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Influence of carbon and nitrogen sources on *Flavobacterium* growth and zeaxanthin biosynthesis

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Growth and production of zeaxanthin by *Flavobacterium* sp were studied using different carbon and nitrogen sources in a chemically defined medium. The best growth was supported by sucrose, but glucose yielded similar carotenoid concentrations. Both asparagine and glutamine stimulated growth and pigment formation. Carotenoid production and glucose consumption increased as a function of asparagine concentration. In the presence of asparagine, high glucose concentrations decreased pigment production without affecting biomass formation. In the absence of glucose, asparagine could not support growth and zeaxanthin production. When compared to the effect of 55 mM glucose, 10 mM oxaloacetate increased growth and carotenoid production. Pyruvate and other intermediates of the citric acid cycle showed a similar stimulatory effect. The intermediates of glycolysis: glucose 6-phosphate and fructose 1,6-diphosphate did not support growth. These results suggest that *Flavobacterium* sp utilizes asparagine primarily as a nitrogen source for growth and production of zeaxanthin.

Keywords: zeaxanthin; carotenoids; pigments; *Flavobacterium*

Introduction

Zeaxanthin (3,3′-dihydroxy-beta-carotene) is a yellow xanthophyl which belongs to the carotenoid family of pigments. From a commercial standpoint, zeaxanthin has been used as a natural food colorant for fish (skin pigmentation) and poultry (yolk and skin pigmentation) [12]. This compound occurs widely in nature and is synthesized by a wide variety of microorganisms [1]. Among the sources of microbial xanthophyls, McDermott *et al* [9] reported that *Flavobacterium* sp produces zeaxanthin as essentially its only carotenoid. This has stimulated nutritional and biosynthetic studies on zeaxanthin formation by *Flavobacterium* [9,10]. Although several fermentation studies have been conducted for zeaxanthin production in complex media [5,14,15,17], only limited information is available concerning pigment regulation by nutritional factors. The purpose of this work was to study the effect of various carbon and nitrogen sources on zeaxanthin production by *Flavobacterium* sp in a chemically defined medium able to support rapid growth and zeaxanthin production. In addition, it provides a basis for further studies on the regulation of zeaxanthin biosynthesis by *Flavobacterium* sp under well-controlled conditions.

Materials and methods

Microorganism

The type strain of *Flavobacterium* sp (ATCC 25582) was obtained from the American Type Culture Collection. Zeaxanthin constitutes 95% of the total carotenoids produced by this strain.

Cultures

Stock cultures were prepared from yeast-tryptone-sodium (YTN) slants [4] previously grown for 48 h and maintained in 40% glycerol at −20°C. Seed cultures were developed by inoculating 1 ml of a cell suspension (OD_{540} adjusted to 0.3 with saline solution) into 250-ml Erlenmeyer flasks containing 50 ml YTN. The cultures were incubated at 29°C on a rotary shaker (180 rpm) and after 24 h, pelleted, washed with saline and diluted to an optical density (OD_{540}) of 1.0. For pigment production, 250-ml Erlenmeyer flasks containing 25 ml of a chemically defined medium were inoculated with 5% (v/v) of the washed seed culture and allowed to grow for 48 h (stationary growth phase) with agitation (180 rpm). The chemically defined medium (CD-medium) was designed and optimized in our laboratory (Bautista *et al*, in preparation) and contained per liter: 30 g NaCl; 1.74 g K_2HPO_4; 0.74 g $MgSO_4 \cdot 7H_2O$, 1 ml of an inorganic salt solution and the desired carbon and nitrogen source. The salt solution contained per liter: 2.85 g H_3BO_3: 1.8 g $MnCl_2$; 1.36 g $FeSO_4 \cdot 7H_2O$; 1.77 g sodium tartrate; 0.74 mg $MgSO_4 \cdot 7H_2O$; 26.9 mg $CuCl_2 \cdot 2H_2O$; 20.8 mg $ZnCl_2$; 40.4 mg $CoCl_2 \cdot 6H_2O$ and 25.2 mg $Na_2MoO_4 \cdot 2H_2O$. The carbohydrates and amino acids were sterilized separately by heat (autoclaved) and filtration (Millipore membranes type HA), respectively, and added before inoculation.

Analysis

Microbial density was determined in a spectrophotometer at 540 nm. Zeaxanthin was determined at different time points. For this purpose, 2-ml samples of the culture medium were centrifuged, the cells washed and the pigments extracted according to Britton [3]. After extraction

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the samples were resuspended in 2.5 ml ethanol and their zeaxanthin concentrations were estimated at 450 nm using an extinction coefficient of 1540 (1%, 1 cm) and the formula provided by Britton [3]. Zeaxanthin was also visualized by thin layer chromatography (TLC). For this purpose, the extracted samples were evaporated to dryness with nitrogen. The samples were resuspended in acetone (0.5 ml), applied on TLC 60 plates (Merck de Mexico, SA de CV), eluted with dichloromethane-ethyl acetate (80:20) and quantified using a CAMAG TLC Scanner II (Muttenz, Switzerland), using pure zeaxanthin as standard (Hoffmann-La Roche & Co, Basel, Switzerland).

Glucose and ammonium determination

The glucose concentration was estimated directly from the fermentation medium by the enzymatic method reported by Trinder [8]. Ammonium concentration in the culture media was determined according to the Berthelot method [11].

Results and discussion

Effect of different carbon sources on growth and pigment production

Growth and pigment production by *Flavobacterium* sp were studied using different sugars as carbon sources. With the knowledge that glucose, sucrose and xylose support rapid growth and pigment production by *Flavobacterium* sp in complex media [4,14,15,17], the influence of these carbon sources on growth and pigment production was tested in a CD-medium supplemented with 20 mM NH_4Cl. As shown in Table 1, glucose and sucrose (55 mM) supported limited growth and zeaxanthin production compared to yeast extract, used as a positive control. Among these sugars, the best growth was supported by sucrose, but glucose promoted similar levels of carotenoid production. The poorest response was observed by adding xylose. This carbohydrate failed to promote any more growth and pigment production than that found without addition of a carbon source. Utilization of carbon sources for growth appears to be strain-specific, since other flavobacteria strains grow well on glucose and sucrose [15].

Table 1 Effect of different carbon and nitrogen sources on growth and zeaxanthin production

Conditions	Growth (OD at 540 nm)	Zeaxanthin (μg ml^{-1})
Carbon sources[a]		
Yeast extract (1%)	2.38 ± 0.070	0.75 ± 0.025
d-Glucose (55 mM)	1.55 ± 0.066	0.15 ± 0.020
Sucrose (55 mM)	1.82 ± 0.102	0.15 ± 0.027
Xylose (55 mM)	0.51 ± 0.032	0.07 ± 0.017
No carbohydrate	0.46 ± 0.055	0.07 ± 0.011
Nitrogen sources (7.5 mM)[b]		
l-Asparagine	3.74 ± 0.050	0.38 ± 0.016
l-Glutamine	2.96 ± 0.119	0.36 ± 0.011
l-Cysteine	1.89 ± 0.090	0.07 ± 0.009
l-Methionine	1.85 ± 0.015	0.08 ± 0.0095
Glycine	0.60 ± 0.020	0.03 ± 0.007
l-Asparagine without glucose	0.44 ± 0.101	0.08 ± 0.009
l-Asparagine without $MgSO_4$	0.55 ± 0.030	0.03 ± 0.006
l-Asparagine + l-cysteine	2.31 ± 0.076	0.28 ± 0.003

[a]Fermentations were performed for 48 h at 29°C and 180 rpm in 250-ml Erlenmeyer flasks containing 50 ml CD-medium supplemented with 20 mM NH_4Cl.
[b]Fermentations were performed in CD-medium supplemented with 110 mM glucose.

Effect of amino acids on growth and zeaxanthin formation

There have been very few studies on the effect of specific amino acids on flavobacteria. Sheperd *et al* [15] found that addition of methionine or cysteine to complex media enhanced specific production of zeaxanthin. To study the effects of various amino acids on growth and zeaxanthin production, we used the CD-medium supplemented with 110 mM glucose. Based on studies by Sherman *et al* [16], nine different amino acid combinations, using a total of 20 individual amino acids, were tested. The most effective combination for both growth and pigment production contained asparagine, glutamine, methionine, cysteine and glycine. Limited growth was observed with other amino acid combinations (data not shown). In order to distinguish the individual effects of the best amino acid combination, we tested each one of them (7.5 mM) on growth and carotenoid production in CD-medium supplemented with 110 mM glucose. Both growth and pigment formation were better on asparagine or glutamine than on cysteine or methionine (Table 1). The poor growth observed with glycine, was possibly due to the effect of this amino acid on the peptidoglycan composition of the cell wall in bacteria [5]. In the absence of glucose, asparagine could not support good growth and zeaxanthin production, suggesting that the amino acid served only as nitrogen source. Poor growth and low pigment formation were observed with asparagine, in the absence of $MgSO_4$. However, the sulfate deficiency was partially complemented by cysteine (Table 1). Similar results were obtained with the use of methionine (data not shown). This indicates the importance of sulfur for good growth and pigment production in *Flavobacterium* sp.

To characterize the asparagine stimulation, we tested a range of concentrations up to 22.7 mM. As shown in Figure 1, growth and pigment production increased with increasing asparagine concentrations. Glucose consumption was directly proportional to the amino acid concentration. The increase of cell growth and glucose consumption as a function of asparagine concentration suggests either a limitation in growth and carbon consumption due to nitrogen deficiency or the production of easily metabolized compounds from this amino acid. In asparagine concentrations of 13.3 mM and higher, the cells excreted ammonium into the medium. This is probably due to asparagine catabolism into aspartate and ammonium, as has been shown for enteric bacteria [7]. As expected, higher levels of ammonia were excreted when higher levels (22.7 mM) of asparagine were present. These results further supported the hypothesis that asparagine was utilized primarily as nitrogen source and is probably capable of producing catabolites which function as growth and pigment promoters in *Flavobacterium* sp.

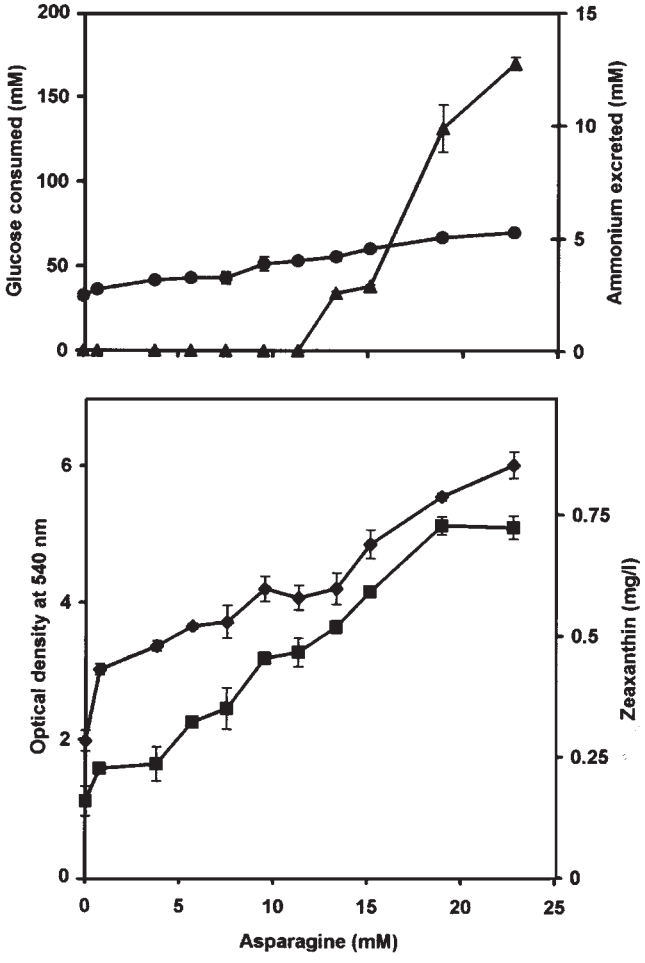

Figure 1 Effect of asparagine concentrations on growth (◆), glucose consumption (●), zeaxanthin production (■) and excretion of ammonia (▲). Fermentations were performed for 48 h at 29°C and 180 rpm in 250-ml Erlenmeyer flasks containing 50 ml CD-medium supplemented with 110 mM glucose.

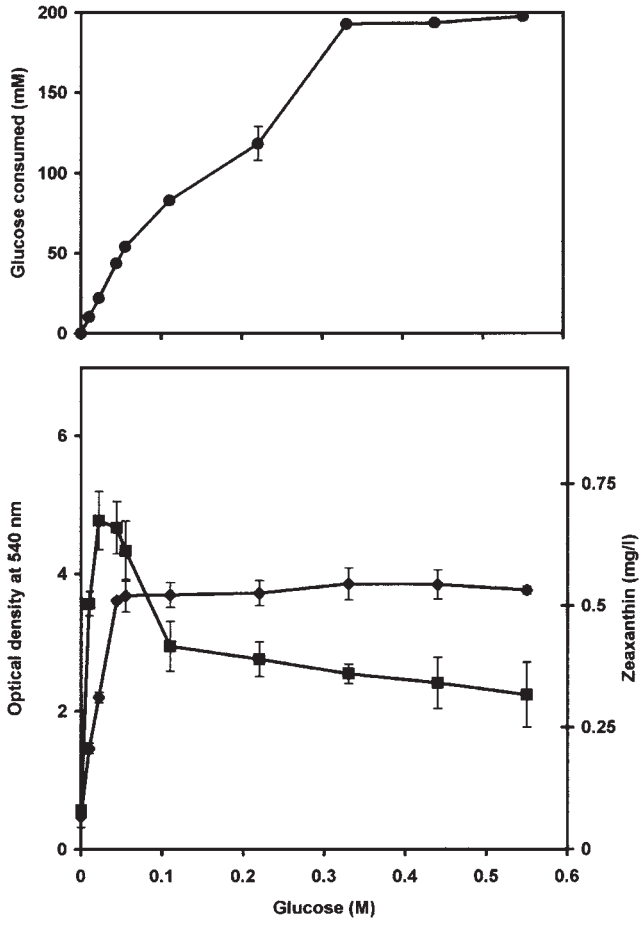

Figure 2 Effect of glucose concentrations on growth (◆), glucose consumption (●) and zeaxanthin formation (●). Fermentations were performed as described in Figure 1 in CD-medium supplemented with 7.5 mM asparagine.

Influence of glucose and ammonium concentrations in the presence of asparagine

Growth and pigment production were studied in a wide range of glucose concentrations in CD-medium containing asparagine (7.5 mM). At glucose concentrations above 10 mM both growth and zeaxanthin production increased. Above 55 mM glucose, the biomass remained constant while specific zeaxanthin production decreased steadily (Figure 2). In the presence of ammonium (20 mM) as nitrogen source, high glucose concentrations also decreased specific production of zeaxanthin without a reduction in growth (not shown). These data suggest a repressive effect of glucose on carotenoid production. At concentrations up to 330 mM glucose in the presence of asparagine, glucose consumption was partially proportional to its own concentration present in the broth. Similar results on glucose consumption were observed when 20 mM ammonium was used as nitrogen source.

To study the effect of NH_4Cl concentration on growth and pigment production in *Flavobacterium* sp, we tested a series of concentrations up to 150 mM NH_4Cl. The medium utilized was supplemented with 7.5 mM asparagine and 110 mM glucose. Above 10 mM ammonium, the zeaxanthin production decreased steadily. However, concentrations of NH_4Cl 40 mM and higher suppressed cell growth. Therefore a specific negative effect on zeaxanthin production could not be ascribed to this ion. Above 10 mM NH_4Cl, the pH of the media was increased, which may explain the pigment and biomass reduction (data not shown). In *Monascus* sp, the utilization of different nitrogen sources produces different pH patterns in fermentation, which affect growth and pigment production [18].

Effect of intermediates of glycolysis and the citric acid cycle

l-Asparagine utilization involves its conversion to aspartate and ammonia by asparaginase [7]. Later, transamination of aspartate with α-ketoglutarate by l-aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1) produces oxaloacetate and glutamate [6]. Our results suggest that asparagine is used only as nitrogen source, and may be capable of producing catabolites that function as growth and pigment promoters. Therefore, we set out to determine the effect of oxaloacetate, and other intermediates of the citric acid cycle, on growth and pigment production. When compared to the effect of 55 mM d-glucose alone, 10 mM oxaloacetate

Table 2 Effect of citric acid and glycolysis intermediates (10 mM) on growth and zeaxanthin production[a]

Conditions	Growth (OD at 540 nm)	Zeaxanthin (μg ml^{-1})
Control (55 mM glucose)	1.53 ± 0.13	0.09 ± 0.004
Oxaloacetate	3.06 ± 0.051	0.49 ± 0.007
Citrate	2.59 ± 0.037	0.63 ± 0.015
α-Ketoglutarate	2.61 ± 0.084	0.52 ± 0.014
Malate	3.00 ± 0.051	0.46 ± 0.018
Pyruvate	2.78 ± 0.160	0.34 ± 0.018
Glucose 6-phosphate	1.42 ± 0.042	0.08 ± 0.005
Fructose 1,6-diphosphate	0.73 ± 0.100	0.07 ± 0.011

[a]Fermentations were performed as described in Table 1 in CD-medium supplemented with 20 mM NH_4Cl.

increased growth and volumetric zeaxanthin production in CD-medium supplemented with 20 mM NH_4Cl (Table 2). Other intermediates of the citric acid cycle, such as citrate, α-ketoglutarate and malate, showed a similar effect. Under the same conditions, the intermediates of glycolysis:glucose 6-phosphate and fructose 1,6-diphosphate did not support growth of *Flavobacterium* sp likely due to assimilation problems. Pyruvate, the final product of glycolysis, showed the same effect as the citric acid cycle intermediates; cultures reached growth and zeaxanthin levels of approximately OD_{540} = 2.8 and 0.34 mg L^{-1}, respectively (Table 2). These results suggest that asparagine stimulates growth and production of carotenoids through the formation of oxaloacetate. In turn, oxaloacetate could be a source of pyruvate, phosphoenolpyruvate and acetyl-CoA by the anaplerotic function of phosphoenolpyruvate carboxylase [PEPC; orthophosphate: oxaloacetate carboxy-lyase (phosphorylating); EC 4.1.1.31] described in bacteria [2]. Acetyl-CoA is a primary precursor of 3-isopentenyl pyrophosphate, which feeds the carotenoid biosynthetic pathway in *Flavobacterium* sp [13]. If addition of asparagine leads to oxaloacetate production and oxaloacetate can function as a carbon source for growth and zeaxanthin production, why then cannot asparagine also serve as carbon source? A possible explanation may be related to limiting α-ketoglutarate concentrations. Indeed, we could predict that in the absence of glucose, the pools of α-ketoglutarate and other intermediates of the Krebs cycle would be at their basal levels. Under these conditions, the aspartate derived from asparagine catabolism would not find enough α-ketoglutarate to transaminate in order to form oxaloacetate and glutamate [19].

Pigment production in a defined medium

Based on our results, we supplemented the CD-medium with 44 mM citrate and 20 mM ammonium chloride as carbon and nitrogen sources, respectively. We obtained a maximum volumetric zeaxanthin production of 1 mg L^{-1} and OD_{540} = 2.3. This medium seems to be appropriate for producing zeaxanthin for regulatory studies in which a chemically defined medium is necessary.

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