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Influence of culture conditions of *Gordonia jacobaea* MV-26 on canthaxanthin production

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Summary. Commercial interest in the use of natural pigments isolated from microorganisms has increased in recent years; hence, molecules belonging to the polyisoprenoid group (i.e. β-carotene, astaxanthin, and canthaxanthin) have been the focus of much attention. The bacterium *Gordonia jacobaea* readily synthesizes and accumulates large amounts of canthaxanthin (β-β´-carotene-4,4´-dione), which is widely used in the food and cosmetics industries. In the present work, the effects of different low-cost raw materials on fermentation and canthaxanthin accumulation by a hyperpigmented strain of *G. jacobaea* were studied. Canthaxanthin production and peak levels of accumulation varied according to the different media used. [**Int Microbiol** 2005; 8(1):55-58]

Key words: *Gordonia jacobaea* · canthaxanthin · carotenoids · soy-meal · fermentation

Introduction

Canthaxanthin (β-β´-carotene-4,4´-dione) is a ubiquitous keto-carotene that is of considerable industrial interest because of its widespread use in both the food and cosmetic industries [12]. While synthetic forms of canthaxanthin and many other pigments are currently available, their use in the food industry has been questioned by consumer agencies, so that the development of new sources of natural pigments has become essential to meet the increasing demand.

Due to their ease of manipulation, microorganisms provide an excellent system for the large-scale production of carotenoids, as has been shown with the yeast *Phaffia rhodozyma* [13] and the bacterium *Brevibacterium* KY-4313 [14], which accumulate the natural carotenoids astaxanthin and canthaxanthin, respectively. However, the inability of these two sources to meet world-wide demand has spurred research aimed at finding new sources (Table 1) and at optimizing fermentation technologies.

Gordonia jacobaea MV-1 (gram-positive, catalase negative, G + C 61%), which was isolated in this laboratory during a routine screening of pigmented microorganisms, is able to accumulate several carotenoids, including the keto-carotenoid *trans*-canthaxanthin [8]. However, its low carotenoid content (200 μg/g dry weight) does not support its industrial application. After several rounds of mutations, a hyperpigmented mutant (MV-26) with enhanced canthaxanthin and β-carotene accumulation was obtained. This mutant accumulates six-fold more canthaxanthin than the wild-type strain [9].

The influence of growth conditions and medium composition on the carotenoid synthesis pathway has been reported previously. This pathway begins with isoprenyl pyrophosphate (IPP), which may be formed either from mevalonic acid (MVA) or through the glyceraldehyde phosphate/pyruvate pathway [4,11]. Thus, media rich in these or related pre-

Table 1. Microbial sources of carotenoids

Microorganism	Main carotenoid	Yield	Reference
Haematococcus pluvialis	Astaxanthin	1.30 mg/l	[16]
Phaffia rhodozyma	Astaxanthin	30 µg/g	[13]
Halobacterium salinarium	Astaxanthin	265 µg/g	[7]
Dictyococcus cinnabarinus	Canthaxanthin	1 mg/g	[14]
Brevibacterium KY-4313	Canthaxanthin	2 mg/l	[14]
Haloferax alexandrinus	Canthaxanthin	2156.67 µg/g	[2]
Muriellopsis sp.	Lutein	22.7 mg/g	[10]
Blakeslea trispora	Lycopene	40 mg/l	[6]
Flavobacterium sp.	Zeaxanthin	0.09 µg/l	[1]
Dunaliella salina	ß-Carotene	2.12 mg/l	[15]
Dunaliella bardawil	ß-Carotene	20.1 pg/cell	[3]

cursors can be used to increase the carotenoid yield in industrial fermentations. In the present study, different media and low-cost raw materials were assayed in order to determine the optimal conditions of canthaxanthin production by *G. jacobaea* MV-26.

Materials and methods

Strains and culture conditions. The strain employed was the hyperpigmented *Gordonia jacobaea* MV-26 [9]. The bacterium was grown in different commercial media: yeast extract peptone dextrose (YPD) (20 g peptone/l, 10 g yeast extract/l, 20 g glucose/l), tryptone soy-meal broth (TSB) (3 g soy-meal peptone/l, 2.5 g glucose/l, 17 g casein peptone/l, 5 g dipotassium hydrogen phosphate/l, 5 g NaCl/l) and brain heart infusion broth (BHIB) (12.5 g calf-brain infusion solids/l, 5 g beef-heart infusion solids/l, 10 g protease peptone/l, 2 g glucose/l, 5 g NaCl/l, 2.5 g di-sodium phosphate/l). Low cost media consisting of different proportions of soy meal (0.5%–2%), beet molasses (0.5%–5%), and a mixture of soy meal (0%–5%) and glucose (0%–10%) were also assayed.

G. jacobaea MV-26 was grown in 1-l flasks containing 250 ml of the appropriate medium at 30ºC or 37ºC in a rotary shaker (150 rpm) for 8 days. In the case of YPD medium, screening was implemented for 10 days. Every other day, aliquots were withdrawn, and the levels of β-carotene and canthaxanthin (Fig. 1) were evaluated. After this first screening, *G. jacobaea* was grown in 2 l of each medium: BHIB, TSB, YPD, 0.5% soy meal, and 1.5% glucose/2% soy meal at 30ºC in a rotary shaker (150 rpm) since these media resulted in the highest levels of canthaxanthin production. When necessary, *G. jacobaea* was plated on medium supplemented with agar (2%).

Growth curve of *Gordonia jacobaea* MV-26. Growth curves were established either by determining the optical density at 600 nm in a Beckman DU-40 spectrophotometer or by plate counting (colony-forming units, CFU) when complex media were used (i.e. soy-meal-based).

Analysis of pigment production. Samples (3 ml) were withdrawn every 24 h and 1 ml of 0.1 M potassium phosphate buffer (pH 7) and 3 ml hexane (Merck) were added. After vigorous vortexing, the samples were centrifuged for 10 min at 5000 rpm to allow phase formation. The organic phase was filtered through a 0.22-µm filter (Gelman Sciences) and the pigment content was evaluated. When pigment production was analyzed in 2-liter cultures, an additional ethanol extraction was performed. In this case, following maximum canthaxanthin production, the biomass was harvested at 4ºC by means of continuous-flow centrifugation at 15000×*g*. The pigments were subsequently extracted by resuspending the cells in pure ethanol.

Carotenoid pigments were analyzed by HPLC using a silica-gel column (Teknokroma, 5-µm pore size, 25-cm length and 45-mm diameter). The mobile phase was hexane/ethyl acetate (1:1 v/v) (Romil). The flow was 1 ml/min and the pressure was 0.4 kpsi; the injection volume was 30 µl and the temperature was 25ºC. HPLC analysis of carotenoid pigments extracted with ethanol was carried out by adding one volume of hexane plus 1 ml 0.1 M potassium phosphate buffer (pH 7). After centrifugation for 10 min at 5000 rpm, the hexane phase was recovered and filtered as described previously. The peaks were evaluated based on their absorption at 480 nm. Retention times and concentrations of the samples were compared with pure standards of β-carotene and canthaxanthin.

Statistical analysis. The results of the influence of the growth media on canthaxanthin production were subjected to statistical analysis with the SPSS 12.0 program.

Results and Discussion

G. jacobaea MV-26 grew in all of the media, except those containing only beet molasses. While in all media supporting growth the production of carotenoids varied, maximum canthaxanthin accumulation consistently occurred in the stationary phase of growth (Fig. 2). Moreover, pigment production in *G. jacobaea* followed a pattern in which peak canthaxanthin production was inversely correlated with peak β-carotene production. Thus, as observed in other organisms [5], the keto-carotene canthaxanthin is the end-product of the carotenoid pathway, and β-carotene is an intermediate.

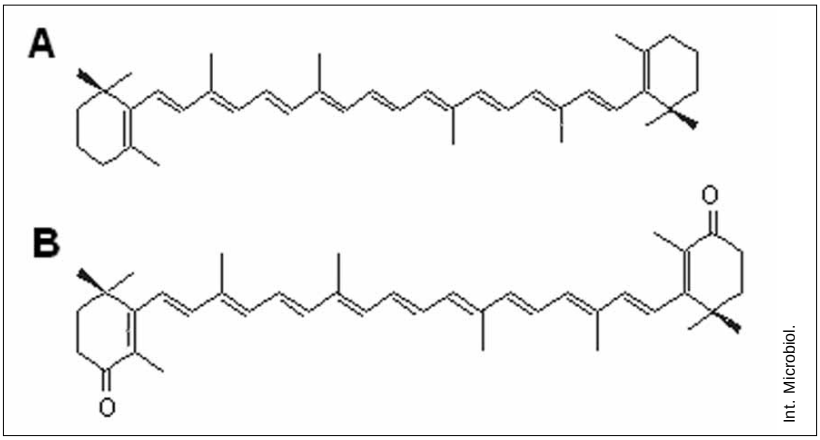

Fig. 1. β-Carotene (**A**) and canthaxanthin (**B**) molecules.

Standard calibration curves prepared with known amounts of pure β-carotene and *trans*-canthaxanthin allowed quantification of these pigments in the different media (Table 2). The highest levels of canthaxanthin were produced in soy-meal-based media, especially when supplemented with glucose as carbon source. In a previous study [8], an effect of the carbon source on carotenoid synthesis and accumulation was observed. Among all the carbon sources tested, glucose induced the highest level of canthaxanthin synthesis, probably by favoring the overproduction of mevalonic acid, which is a key metabolite in the synthesis of polyprenoid-derived carotenoids. Accordingly, beet molasses should have been a good medium for canthaxanthin production. Thus, the inability of *G. jacobaea* to grow on molasses-based media was most likely due to the presence of an inhibitor in the industrial raw material.

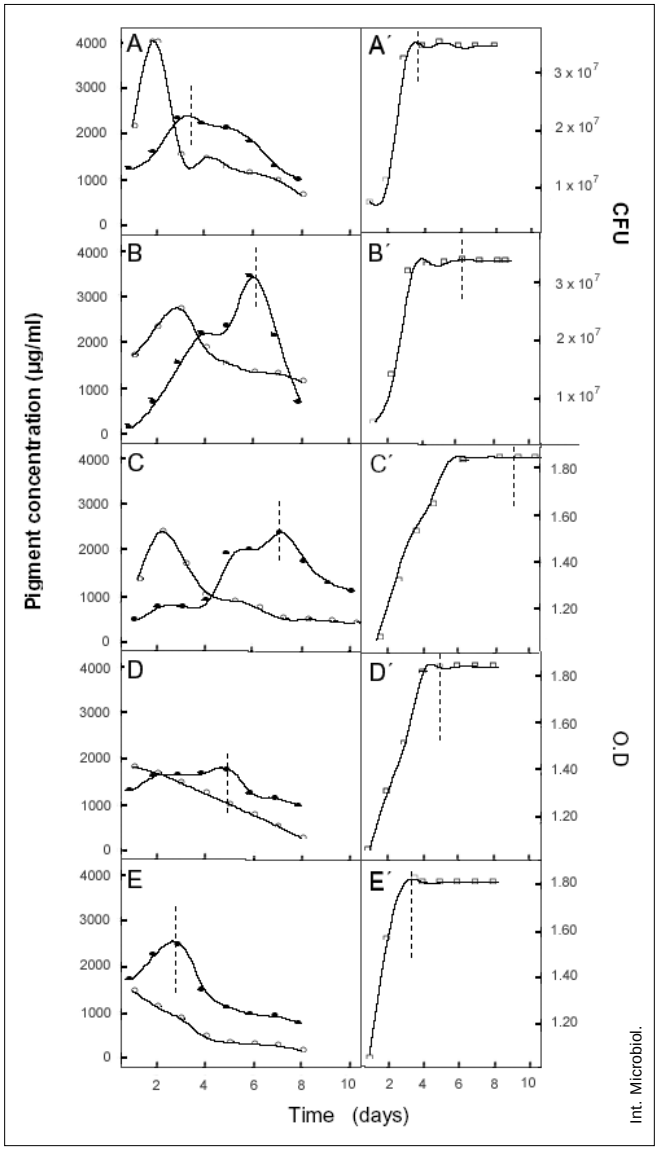

Fig. 2. Concentrations of canthaxanthin (µg/ml) (•) and ß-carotene (µg/ml) (o) produced by *Gordonia jacobaea* MV-26 in different production media: (A) 0.5% soy meal/0% glucose; (B) 2% soy meal/1.5% glucose; (C) YPD; (D) TSB; (E) BHIB. (A´-E´) represent the evolution of growth of *G. jacobaea* (□) in the different media. The dashed vertical line marks the peak of production and the bacterial growth phase.

The production profile varied both in quantity and timing depending on the medium used. Thus, in BHIB media the maximum pigment concentration (2600 µg/ml) was measured on day 3 of fermentation (stationary phase), whereas in TSB medium the maximum (1500 µg/ml) was attained on day 6 (stationary phase). In the case of YPD medium, a maximum yield was obtained on day 9 (stationary phase), but it was always lower than the yield from BHIB medium. When soy-meal-containing media were compared, 5% soy meal/1% glucose was found to elicit a peak of around 13000 µg/ml, while 2% soy meal/1.5% glucose afforded a peak of nearly 3500 µg/ml. However, despite the higher level of canthaxanthin production, the use of 5% soy meal/1% glucose medium was ruled out because of the difficulties involved in handling this mixture. Instead, it was concluded that the addition of 0.5% soy meal/0% glucose could be implemented in order to increase canthaxanthin production. The ability of soy meal to increase pigment production was most likely due to the presence of precursors of the carotenoid pathway, such as mevalonic acid or related substances, which, together with the extra amounts of glucose present in the media, could have increased canthaxanthin production. These putative precursors might also have been responsible for the increases in β-carotene and canthaxanthin that occurred during the first few days of the stationary phase of growth, an effect that was particularly observable using BHIB medium, which is rich in terpenoid precursors, this leading to the emergence of an earlier peak of pigment production.

Varying the temperature from 30ºC to 37ºC had little effect on canthaxanthin production (data not shown). However, a larger range of temperatures should be tested before an effect of temperature on canthaxanthin synthesis can be completely ruled out.

Statistical analysis, based on Student's *t*-test (95% confidence interval), confirmed the observations on the differential effects of the tested media on pigment production by *G. jacobaea* MV-26.

Table 2. Yields of canthaxanthin production by *Gordonia jacobea* MV-26 in different production media

Medium	Canthaxanthin (µg/ml)
YPD	2140
BHIB	2489
TSB	1760
10% glucose/0% soy	1800
0% glucose/0.5% soy	2340
5% glucose/1% soy	1000
2% glucose/1.5% soy	2650
1.5% glucose/2% soy	3440
1% glucose/5% soy	13373

A comparison of the abilities of hexane and ethanol to extract canthaxanthin showed that the extraction capacity of hexane was 50% higher than that of ethanol. However, since hexane is not allowed in foods, ethanol extraction must be optimized for *G.. jacobaea*. Nonetheless, ethanol extraction of carotenoids from this bacterium is an advantage compared to other carotenoid-producing microorganisms, such as *Phaffia rhodozyma*, in which this solvent is ineffective unless the yeast has previously been disrupted. Moreover, the use of ethanol in pigment extraction lowers the possibility of toxicity and animal intoxication due to contamination with residual organic solvents in downstream processes.

Taking into account that higher concentrations of soy meal hampered fermentations because of its insolubility, the medium containing 0.5% of soy meal was chosen as the optimum to obtain the highest and the more profitable pigment production. The production profiles in higher volumes were similar to those observed for lower volumes. In general, soy meal alone, even without glucose added, promoted pigmentation and the amount of carotenoids was also higher.

The current widespread concern about the use of genetically modified organisms (GMO) in processes related to the food industry makes classical approaches, such as the one described here, of commercial relevance as the best and safest way for increasing the concentrations of important biomolecules.

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Influencia de las condiciones de cultivo de *Gordonia jacobea* MV-26 en la producción de cantaxantina

Resumen. El interés comercial del uso de pigmentos naturales aislados a partir de microorganismos se ha incrementado en los últimos años y las moléculas pertenecientes al grupo de los poliisoprenoides (p.e. β-caroteno, astaxantina y cantaxantina) se han convertido en un foco de atención. La bacteria *Gordonia jacobaea* es capaz de sintetizar y acumular grandes cantidades de cantaxantina (β-β´-caroteno-4,4´-diona), muy usada en la industria alimentaria y de cosméticos. En este trabajo estudiamos la influencia de diferentes materias primas de bajo coste en la fermentación y la acumulación de cantaxantina por una cepa mutante hiperpigmentada de *G. jacobaea*. Se ha observado que la producción de cantaxantina y el momento en el que se alcanza la máxima producción varía según los diferentes medios empleados. [**Int Microbiol** 2005; 8(1):55-58]

Palabras clave: *Gordonia jacobaea* · cantaxantina · carotenoides · medio de soja · fermentación

Influencia das condições de cultivo de *Gordonia jacobaea* MV-26 na produção de cantaxantina

Resumo. O interesse comercial do uso pela indústria alimentar de pigmentos de origem natural isolados a partir de microorganismos experimentou um aumento consideravel nos últimos anos, em especial aqueles com uma estructura carotenóide (β-caroteno, astaxantina e cantaxantina) A bacteria *Gordonia jacobaea* presenta uma grande capacidade para produzir e acumular grandes quantidades de cantaxantina (β-β´-carotene-4,4´-dione), muito usada pela indústria alimentar e cosmética. O presente trabalho utiliza cepas hiperpigmentadas de *Gordonia jacobaea* modificadas geneticamente para analizar a influencia de diferentes matérias primas de baixo custo, na fermentação e acumulação de cantaxantina. A produção de cantaxantina e o momento em que esta é máxima variou consoante o meio de cultivo utilizado. [**Int Microbiol** 2005; 8(1):55-58]

Palavras chave: *Gordonia jacobaea* · cantaxantina · carotenóides · meio de cultivo de soja · fermentação