to impose the conditions that would be favourable only for bacteria able to consume carvacrol. Furthermore, the concentration of the carvacrol employed in this medium (0.3 g/L) was the highest one that bacteria could tolerate (personal communication with Prof. D. W. Ribbons).

The growth of the six bacterial isolates is shown in Fig. 2. All cultures, apart from the $8B_{CA}$, grew till the third day of cultivation, demonstrating the average cell mass productivity of up to 0.76 g/L per day. The isolates $3B_{CA}$ and $5B_{CA}$ grew further till the fifth day. The isolate $8B_{CA}$ manifested a 3-day long lag phase. After five days of cultivation the isolated bacteria consumed 12.5 to 22.0 % of the total amount of the carvacrol present in the medium (Fig. 3). The highest amount of carvacrol consumption was observed in $3B_{CA}$, followed by the isolate $8B_{CA}$ with 20 %. The cultures $5B_{CA}$ and $7B_{CA}$ showed similar consumption of about 19 %. Although the isolates from thyme ($1B_{CA}$) and oregano ($2B_{CA}$) grew well on agar plates, in liquid medium, they were somehow less active in carvacrol consumption.

Three of the pure bacterial cultures, cultivated under the same conditions as the isolates, consumed the carvacrol (Fig. 3). *Bacillus* sp. 102 neither grew nor consumed it. The carvacrol consumption by both *Pseudomonas* sp. 104 and 107 of about 19 % was less than that consumed by the isolate $3B_{CA}$ (22 %). However, the highest accumulation of the cell mass, 3.4–3.7 g/L, as well as the highest productivity, 1.1–1.2 g/L per day, for the first three days, were observed exactly in these two bacteria (Fig. 4). Since these bacteria were already adapted for growing on phenolic compounds, it was expected that they would be able to consume carvacrol to a greater extent than the bacterial isolates.

Dangel *et al.* (*12*) while working with the denitrifying *Pseudomonas* sp. found that it depleted cyclohexanol (1.5 mM or 0.15 g/L) anaerobically within 48 hours. The same culture degraded 1mM phenol anaerobically with-

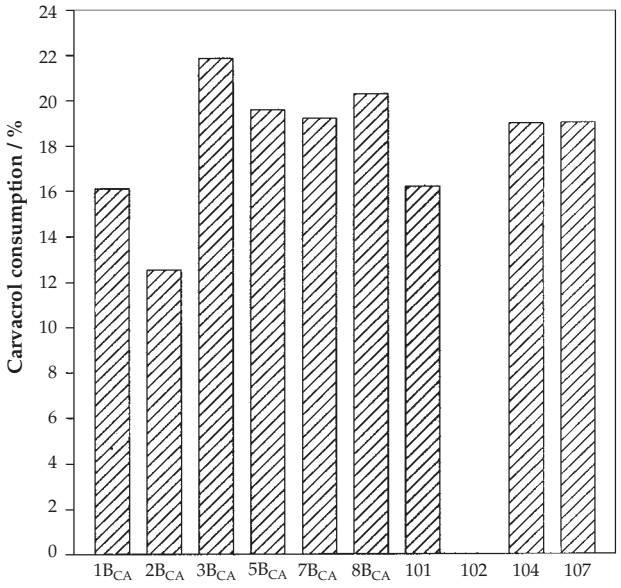

Fig. 3. Carvacrol consumption after 5 days of cultivation by the isolates 1B$_{CA}$ – 8B$_{CA}$ and pure cultures of *Bacterium* sp. 101, *Bacillus* sp. 102, *Pseudomonas* sp. 104 and 107

in the same time, having doubled time of about 20 hours and no exponential phase due to the toxicity of the phenol (*13*). Our *Pseudomonas* sp. 104 displayed the shortest doubling time of 14 h among all tested bacteria. When investigating thymol metabolism by *Pseudomonas*, Chamberlain and Dagley (*9*) cultivated the bacteria in a mineral medium with 0.3 g/L (2 mM) thymol and it was converted into 3-hydroxythymo-1,4-quinone within the first 24 hours. *Pseudomonas putida* exhausted orcinol (3,5-dihydrotoluol) in concentration of 0.5 g/L, also after 24 h of cultivation (*14,15*). On the other hand, for the same period, *Pseudomonas* sp. 104 and 107 consumed, respectively, only 3.6 and 7.6 % of the carvacrol. All cultures investigated in this work demonstrated lower carvacrol consumption within the time of cultivation.

Our quest for fungi capable of assimilating carvacrol resulted in only two isolates. During 13 days of cultivation, the isolate from oregano consumed 11.4 % and that from the pine needles assimilated 7.1 % of the initial carvacrol amount (Fig. 5). Both fungi accumulated negligible amount of biomass (data not shown). The fungal isolates were also cultivated in mineral Czapek medium. In this medium, as can be seen from Fig. 5, they consumed the carvacrol to a lesser extent. The oregano isolate consumed 68 % while the pine isolate 74 % of the carvacrol, compared to the uptake in the peptone medium.

Of the pure cultures, only *Aspergillus niger* ATCC 60363 grew on a potato agar medium with 0.3 g/L carvacrol, although slower than the isolates. However, when cultivated in a liquid peptone medium with 0.3 g/L carvacrol, the fungus did not germinate and no carvacrol consumption was observed even during 13 days of cultivation.

In our further experiments we will focus, firstly, on identification of the best carvacrol consumers among the isolates, and secondly, on improvement of the properties of the selected microorganisms through optimization of the medium composition and environmental conditions.

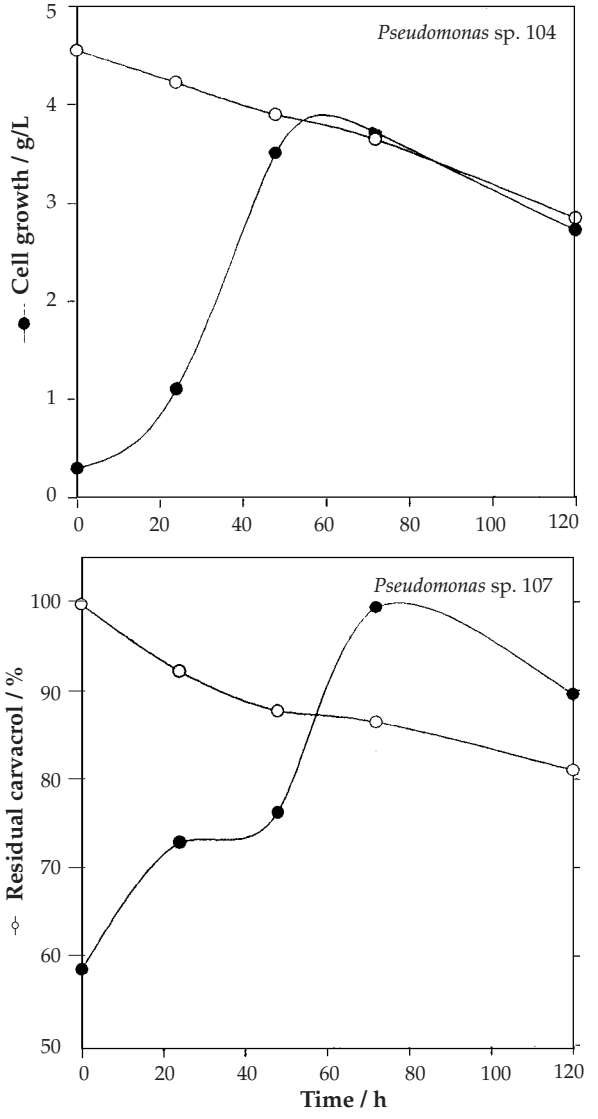

Fig. 4. Cultivation of *Pseudomonas* sp. 104 and 107 in liquid mineral medium; dynamics of growth (●–●); supstrate consumption (❍–❍)

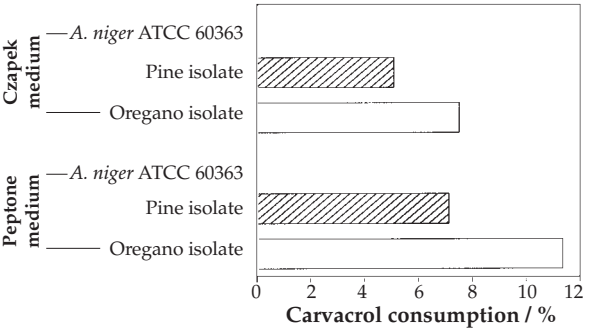

Fig. 5. Comparison of carvacrol consumption by the fungi cultivated in liquid peptone and Czapek medium

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Izolacija mikroorganizama sposobnih za asimilaciju karvakrola

Sažetak

Izolirano je nekoliko bakterijskih kultura i kultura plijesni sposobnih da asimiliraju karvakrol. Za izolaciju kultura upotrijebljene su biljke origano, majčina dušica i vrijesak, a i dijelovi bora (smola, kora i iglice drveta). Bakterijski su izolati uzgajani u tekućem mediju s karvakrolom kao jedinim izvorom ugljika. Izolirane kulture s vrijeska i bora iskoristile su najveći udjel karvakrola (od 19 do 22 %). Fungalni izolati rasli su puno sporije od bakterijskih i nakon 13 dana uzgoja iskoristili su samo 7,1–11,4 % karvakrola.

Sposobnost asimilacije karvakrola isto je tako ispitivana u nekim čistim kulturama bakterija koje pripadaju u rodove *Bacterium*, *Bacillus* i *Pseudomonas*, te plijesni iz rodova *Aspergillus*, *Botrytis* i *Geotrichum*. Ta je sposobnost primijećena samo u kultura *Bacterium* sp. i *Pseudomonas* sp. Obadva soja *Pseudomonas* sp. 104 i 107 iskoristila su 19 % od supstrata. Ti su sojevi postigli najveći prinos stanične biomase i najveću produktivnost (1,1–1,2 g $L^{-1}dan^{-1}$).