ACTA ABP BIOCHIMICA POLONICA

Vol. 59, No 1/2012
151–153
on-line at: www.actabp.pl

Communication

Agro-food wastes utilization by *Blakeslea trispora* for carotenoids production*

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The all-trans-β-carotene is a natural pigment used in various industrial fields (food, cosmetics, pharmaceuticals, etc) and possesses the higher provitamin A activity, in respect to other carotenoids. All-trans-β-carotene is produced industrially by chemical and biotechnological means. For β-carotene biotechnological production in industrial scale mated cultures of *Blakeslea trispora*, a heterothallic fungus, are mainly used. Despite the intense research for β-carotene production by *B. trispora*, natural substrate utilization has not been extensively studied. Solid agro-food wastes such as cabbage, watermelon husk and peach peels from northern Greece as main carbon source into submerged *B. trispora* cultures for carotenoids production, was examined. The media containing only the agro-food waste (2–4) gave a biomass accumulation 7.77±0.4 g/L, while a reference medium 1 with glucose (10 g/L) gave 4.65±0.21 g/L. In another experiments series agro-food wastes were used with corn steep liquor and thiamine (media 6–8), giving a biomass accumulation and total carotenoid volumetric production 10.2±2.41 g/L and 230.49±22.97 mg/L, respectively. These are the higher values reported for solid wastes so far in respect to those obtained from a synthetic medium, with higher glucose concentration of 50 g/L where the correspondent values were 9.41±1.18 g/L and 45.63 mg/L respectively. The results support that *B. trispora* is able to utilize, almost equivalently, different origin agro-food wastes for carotenoids production. Furthermore, β-carotene percentage in all examined cases was over 76%, as it was estimated by HPLC analysis, suggesting that these agro food wastes may be used for high purity, large scale β carotene production.

Key words: biotechnologically produced carotenoids, β-carotene, natural substrates, *Blakeslea trispora*, agro-food wastes

Received: 17 October, 2011; accepted: 01 March, 2012;
available on-line: 17 March, 2012

INTRODUCTION

Carotenoids are naturally occurring pigments formed through the isoprenoid pathway that impart attractive colour to animals and plants, and also have essential biological functions as antioxidants, membrane stabilizers, and precursors to essential metabolites, such as vitamin A. Various algae and fungi have the ability to produce and accumulate intracellular high levels of carotenoids, including β-carotene, lycopene, and astaxanthin and thus appear promising for industrial carotenoids production. Scientific interest in carotenoids has also increased the last years, mainly because of the antioxidant activity of certain carotenoids *in vitro* and *in vivo* (Palozza & Krinsky, 1992) and their potential to prevent chronic diseases associated with reactive oxygen species (ROS), including certain cancers, macular degeneration and cataract formation, cardiovascular diseases, certain infections and other associated with aging (Halliwell, 1997). Carotenoids have also been postulated to enhance immune responses, to alter gene regulation, to induce apoptosis in cancer cell lines, and to have other biological effects in humans and animals (Bendich & Olson, 1989). The overall interest in carotenoids has been increased in the last few years due to an increasing demand for these compounds in the food, pharmaceutical, cosmetic, and animal feed industries (Valduga *et al.*, 2009), although most carotenoids used industrially are principally manufactured using chemical synthesis or obtained from agricultural extracts. There is much interest in biotechnological production due to consumer demand for high quality and "natural" food additives, environmental concerns associated with chemical manufacture and solvent expenditure for carotenoids extraction from low in content agricultural residues (Echaverri-Erasum & Johnson, 2002). The development of biotechnological processes aim at increasing the carotenoid yield and reduce process costs, by using low-cost agro substrates rich in sugars, organic compounds and inorganic minerals. The types of carotenoid and their relative amounts produced vary depending on the microorganism, the culture medium, and the operation conditions (temperature, pH, aeration rate, and luminosity). Most of the carried out studies aimed to optimize the culture conditions that directly affect the growth of the microorganism and the carotenoids production (Valduga *et al.*, 2009).

Today is also observed a continuously growing concern about the environmental protection, so there are a lot of efforts for disposal mitigation of solid agro-food wastes residues. At present there are a few possibilities for the utilization or recycling the most of these wastes, which are disposed off or used as animals feed. The transport costs and sales problems, due to the low quality and prone to spoilage by microorganisms of the re-

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*Presented at the 16th International Symposium on Carotenoids, 17–22 July, 2011, Kraków, Poland

Abbreviations: ROS, reactive oxygen species; MeOH, methanol; HPLC, high performance liquid chromatography; RP-HPLC-DAD, reverse phase high performance liquid chromatography with diode array detector; SmF, submerged fermentation; SSF, solid state fermentation.

Table 1. Medium composition

Medium #	1	2	3	4	5	6	7	8
Composition (g/L)[a]								
Glucose	10	–	–	–	50	–	–	–
Cabbage	–	100	–	–	–	100	–	–
Watermelon husk	–	–	100	–	–	–	100	–
Peach peels	–	–	–	100	–	–	–	100
Corn steep liquor	–	–	–	–	80	80	80	80
Thiamin–HCl[b]	–	–	–	–	5	5	5	5

[a]All media also contained (g/L): Span 20 10.0, Tween 80 0.1, L-asparagine 2.0, KH_2PO_4 1.5, $MgSO_4x7H_2O$ 0.5, and 2% (v/v) linoleic acid; [b]The amounts given are in mg/L

sidual matter have led to alternative utilization concepts. One such concept is to turn them by mild biotechnological means into usable products, moderating thus the pollution problems and leading to a more stable and with smaller volume waste. The use of agro-food wastes in agriculture by composting is also commonly used, but in such case the value of the final product is not high enough to sustain the overall process and the time consumed is quite long.

Vegetable and fruit processing wastes are composed mainly of organic matter such as starch, cellulose, soluble sugars and organic acids (Stabnikova *et al*., 2005). Agro-industrial residues are generally considered as the best substrates for the solid-state fermentation (SSF) processes. SSF has been used for decades to convert moist agricultural polymeric substrates like wheat, rice, soy, cassava, *etc*. into fermented food products. The solid substrate in SSF acts as a source of carbon, nitrogen, minerals and growth factors, and has a capacity to absorb water, necessary for microbial growth. On the other hand, industrially important products have traditionally been obtained from submerged fermentation (SmF), due to the ease in handling and greater control of environmental factors such as temperature and pH (Özdemir *et al*., 2009). Diverse food wastes, apple, orange and potato, have been already screened for laccase production, under SSF conditions, by the white-rot fungus *Trametes hirsute* (Rosales *et al*., 2002). The production of extracellular amylase by *Bacillus subtilis* has been studied in SSF with different solid substrates such as banana husk, water melon husk, lentil bran, wheat bran, melon husk and maize oil cake (Özdemir *et al*., 2009). On the other hand, water extracts of cabbage and watermelon have already been successfully used for SmF yeast production (Stabnikova *et al*., 2005).

In respect to above, three agro-food wastes (cabbage, watermelon husk and peach peels) have been selected according to their high availability in the area of northern Greece in addition to their relative low cost, and examined for carotenoid production by *B. trispora*.

MATERIALS AND METHODS

Materials. KH_2PO_4 and $MgSO_4{\times}7H_2O$ were purchased from Scharlau, L-asparagine from Fluka, Tween 80 and 2-propanol were obtained from Mallinckrodt J.T. Baker. Span 20, PDA, thiamin-HCl, all-trans β-carotene (type II synthetic), lycopene (from tomato), and linoleic acid were from Sigma-Aldrich. Corn Steep Liquor was from Tyte & Lyle, Thessaloniki, Greece. D-glucose was purchased from Riedel-deHaën. MeOH, HPLC grade (LiChrosolv), was from Merck. Peaches, cabbages and watermelons were obtained from local market of northern Greece.

Microorganism. *Blakeslea trispora* ATCC 14271, mating type (+), and *Blakeslea trispora* ATCC 14272, mating type (–).

Inoculum preparation and cultures. The inoculum preparation is given elsewhere (Papaioannou & Liakopoulou, 2010).

The batch cultivation experiments were performed in 500 ml Erlenmeyer flasks with a working volume 100 mL, incubated at 26°C in a rotary shaker at 200 rpm.

Eight different media were investigated and their compositions are given in Table 1. The agro-food wastes water content was ≈91.5±3.3%.

In all experiments the initial pH was adjusted to 7.5.

All experiments were performed in triplicates and the values reported here are mean values.

Total carotenoid extraction and determination. Total carotenoids extraction and quantification with UV-Vis was performed according to Papaioannou *et al*. (2008).

RP-HPLC-DAD analysis of main carotenoids. The concentration of the three main carotenoids (lycopene, γ-carotene and β-carotene) contained in total carotenoids extracts was determined by HPLC protocol, as previously described (Papaioannou & Liakopoulou, 2010).

RESULTS AND DISCUSSION

In case of media (**2**–**4**) *B. trispora* biomass accumulation and total carotenoids volumetric production was about 7.77±0.44 g/L and 38.2±4.83 mg/L, respectively (Fig. 1). The maximum biomass accumulation for the reference medium **1** containing only glucose (10 g/L) was achieved after three days, whereas the maximum carotenoid volumetric production was observed after five days (Fig. 1). The same behaviour was observed in case of media (**2**–**4**) with agro-food wastes. Biomass accumulation in case of agro-food wastes (media **2**–**4**) is almost 1.7 times higher than that of medium **1** (4.65±0.21 g/L (Fig. 1). The total carotenoids production in case of agro-food wastes (media **2**–**4**) is slightly higher than that of medium **1** (about 1.27 times higher). The higher total carotenoids accumulation 42.2±1.31 mg/L was observed in case of cabbage (medium **2**) after 10 days cultivation. Thus, in accordance to the above results, is assumed that the agro-food wastes (media **2**–**4**) are stimulating both the fungal growth and the carotenoids production.

HPLC analysis (not shown) of the total carotenoids extracts from the above fungal biomass showed that the

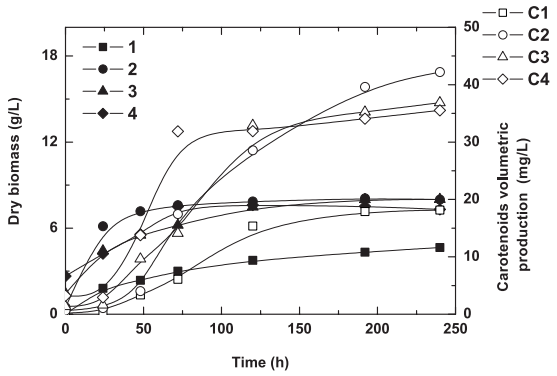

Figure 1. *Blakeslea trispora* growth curve (solid symbols) and total carotenoids volumetric production (open symbols) for media (2–4):
cabbage (●), watermelon husk (▲), peach peels (◆) and reference glucose medium **1** (■).

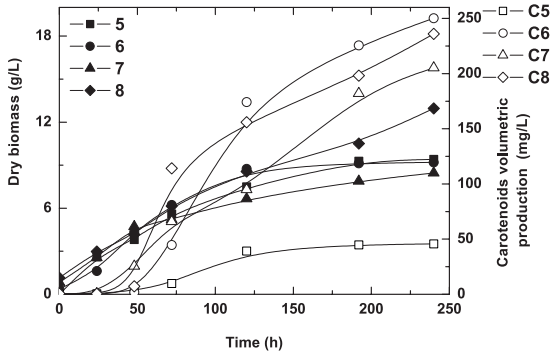

Figure 2. *Blakeslea trispora* growth curve (solid symbols) and total carotenoids volumetric production (open symbols) for enhanced agro-food media (6–8):
cabbage (●), watermelon husk (▲), peach peels (◆) and reference glucose medium **5** (■).

main carotenoid produced in cases of media **2–4** is the all-trans-β-carotene (>76%).

In another experimental series, has been examined the enhancement of the agro-food media with corn steep liquor and thiamine. The resulted media (media **6–8**) and another synthetic medium **5**, that has been used in previous studies (Papaioannou & Liakopoulou, 2010), biomass accumulation and total carotenoids volumetric production are given in Fig. 2. In the enhanced media (**6–8**) a delay was observed as far as biomass accumulation and carotenoids volumetric production (after five and eight days respectively) in relation to agro-food (**2–4**, Fig. 1) media. The biomass accumulation and carotenoids volumetric production in case of media **6–8** are 10.2±2.41 g/L and 230.49±22.97 mg/L respectively (Fig. 2). As it is shown, cabbage waste (medium **6**) gave the higher total carotenoids accumulation 250.25±10.56 mg/L after 10 day cultivation, while the lower observed in case of the watermelon 205.28±3.48 mg/L (medium **7**). The biomass accumulation in case of media **5** is almost the same (9.41±1.18 g/L) with the enhanced agro-food wastes (media **6–8**) as shown in Fig 2. Though, the total carotenoids accumulation in case of enhanced agro-food wastes (**6–8**) is almost 4-6 time higher than this of medium **5** (Fig. 2). The results given in Fig. 2 show that the enhanced agro-food wastes (**6–8**) in the presence of corn steep and thiamine have a more intense stimulating result in both the fungal growth and carotenoids production than the simple agro-food wastes media (**2–4**) examined above (Fig. 1).

The HPLC analysis (not shown) and in this case showed as well that the main carotenoid produced in these cases for the enhanced agro-food wastes (**6–8**) is the all-trans-β-carotene (>80%), in contrast to medium **5** that gives a β-carotene accumulation about 63%.

CONCLUSIONS

B. trispora is able to grow into submerged cultures of different origin solid agro-food wastes and produce significant higher amounts of carotenoids, in respect to reference media containing glucose as main carbon source. This is significant for lowering the overall carotenoids biotechnological production cost and mitigating at the same time the wastes disposal environmental problems. Furthermore, the higher β-carotene percentage (over 76%) in all examined cases in respect to reference media, suggests that these agro-food wastes may be used for high purity, large scale β carotene production. In view of the above encouraging results the studies with these wastes will be continued to further optimize the operation conditions (enhancement of the agro-food culture medium with minerals and nutrient, initial concentration of raw agro-food waste, etc.) and further increase carotenoids production.

Acknowledgment

Dr Papaioannou EH is grateful to the State Scholarships Foundation of Greece (IKY) for his postdoctoral scholarship.

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