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Commercial opportunities for carotenoid production by biotechnology

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Abstract: Over 600 different carotenoids have been found in natural sources. These compounds are normal constituents in microorganisms, algae, and higher plants as well as a number of animal species. With the inherent carotenoid biosynthetic capability of natural sources, the commercial biosynthesis of these compounds by appropriate organisms is a way to produce carotenoids that cannot easily be synthesized or cannot be produced economically by chemical synthetic techniques. With the new power of biotechnology and recombinant DNA technology, the biosynthetic capability of organisms to produce carotenoids can be enhanced. This paper reviews previous and current efforts to produce carotenoids by biotechnology. In addition, the use of recombinant DNA technology to increase or modify the carotenoid biosynthetic capability of different organisms is discussed.

INTRODUCTION

The production of carotenoids from biological sources has been an area of intensive investigation. Because of the inherent biosynthetic capacity of a variety of different organisms, there has been a considerable effort to develop systems to produce carotenoids commercially from biological hosts. To date this research effort has met with only marginal success. There are currently only two carotenoids actually being produced from biological sources. Nevertheless considerable interest remains in developing biological systems to produce carotenoids. With the modern techniques of bioprocessing and recombinant DNA technology a number of new efforts are underway to develop systems for carotenoid production by biological systems. This paper reviews the advantages and disadvantages of biological production of carotenoids compared to traditional chemical syntheses. In addition, issues that have limited the widespread use of biological production of carotenoids are discussed. Finally, research programs to develop systems for carotenoid biosynthesis are reviewed along with the use of new recombinant DNA technology to increase the efficiency of carotenoid production.

CHEMICAL AND BIOLOGICAL SYNTHESIS OF CAROTENOIDS

There are several advantages of chemical synthesis for carotenoid production. Chemical synthetic technology has been developed for many carotenoids. The syntheses produce carotenoids of exceptional purity and consistency, and the overall costs of production of these carotenoids are quite low. There are also several disadvantages for production of carotenoids by chemical synthesis. The synthesis of certain carotenoids is very complex. Knowledge and technology developed for the synthesis of one carotenoid may be applicable to the synthesis of other carotenoids, but often the synthesis of a new carotenoid requires the development of a new chemical route. Finally, chemical synthesis produces mixtures of stereoisomers, some of which may not be found in Nature, may not be as active as the naturally occurring carotenoid isomer, may not be desired by the consuming public, or may have undesired side effects.

There are several distinct advantages in the biological production of carotenoids. With the natural production of over 600 different carotenoids, there is a wide range of biosynthetic capability. Because of the characteristics of the biosynthesis of carotenoids, the knowledge of the biosynthesis of one carotenoid is applicable to other carotenoids. With biological production, only the naturally occurring stereoisomers are produced. Finally all the modern tools of bioprocessing and recombinant DNA technology can be used to develop systems for the production of carotenoids.

As with any technology there are also disadvantages for the biosynthesis of carotenoids. Mixtures of carotenoids are often produced in biological systems and may require further processing and purification. In many cases the technology is new and requires considerable research to refine the system. Finally, the overall cost of production is higher than that of a chemical synthetic process.

Research to develop a process for the biosynthesis of carotenoids needs to take account of the inherent advantages and disadvantages of this approach.

Many processes have been developed to use biological production as a way to produce a variety of different products, including enzymes, therapeutic proteins, amino acids, and many other compounds. These existing processes use the host cells as a chemical reactor. The product of interest is often produced in fermentation systems, and the product of interest in most cases is excreted into the medium after it is produced. Excretion into the medium is a key factor in the success of these processes for the following reasons:

- Cells do not accumulate large amounts of the product within the confines of the cells. High concentrations of the product in the cell can lead to a negative feedback on the enzymes responsible for the synthesis of the product. In addition high concentrations of the product in the cell may be toxic and lead to an overall lower metabolism or viability of the cells.
- If the product requires further processing and purification, the cost of purification is lower when the product is present in the medium than if it were a constituent inside the cells. There are fewer components that have similarities to the desired product when the product is found in the medium.

The productivity of the synthesis in these processes is dependent on the metabolic activity of the cells and the cost of the biomass production is not a key determinant in the overall productivity and cost of the process.

Carotenoids are intra-cellular components and cannot be excreted into the medium in a fermentation process. There are several key consequences of this physiological situation. First, the key productivity parameters for biological carotenoid production are the cost of the biomass production, the concentration of the carotenoid of interest inside the cell, and the metabolic activity of the cells producing the carotenoids. Further, if a purified carotenoid is required for the particular application, then the costs of processing and purification are high. Strategies to produce carotenoids in biological systems must take account of the inherent biochemical and physiological constraints of the system.

PRODUCTION OF CAROTENOIDS BY BIOTECHNOLOGY

Carotenoids are currently being produced for animal and human consumption. Carotenoids are used as pigments to color the skin or egg yolks in poultry, to color the flesh of fish grown under aquaculture conditions, and to color the shells of crustaceans. Carotenoids are also widely used as colorants in food for human consumption and also as constituents in vitamins and dietary supplements. There are increasing awareness and opportunities for the expanded use of carotenoids for vitamin and dietary supplement formulations. Convincing evidence has been seen in a number of epidemiological studies and direct clinical studies that suggest a central role for carotenoids as a means of reducing chronic diseases (ref. 1). These studies have shown that a diet rich in carotenoids can lead to a reduced risk of heart disease, cancer, eye disease, and other diseases. With this increased awareness by the vitamin manufacturers as well as the consuming public, there is increasing interest in the biological production of carotenoids by many companies.

There are several commercial operations currently used to produce carotenoids for human as well as animal consumption. The production of β-carotene by the alga *Dunaliella sp.* is a well developed technology.

There are several commercial operations producing β-carotene around the world. The technology capitalizes on the inherent biochemistry of *Dunaliella*. This unicellular alga lacks a cell wall and produces high levels of β-carotene when grown under high salt and high light conditions. Research has been performed to develop specialized strains amenable to large-scale production. In addition processes have been developed for the efficient extraction of β-carotene from the algal cells. This technology and the businesses developed from it have become well established.

Considerable research has been performed to develop other biological systems for the production of β-carotene. In addition, a number of patents have been applied for or granted for methods to develop strains of fungi, algae, or other organisms to produce β-carotene (refs. 2-4). While this research has lead to an increased understanding of the processes needed to produce β-carotene from these organisms, this effort has not yet been successful in establishing new production processes, facilities, or businesses for β-carotene production.

The production of astaxanthin from *Phaffia sp.* has also been extensively studied. *Phaffia* is a yeast that naturally produces astaxanthin. *Phaffia* cells normally produce about 300 ppm per dry mass. This level of production is too low to develop a commercially viable sÿnthesis. Considerable research has been performed to increase the productivity of astaxanthin synthesis in *Phaffia* (refs. 5-7). The result of this effort has been that several companies in the world are producing or about to produce a *Phaffia* product containing astaxanthin for the fish and shellfish aquaculture industry. Research has shown that fish or shell fish can efficiently utilize disrupted cells containing the astaxanthin. Therefore, there is no need to purify the astaxanthin away from the other constituents of the *Phaffia* cells, and processes have been developed that efficiently disrupt *Phaffia* cells.

The single-celled alga *Haematococcus pluvialis* has also been extensively studied as a host to produce astaxanthin (ref. 8). Technology has been developed to take advantage of the physiology of this alga. Under growing conditions this alga does not produce astaxanthin. However, when the culture is subjected to stress in which nutrients are eliminated from the growth medium, then the alga produces and accumulates astaxanthin. The levels of astaxanthin can be very high, and there are reports of astaxanthin accumulation of greater than 4% per dry mass. Under most conditions this level of astaxanthin synthesis and accumulation occurs only after several weeks of growth. Methods to reduce the time required to produce astaxanthin are currently under extensive study by several groups, and pilot scale production is under way at several sites. As with *Phaffia,* cells of *Haematococcus pluvialis* have been found to be a suitable delivery vehicle for astaxanthin for aquaculture and no further purification of the astaxanthin is required.

A number of groups have investigated a variety of organisms and systems to produce various carotenoids. The plant *Adonis aestivalis* produces astaxanthin in the petals of the flower. Researchers have developed varieties of *Adonis* with an increased astaxanthin content (ref. 9). Canthaxanthin is an intermediate in the synthesis of astaxanthin. Mutated strains of *Phaffia rhodozyma* were developed that produce canthaxanthin (ref. 10). Several new species of bacteria have recently been discovered that produce carotenoids. One strain produced astaxanthin, canthaxanthin, β-carotene, and zeaxanthin (ref. 11). These strains were developed to produce higher levels of these carotenoids than is normally found in the wild-type bacterial cells. A new strain of *Corynebacterium sp.* was found to produce canthaxanthin (ref. 12). Strains with increased biosynthetic capacity as well as optimized fermentation systems have increased productivity above that of the wild-type strain. Two new bacteria, *Altermonas sp.* and *Flexibacter sp.,* were discovered to produce zeaxanthin (refs. 13-14). Again strains with improved biosynthetic capacity and productivity were developed. The green alga *Neospongiococcum excentricum* was shown to produce zeaxanthin (ref. 15). High producing strains of this alga coupled with an optimization of the fermentation conditions led to the development of a process to produce 0.65% xanthophylls (dry mass basis). *Flavobacterium multivorum* has been known for many years to produce zeaxanthin. An improved zeaxanthin-producing strain of this bacterium was recently developed which coupled high zeaxanthin productivity with high biomass in a two-day fermentation period (ref. 16).

As can be seen from the discussion above, there has been considerable research and attention to try to develop strains of organisms to produce a variety of carotenoids. The current status of this endeavor is that

there are commercial operations for β-carotene and for astaxanthin. The production cost for these and for the other systems that have been developed is greater than that of the corresponding chemical synthesis. To date the biological production of carotenoids cannot directly compete with chemical synthesis in cost of production.

THE USE OF RECOMBINANT DNA TECHNOLOGY TO PRODUCE CAROTENOIDS

To increase the efficiency of carotenoid production by biological systems, there are currently only two strategies: to increase the efficiency of biomass production and to increase the efficiency of carotenoid synthesis. Improvements can no doubt be made to increase the efficiency of biomass production in selected hosts. However, the improvement is likely to be only comparatively small and not sufficient to lower the overall production costs significantly.

Carotenoid synthesis is governed in the cells by the level and the activity of the carotenoid biosynthetic enzymes. The use of recombinant DNA technology can alter the level and the activity of enzymes. The application of recombinant DNA technology can be used to increase carotenoid productivity, and it is the tool most likely to lead to an increased carotenoid production in biological systems.

There are two requirements for the use of recombinant DNA technology for the production of any compound. The first is the availability of genes and promoters for the enzymes to produce that compound. This requires the isolation and characterization of the genes responsible for the synthesis of the compound as well as promoters that will be active when the genes need to be expressed maximally. The second is a system to introduce the genes into the desired host and have those genes incorporated and expressed in a stable manner through many generations of the cells.

Carotenoid synthesis can be considered in two different parts, the early stages and the later stages. Carotenoids are isoprenoid compounds. There are seven biochemical conversions required to transform the precursor of all isoprenoids, acetyl Co-A, into the C_{15} molecule farnesyl diphosphate. The genes for all these seven enzymes have been isolated and characterized (ref. 17). The genes for the enzymes to convert farnesyl diphosphate to the C_{20} compound geranylgeranyl diphosphate and then on to the first specific carotenoid precursor, phytoene, have been isolated and characterized. A number of different carotenoid biosynthetic genes encoding enzymes that convert phytoene into lycopene, neurosporene, and ζ-carotene have been isolated and characterized as have the genes encoding enzymes that convert these compounds into β-carotene and α-carotene. Finally genes that encode enzymes to convert β-carotene into zeaxanthin, canthaxanthin, astaxanthin, capsorubin, and capsanthin have been isolated and characterized (ref. 17).

Industrial research groups, including the Amoco Biotechnology, Kirin Research, and ICI/Zeneca Seed Research groups, were among the first to recognize the importance of the isolation and characterization of the genes for the carotenoid biosynthetic enzymes, and they led the way in the initial research.

Concurrent with the isolation of the genes for the carotenoid enzymes, several groups developed transformation systems in organisms that are desirable hosts for production of carotenoids. The fungus *Phycomyces blakesleeanus* normally produces β-carotene. A transformation system was developed in which DNA from one strain of *Phycomyces* could be introduced in a stable manner into a different strain by micro-injection (ref. 18). A transformation system was developed for the stable incorporation of foreign DNA into the astaxanthin-producing yeast *Phaffia rhodozyma* (ref. 19). In addition there are currently well established transformation systems in many bacteria and fungi, including those such as *Pichia* and *Saccharomyces*, that are amenable to large scale fermentation, as well as higher plants.

All the tools then are in place to use recombinant DNA technology to increase the productivity of carotenoids. These tools include transformation systems to introduce foreign DNA into desired host organisms, genes encoding enzymes from the core isoprenoid biosynthetic pathway, and genes encoding enzymes for carotenoid biosynthesis.

There are several examples of the use of recombinant DNA technology to increase carotenoid productivity. Research was performed in which the genes for the entire carotenoid biosynthetic pathway from geranylgeranyl diphosphate to zeaxanthin were introduced into baker's yeast (*Saccharomyces cerevisiae*). Additional genes from the core isoprenoid enzymes were added to the same strain. The new strains were subjected to classical strain improvement and a fermentation was developed in which more than 5% carotenoids (dry weight basis) were produced in these cells (ref. 20). Other research targeted the production of carotenoids in higher plants. Increased amounts of the gene for phytoene synthase was found to lead to increased carotenoid levels in higher plants (ref. 20).

The gene for phytoene synthase from tomatoes was isolated and characterized and, when reintroduced into tomatoes, caused an increase of carotenoid synthesis in tomato fruits (ref. 21). Genes for the synthesis of carotenoids in the bacterium *Erwinia uredovora* were also isolated and characterized (ref. 22). These genes were used to produce carotenoids in *E. coli, Zymomonas mobilis*, and *Saccharomyces cerevisiae*. None of these efforts, however, has led to the development of a process to produce carotenoids. It remains to be seen if recombinant DNA technology will finally allow the development of a system for biological production of carotenoids competitive with that of chemical synthesis.

In conclusion processes exist for the biological production of selected carotenoids, but these production methods do not directly compete with the production of the same carotenoids by chemical synthesis. There is increased commercial interest in carotenoids other than β-carotene and a number of research programs are in place in industry and in academia to develop processes to produce carotenoids biologically. All the tools for the use of recombinant DNA technology to produce carotenoids have been identified and are in place to be used to develop biological production processes. These tools include the availability of a number of genes that encode carotenoid biosynthetic enzymes and of transformation systems to introduce these genes into desired hosts. However, the simple application of recombinant DNA technology to increase carotenoid production is not likely to lead to the development of a process for carotenoid production. Strategies need to be developed to target specific carotenoids of interest and to target hosts that have the capabilities of supporting high carotenoid synthesis coupled with low cost biomass production. In addition these targeted carotenoids should be those that lend themselves best to biological production and not those for which efficient chemical syntheses exist. Finally, the production process should take into account the need for purification and must consider how purification costs could be lowered by selecting a host amenable to carotenoid isolation.

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