Cloning, sequencing and expressing of the carotenoid biosynthesis genes of ß-carotene from epiphytic bacteria *Erwinia uredovora* in non carotenogenic bacteria *E. coli.*

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Abstract: Four genes which encode the enzymes of ß-carotene in the epiphytic bacteria *Erwinia uredovora* have been designated as *crt*E, *crt*B, *crt*I and *crt*Y. These genes were cloned in *Eschershia coli* BL21 and located on a 4467-bp fragment whose nuclotide sequence was determined. *E. coli* does not naturally synthesize carotenoids but, by using the carotenogenic genes recombinant strains accumulated ß-carotene where the four genes were expressed as follows: farnesyl diphosphate (FPP) *crt*E geranylgeranyl diphosphate (GGPP) *crt*B phytoene crtI lycopene *crt*Y ß-carotene. *E. coli* BL21 acquired the yellow pigments due to the prescence of ß-carotene after transformed by pGEX-5X-3-*crt*EBIY.
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Introduction:

1. Introduction

Recent developments in the molecular biology of carotenoid biosynthetic from organisms that accumulate different carotenoid product have been provided a variety of genes **(Sandmann, 1994)** that can be employed as a tool for new strategy of heterologous expression in different host organisms. This strategy was used by different researchers to synthesis carotenoid pigments in microorganisms that cannot produce carotenoid naturally such as bacteria (*Escherishia coli*) and yeasts as (*Saccharomyces* and *Candida*). This strategy only works, however, when the substrate specifity of the enzyme is such that does not need to recognize the entire substrate molecule but only certain regions of it that are suitable for conversion. Genes controlling the synthesis of these pigments have been studied in several organisms such as *Erwinia* species **(Misawa *et al*. 1990), (Perry *et al.* 1986, and Schnurr *et al.* 1996),** *Mycobacterium auraum* **(Hossaini-Iraqui *et al*. 1992), Viveiros *et al*. 2000)**, *Xanthophylomyces drorhous* **(Verdoes *et al*. 1999)** and *Brevibacterium linens* **(Krubasik and Sandmann, 2000).**

The aim of metabolic engineering is defined as the purposeful modification networks in living cells to produce desirable chemicals with superior yield and productivity by using recombinant DNA technologies **(Bailey, 1991)** and **(Stephanopoulos, 1994).** Its main field should be investigations undertaken to produce chemicals interest efficiently and abundantly by using appropriate microorganisms **(Ikeda and Katsumata, 1992).** It has traditionally been postulated that microbes naturally synthesizing desirable chemicals should be used as hosts. However, the use of suitable microorganisms which have the ability to produce the precursor for desirable chemicals with superior yield and high levels of productivity is also feasible **(Miura *et al*. 1998).**

This notion significantly extends the range of microbes used as productive hosts. In order to achieve these objectives, three main research approaches are usually employed (i) introducing exogenous genes which convert the final precursor of host organism to a desirable chemical, at a viable yield; (ii) enhancing the metabolic final precursor (this may, for example, be achieved by amplifying rate limiting reactions or eliminating mechanisms feedback inhibition; and (iii) increasing precursor by minimzing metabolic flow to biosynthetically related products **(Shimada *et al.* 1998).**

Gene clusters responsible for the synthesis of carotenoids have been isolated from various carotenogenic bacteria including *Erwinia* species and

the marine bacteria *Agrobacterium auranticum,* and the functions of the genes have been elucidated **(Misawa, 1997).** The first substrate of the enzymes encoded by the *Erwinia* carotenogenic gene clusters is farnesyl pyrophosphate (FPP) which is the common precursor not only for carotenoid biosynthesis but also for numerous other compounds such as sterols, dolichols, hopanols and quinines.

Misawa *et al.* (1990) illustrated that the enzymes and genes which mediate the biosynthesis of cyclic carotenoids such as ß-carotene are virtually unknown. They have elucidated for the first time the pathway for biosynthesis of these carotenoids at the level enzyme-catalyzed reaction, using bacterial carotenoids biosynthesis genes. These genes were cloned from a phytopathogenic bacterium, *Erwinia uredovora* 20D3 (ATCC 19321), in *E. coli* and located in a 6.918- Bp fragment whose nucleotide sequence was determined. Six open reading frame were found and desingnated the C*rtE,* C*rtX,* C*rtY,* C*rtI,* C*rtB* and C*rtZ* genes in reference to the carotenoid biosynthesis genes of photosynthetic bacterium, *Rhodobacter capsulatus*; only C*rtZ* had the opposite orientation from the others. The carotenoid biosynthetic pathway in *Erwinia uredovora* was clarified by analyzing carotenoid accumulated in *E. coli* transformants in which some of these six genes were expressed, as follows: geranylgeranyl pp1 *crt*B prephytoene ppi *crtE* phytoene *crt*I Lycopene c*rt*Y ß-carotene *crtZ* Zeaxanthin *crt*X Zeaxanthin-B-Diglucosidase. The carotenoids in this pathway appear to be close to those in higher plants rather than to those in bacteria.

Wurtzel *et al.* (1997) used the carotenoid biosynthetic gene cluster of *Erwinia uredovora* to condition the expression of colored carotenoids in various heterologous systems including *Escherishia coli*. They assessed fourteen *E. coli* strains for level and stability of expression of *Erwinia uredovora* carotenoid genes. Lycopene and zeaxanthin producing strains were examined for colony pigmentation and for accumulated carotenoids extractable from liquid cultures. *E. coli* Top10F´ culture accumulated almost twice as much as lycopene (approximately 100 µg/g wet cell weight) as compared to *E. coli* SURE cells.

Misawa and Shimada (1998) introduced the *Haematococcus pluvialis ipi* gene and the bacterial *crt* genes (*Erwinia sp.* and *Agrobacterium auranticum*), responible for the biosynthesis of carotenoids to non carotenogenic bacteria (*E. coli*). They found that *E. coli* accumulated more than 1mg/g (0.1%) of lycopene, ß-carotene, zeaxanthin and canthaxanthin, and more than 0.5 mg/g (0.05%) of astaxanthin, on a dry weight basis. If compared in total amounts of the carotenoids synthesized in the cells, the amount in these *E. coli* transformants correspond to tenfold levels of the carotenoids produced by the carotenogenic bacterium *Erwinia uredovora* or *Erwinia herbicola.* Moreover **Sandmann (1999)** mentioned that *E. coli* was found to be very convenient host for heterologous carotenoid production. Most of the carotenogenic genes from bacteria, fungi, and higher plants can be functionally expressed in the bacterium. Furthermore, plasmids belonging to different incompatability groups with different antibiotic resistant markers were used. Meanwhile, **Albrecht *et al.* (1999)** noticed that carotenoid production of *E. coli* can be increased by metabolic engineering of the supply of prenyl pyrophosphate as a precursor for carotenoid production. Transformation with the genes for overexpression of 1-deoxy-D-xylose-5-phosphate reductoisomerase, and isopentenyl pyrophosphate isomerase stimulated carotenogenesis up to 3.5-fold to a final yield of 1.5 mg/gdry weight.

Verdoes *et al.* (1999) showed that the red yeast heterobasidiomycetous *Xanthophyllomyces dendrorhous* (perfect state of *Phaffia rhodozyma*) contain a novel type of carotenoid biosynthetic enzyme. Its structural gene, designated C*rtB*, was isolated by functional complementation in a genetically modified carotenogenic *Escherishia coli* strain. They demonstrated also that the C*rtB* gene encodes a bifunctional protein involved both in synthesis of phytoene from geranylgeranyl diphosphate and in cyclisation of lycopene to ß-carotene. By sequence comparison with other phytoene synthesis and complementation studies in *E. coli* with various deletion dervatives of the C*rtYB* gene, the regions responsible for phytoene synthesis and lycopene cyclisation were located within the protein.

Matthews and Wurtzel (2000) indicated that, carotenoids were not ordinarily present in *E. coli.* Co-overexpression of *E. coli* D-l-deoxyxylulose 5-phosphate synthase (dxs) with *Erwinia uredovora* gene clusters encoding carotenoid biosynthetic enzymes led to an increase accumulation of the carotenoid lycopene or zeaxanthin. Thus, rate controlling enzymes encoded by the carotenogenic gene clusters were responsive to an increase in isoprenoid precursor pools. They found that level of accumulation carotenoid was increased up to 10.8 times when DXS was overexpressed. Lycopene accumulated to a level as high as 1333 µg/g dry weight and zeaxanthin accumulated to a level as high as 592 µg/g dw, when pigments were extracted from colonies. Zeaxanthin producing colonies grew about twice as fast as lycopene- producing colonies throughout a time cource 11 days.

Houssaini-Iraqui *et al.* (2001) declared that, the plasmid PC51, which encodes biogenesis of lycopene in *Mycobacterium aurum* A^+, was partially digested by restriction endonuclease and generated fragments were cloned. After transformation of *E. coli* (colorless bacteria) with the plasmids so constructed, seven orange clones were detected and found to carry the same recombinant plasmid (PC51). *E.coli* containing plasmids synthesized neurosporene, lycopene, and were more resistant to ultraviolet irradiation than non pigmented strain. *E.coli* harboring plasmid pC51 accumulated large amount of lycopene, its yield was 0.25 µg/mg dry wt after 60 h of incubation at 37ºC.

Metabolic engineering of carotenoids and its use in food

Xudong *et al.* (2000) reported that rice (*Oryza sativa*), a major stable food, is usually milled to remove the oil-rich aleurone layer that turn rancid upon storage, especially in tropical areas. The remaining edible part of rice grains, the endosperm, lacks several essential nutrients, such as provitamin A. Thus, predominant rice consumption promotes vitamin A deficiency, a serious public's health problem in at least 26 countries, including highly populated area of Asia, Africa, and Latin America. Recombinant DNA technology was used to improve its nutritional value in this respect. A combination of trans gene enabled biosynthesis of provitamin A in the endosperm. Recently **Paine *et al.* (2005)** indicated that, 'Golden Rice a variety of rice engineered to produce ß-carotene (pro-vitamin A) to help combat vitamin A deficiency, and was predicted that its contribution to alleviating vitamin A deficiency would be substantially improved through even higher ß-carotene content. They hypothesized that the daffodil gene encoding phytoene synthase (psy), one of the two genes used to develop Golden Rice, was the limiting step in ß-carotene accumulation. Through systematic testing of other plant psys, they identified a psy from maize that substantially increased carotenoid accumulation in a model plant system. They went on to develop 'Golden Rice 2' introducing this psy in combination with the *Erwinia uredovora* carotene desaturase (*crt*I) used to generate the original Golden Rice1. They observed an increase in total carotenoids of up to 23-fold (maximum 37 µg/g) compared to the original Golden Rice and a preferential accumulation of ß-carotene.

Fraser *et al.* (2001) mentioned that, the transgenic tomato Lines expressing either with the *Erwinia uredovora* phytoene synthase (*crt*B) or phytoene desaturase (*crt*l) gene and have been produced using the polygalacturonase (PG) or CaMV 35S promoter, respectively. Over three generations, tomato fruits expressing the additional phytoene synthase showed increased total carotenoids approaching twofold. Ripe tomato fruits expressing crtl showed significant increases in beta -carotene (threefold) but a reduction in the total carotenoid content (twofold). The contents of other biosynthetically related isoprenoids, including tocopherols (vitamin E), vitamin A, ubiquinones and plastoquinones, were not altered by manipulations to the carotenoid pathway.

This study was concerned on the microbial sources of carotenoid pigments used in foods. The objectives of this investigation were: Isolation of carotenoid genes from the bacteria *Erwinia uredovora* as *crt*E, *crt*B, *crt*I, and *crt*Y and introducing this genes into pGEX-5X-3 plasmid for protein production; and carotenogensis of *E. coli.*

2. Materials and methods

Bacterial strains: *Erwinia uredovora* (DSMZ; 30080)**,** *Escherishia coli* Top 10, *Escherishia coli* BL21**.**The first bacterial culture was obtained from Deutsche Sammlung für Mikroorganisms und Zellkultur; Germany and used for obtaining the carotenoid genes as *crt*E, *crt*B, *crt*I and *crt*Y. The second culture was obtained from Invitrogen (Munich, Germany) and used as a host for plasmid construction, while the third culture was provided by Amersham, Germany and used for the expression of carotenoid genes. All bacterial strains were grown on Luria Bertani agar medium at 30ºC for 24 hour and was then kept in a refrigerator at 5ºC. Subcultureing was carried out monthly.

Plasmid: pGEX-5X-3 (Amersham, cat., no., 27-4586-01)

Media: The following media were used throughout the present study:

Maintaining media:

***Luria bertani agar medium (LBA),* (Anonymous a, 2002),** This medium was used for maintaining of *Erwinia uredovora* and both strains of *Escherishia coli*. Its composition (g/l) was as the following: Tryptone (10.0), Yeast extract (5.0), NaCl (10.0) Agar (15.0), pH 7.0

Nutrient broth medium: This medium was used for growth of *Erwinia uredovora.* The medium has the following composition (g/l): Peptone (5.0), Meat extract (3.0) NaCl (3.0), pH 7.0

***Luria bertani broth medium (LBA),* (Anonymous a, 2002):** It was used for the growth of all strains of *E. coli*. It has the same composition of medium (2) without the addition of agar.

***SOC medium,* (Anonymous a, 2002):** SOC was used as activating medium for the growth of *E. coli* which transformant with plasmid, the composition of the

medium was as the following (g/l): Tryptone (20.0), Yeast extract (5.0), NaCl (0.5), pH 7.0.

The medium was dispensed into bottles at 100 ml per bottle and autoclaved for 20 min. after cooling, 1 ml of sterile 1 M $MgCl_2$, 1 ml of sterile 250 mM KCl and 2.78 ml of sterile 2 M (36%) glucose were added for each 100 ml of medium.

***2xYT medium,* (Anonymous a, 2002)**

It was used for fusion protein expression in *E. coli* BL21. The medium composed of the following ingredients (g/l): Tryptone (16.0) Yeast extract (10.0), NaCl (5.0), pH 7.0

The medium was prepared and autoclaved at 121°C for 20 minutes. After cooling to 50°C, the medium was aseptically supplemented with 1 ml of 100 mg/ml ampicilline stock solution (final concentration 100µg/ml).

All media used throughout this study were dissolved in distilled water and autoclaved for 20 minutes at 121°C.

Molecular biological methods:

Primers design for PCR reaction

The primers were designated according to the published sequences of genes, c*rtE*, c*rtB*, c*rtI* and c*rtY*, **(Misawa *et al.* 1990);** GenBank accession number D90087) *Erwinia uredovora...* [gi: 22474502]. The designated primers (MWG-Biotech AG; Germany) and its expected size of product were explained in Table (1):

Table (1): primers used in One step - RT-PCR to obtain the expected size of carotenoid genes.

Gene	Primers	Expected size of product
crtE *BamHI*	F., CGC GGA TCC GCG ATG ACG GTC TGC GCA AAA AAA	909 bp
	R., CGC GGA TCC GCG TTA ACT GAC GGC AGC GAG TTT	
crtB *EcoR*I	F., CGC GAA TTC GCG ATG AAT AAT CCG TCG TTA CTC	930 bp
	R., CGC GAA TTC GCG CTA GAG CGG GCG CTG CCA GAG	
crtI *SalI*	F., CGC GTC GAC GCG ATG AAA CCA ACT ACG GTA ATT	1479 bp
	R., CGC GTC GAC GCG TCA TAT CAG ATC CTC CAG CAT	
crtY *Xho*l	F., CGC CTC GAG GCG ATG CAA CCG CAT TAT GAT CTG	1149 bp
	R., CGC CTC GAG GCG TTA ACG ATG AGT CGT CAT AAT	

F: Forward primer; R: Reverse primer

Isolation and purification of total RNA

Total RNA was isolated from *Erwinia uredovora* (DSMZ, 30080) using RNeasy mini kits **(Anonymous a, 2002).** The cells were grown in nutrient broth medium. Bacterial cells were harvested at logarithmic phase (18 hours), and then centrifuged at 4000 rpm for 5 min. at 4ºC. The supernatant was decanted, and all remaining media were removed by aspiration. The bacteria were resuspended thoroughly in 100 µl of the lysozyme (Amersham, Karlsruhe, Germany), (containing Tris-EDTA (TE) buffer) (400 µg/ml) and incubated at room temperature for 3-5 min. To complete digestion of the cell wall, 250 µl buffers RTL was added to the sample and mixed thoroughly using vortex (Vortex genie 2, Roth, Karlsruhe, Germany) vigorously then 250µl ethanol (96-100%) was added to the lysate and mixed throughly by pipetting. The sample (usually 700 µl), including any precipitate that may have formed, was transferred to an RNeasy mini column placed in a 2 ml collection tube (supplied). The tube closed gently, and centrifuged for 15 s at 10,000 rpm (8000 xg). The flow was discarded then, 700 µl buffer RW1 was added to the RNeasy column. The flow was discarded then the Rneasy column was transferred into a new 2 ml collection tube (supplied). A 500 µl buffer RPE was piptted onto the Rneasy column. The tube was closed gently, and centrifuged for 15 s at 10.000 rpm (8000 xg) to wash the column. The flow was discarded then, another 500 µl buffer RPE was added to the RNeasy column. The tube closed gently, and centrifuged for 2 min at > 10,000 rpm (8000 xg) to dry the Rneasy silica gel membrane. The RNeasy column was placed in a new 2ml collection tube centrifuge in a microcentrifuge at full speed for 1 min. To elute, transferred the RNeasy column to a new 1.5ml collection tube (supplied). Pipet 30-50 RNase-free water was pipetted directly onto the RNeasy silica-gel membrane, the tube closed gently, and centrifuged for 1 min 10,000 rpm (8000 xg).

***OneStep RT-PCR* (Anonymous b, 2002)**

The master mixture was prepared as reported in Table (2) after thawing their components:

Table (2): Reaction components for OneStep RT-PCR

Component	Volume/Reaction	Final concentration
Master mixure -RNase-free water(provided)	Variable	-
-5x QIAGEN OneStepRT-PCR		
Buffer	10µl	1x
-dNTP mix containing 10 mM of each		
	2.0µl	40µm
-Primer A	Variable	0.6µm
-Primer B	Variable	0.6µm
-QIAGEN OneStepRT-PCR Enzyme mix	2.0µl	-
-RNase inhibitor (optional)	Variable	5-10 umit/reaction
-Template RNA	Variable	1pg –2µg/reaction
-Total volume -	50.0 µl	-

The master mixture was thoroughly, and appropriate dispensed volumes were placed in a clean 1.5 ml PCR tubes. Template RNA (< 2 µg /reaction) was added to the individual tubes, then heated using a thermal cycler (T3-thermocycler, Biometra, Germany) with a heated lid, programmed the thermal cycler according to the program outlined below; Thermal cycler conditions:

1-Reverse transcriptase	30 min 50°C
2-Intial PCR activation step	15 min 95°
3-Step-cycling	
Denaturation	0.5-1 min 94°C
Anealing	0.5-1 min 50-68°C
Extension	1 min 72°C
Number of cycles	25-40
Final extension	10 min 72°C

RT-PCR program was started while PCR tubes are still on ice and until thermal cycler has reached to 50°C, the PCR tubes were placed in the thermal cycler.

Isolation and Purification of plasmid DNA

Plasmid DNA was isolated from *E. coli* Top 10 F and purified **(Anonymous a, 2002)** using miniprep kits. This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1-5 ml overnight cultures of *E. coli* in Luria bertani (LB) broth medium.

Procedure

The bacterial cell pellets were resuspended in 250 µl buffer (P1) and transferred to a microcentrifuge tube. 250 µl buffer (P2) was added, mixed gently. Then, 250 µl buffer (N3) was added, mixed the tube gently 4-6 times then centrifuge for 10 min at 13.000 rpm (~17.000 xg). The obtained supernatants were applied to QIAGEN Spin column by pipetting and centrifuged for 30-60 s then, the flow was discarded. The QIAGEN Spin column washed by adding 0.5ml buffer (PB) then 0.75ml buffer (PE) by centrifugation for 30-60s was applied after each treatment and the flow was discarded. The QIAPrep columns were placed in a clean 1.5 ml microcentrifuge tubes, then DNA, 50 µl buffer EB (10 mM tris-HCl, pH 8.5) or water were added to the centers of each QIAprep Spin column, let stand for 1 min, and centrifuge for 1 min.

DNA digestion with restriction enzyme (Anonymous b, 2002)

An analytical scale restriction enzyme digest is usually performed in a volume of 20 µl on 0.2-1.5 µg of substrate DNA, using 2 to 10 fold excess of enzyme over DNA. The following example of a typical RE digests. In a sterile tube, assemble in order:

Component	Volume (µl)
Sterile or deionized water	16.5
RE 10x buffer	2
DNA, 1µg/µl	1
Mix by pipetting, and then add:	
Restriction enzyme, 10 u/µl	0.5
Final volume	20

The tube was gently mixed, centrifuged for a few seconds in a microcentrifuge, then incubate at 37ºC for 1-4 hours.

***Restriction digestion of pGEX vectors* (Anonymous a, 2002):**

Reagents:

pGEX DNA, 10x One-phor-All buffere plus (OPA+): 100 mM tris actate, 100 mM magnesium acetate, 500 mM potassium acetate, pH 7.5 (optional) restriction enzyme.

Step:

The recommended final DNA concentration in the reaction mixture was 0.1 µg/µl, 5 µg of pGEX DNA, 5-10 µl of 10x One-phor-all buffer plus (OPA^+) or buffer supplied with enzyme, 5-10 µl of optional components (e.g. BSA, Triton x-100, NaCl etc.), 10-25 units of restriction enzyme, and H_2O to 50 µl. The mixture was incubated at 37ºC for 2-16 h.

5µl aliquot of the reaction was examined in agarose gel electrophoresis to verify that the pGEX DNA has been digested.

***Dephosphorylation of linearized pGEX vector* (Anonymous a, 2002)**

Reagents:

-Calf intestinal alkaline phosphatase

-10x one-phor-all-buffer plus (OPA^+): 100 mM tris acetate, 100 mM magnesium acetate, 500mM potassium acetate, pH 7.5, Phenol: redistilled phenol saturated with TE buffer containing ß-hydroxy Quinoline (17), Chloroform /isoamyl alcohol, mixed 24:1. 3M sodium acetate, pH 5.4, aqueous solution, Ethanol (70, 95%). TE buffer: 10 mM tris-HCl, pH 8.0, 1 mM EDTA

Steps:

Calf intestinal alkaline phosphatase was diluted using 1x OPA^+ (10x OPA^+ was diluted with sterile H_2O) where 1-2 µl of diluted enzyme should provide 0.1 unit to the reaction. 0.1 units (1-2 µl of diluted enzyme) of alkaline phosphatase was added to the digested pGEX DNA and incubated for 30 min at 37°C, then heated at 85°C for 15 min to inactivation the alkaline phoshatase. An equal volume of phenol was added to the aqueous sample, vortex for 1 min and centrifuged for 5 min at full speed to separate the phases. The upper aqueous phase was transferred to a fresh tube, vortex an equal volume of chloroform /isoamyl alcohol was added, for 1 min then centrifuged for 5 min at full speed to separate the phases. The upper aqueous phase was transferred to a fresh tube, and then 0.1 volume of 3M sodium acetate, pH 5.4 and 2.5 volumes of 95% ethanol were added. The tubes were mixed and placed at -20°C for 15 min. The tubes were centrifuged at 4°C for 15 min, the supernatant was removed and the pellet washed with 1 ml of 70% ethanol. Recentrifugation was carried out for 2 min, and then drained thoroughly. The DNA pellet was dissolved in 10-20 µl of TE buffer.

***Ligation of the insert DNA to pGEX DNA* (Anonymous a, 2002)**

The following reaction mixture was prepared, 1-5 µl of linearized pGEX DNA, 1-5 µl of insert DNA, 2 µl of 100 mM ATP, 0.5-15 units of T4 DNA Ligase and H_2O to 20 µl. For Cohesive ends Incubation was carried for 1-4 h at 10°C. The reaction was terminated by heating at 65°C for 10 min.

Transformation of competent cells

The transformation of competent cells was proposed by **Blasco, (2002)** as follows:

For each ligation reaction as well as for uncut vector control and the negative control (untransformated) competent *E. coli* Top 10 F (host cell), 1 ml of freshly prepared comptent *E. coli* host cells was added and to separate prechilled 50 ml sterile disposable centrifuged the tubes then, stored on ice.

20 µl of each ligation reaction or 1 ng of uncut vector was added to the competent cell swirled gently to mix, and place on ice for 45 min. No DNA was added to the negative control but instead 20 µl of sterile distilled H_2O were added. The tubes were incubated in a water bath 42°C for 2 min., and then chilled briefly on ice. For each sample, immediately transformed cells, by adding 900 µl of SOC medium (med.6) prewarmed to 37 °C and incubated for 1 hour at 37°C with shaking (250 rpm). 100 µl of the diluted cells, transformed cells from ligated samples and 10 µl of the diluted transformed cells from the uncut vector sample were plated onto separate LB agar. Also 100 µl of the untransformed competent *E. coli* host cells was plated onto LB agar. The plates were incubated at 37°C overnight, and then proceed to screening using procedures. To prepare a frozen stock culture, 100 µl of the diluted transformed cells containing the pGEX DNA was added to 1 ml of LB broth medium and incubate for 30 min at 37°C with shaking at 250 rpm. After incubation 200 µl of sterile 80% glycerol was added and mixed by a pipette tip then, stored at –70°C.

Screening

Screening is needed to verify that the insert is in the proper orientation and the correct junctions are present such that the reading frame is maintained. Screening was carried out using Polymerase Chain Reaction (PCR) according to the method described by **(**Anonymous, 2002).

The following components were mixed in 0.65 ml tube: 10 µl of 10x Taq polymerase buffer, 16 µl of dNTP mix, 5 µl of pGEX 5´ sequencing primer, 5 µl of crt 3`sequencing primer H_2O to 99.5µl. Gently asterile micropipet tip was taught to the bacterial colony to be screened to the above PCR mixture. Pipettes gently to disperse bacterial cell. 0.5 U/µl Taq DNA polymerase was added. Amplified was done in thermal cycler (Biometra) as the following cycle parameters:-

35 cycles (from step 2 to step 4)

1- 95°C	for 10 min
2- 94°C	for 1min
3- 57°C	for 1min
4- 72°C	for 1min
5- 72°C	for 1min
6- 4°C	end.

Separation of DNA molecules

From the previously prepared PCR, 20-40µl were analyzed by agarose gel according to the method described by **Blasco, (2002).**

Preparation

Agarose powder was added to TAE buffer to a final concentration 1% (W/V). The slurry was heated in a microwave oven (Moulinex) until the agarose was dissolved then cooled to 60°C; 1µl of ethidium bromide (1%) agarose solution was added to 10 ml agarose. The warm agarose was poured into the mold and the comb was properly positioned. After the gel was completely set it was transferred into electrophoresis tank and covered with TAE buffer. DNA samples (20-40µl) were mixed with DNA loading buffer and loaded into slots of gel. The size used standard 1 Kb ladder. Electrophoresis was performed at 100 V for 30 minutes the gel was examined by the ultraviolet light and documented**.**

Storage and cultivation of bacteria

Stab culture

It was prepared according to **Blasco (2002)**. 1 ml LB agar (med.2) was autoclaved in 1.5 ml Eppendorf tube. A single colony of *E. coli* BL21 was picked from agar plate and stabbed by means of a sterile inoculation needle. The vial was stored at room temperature in the dark.

Long term storage of cells

Long term storage of cells was performed as glycerol stock, using the method described by **Blasco, (2002).** One ml of sterile glycerol (87%) was added to 1 ml of overnight culture (LB broth medium). The mixture was vortexed to ensure that the glycerol was evenly dispersed and then stored at –80°C. To recover the bacteria the surface of the frozen culture was scraped with a sterile needle and then streaked onto the surface of LB agar plate containing 100 µg/ml ampicilline.

Cultivation in Erlenmeyer flasks

A single colony of *E. coli*BL21 was picked from an agar plate and inoculated in fresh liquid medium with ampicilline (50 µg/ml). One ml of overnight culture (LB broth medium), was used to inoculate 100 ml fresh medium containing ampicilline (50µg/ml). Liquid cultures were normally incubated at 37°C. For experiment related to *Erwinia uredovora*, nutrient agar medium was used.

Hetrologous expression in E. coli BL21

Over-expression was carried out in *E. coli* BL21 cells and was controlled by the strong *tac* promoter according to the method described by (Anonymous, 2002*)*. Induction was performed by addition of 100 mM Isopropyl ß-D thiogalactoside (IPTG). An overnight culture was used to inoculate 100 ml 2xYT medium with ampicilline (100 µg/ml). The cells were grown at 37 °C and 250 rpm to O.D about 1 and then IPTG 100 mM was added. After induction the culture was incubated at 30°C at shaking rate 150 rpm for 4-6 hours. Finally the culture was centrifuged at 4500 rpm, for 20 min. at 4 °C and the pellet stored at –20 °C until used for lysis**.**

Cell lysis and purification

Cultures were harvested by centrifugation and cell lyses by homogenizer for 60 sec, in lysis buffer (1x PBS, 1% triton-100 and tablet of protease inhibitor (Roche, Germany). Cell debris was removed by centrifugation at 4500 rpm for 15 min. The supernatant containing the protein of interest and also *E. coli* proteins so, the protein must be purified by using gluthathione sepharose 4B medium **(Blasco, 2002).**

Protein separation

It was carried out as recommended by **Blasco, (2002)**, by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to assess the purity and estimate the molecular weight of protein. Protein migration to the gel when an electric current is applied. The rate at which the proteins move in the gel is affected by their size (smaller protein move faster in the gel).

Preparation

After assembling the glass plates properly, the resolving gel was prepared and poured into the gap between the glass plates and allowed to polymerize after overlaying gently with isopropanol. After the separating gel had polymerized, the overlay was decanted, stacking gel was prepared and poured. A comb was inserted into the stacking gel solution. After stacking gel had polymerized the comb was removed and the gel mounted in electrophoresis chamber. The samples 5 µl in SDS gel loading buffer. Finally they were loaded up into the wells of the gel together with low molecular weight standard (LMW). For each gel the electrophoresis was carried out at 10 mA for 10 min and 25 mA for approximately 50 min. After electrophoresis proteins were fixed with the staining solution. The excess of dye (coomassie) was then allowed to diffuse from the gel during a prolonged period of destaining.

3-Results and Discussion

From gene isolation to protein production:

-Purification of total RNA of Erwinia uredovora

Figure (1) shows the total RNA of *Erwinia uredovora* which extracted after the bacteria were grown in nutrien broth medium for 18 hours (logarithmic phase), the bands of RNA were shown under U.V lamp after loaded on a 1% agarose gel and stained with ethidiuom bromide.

pGEX-5X-3 Expression vector

Figure (2) shows the map of the plasmid pGEX-5X-3 which was used in this study. This vector conatined GST fusion protein and constructed by inserting a gene or gene fragment into the multiple cloning site of it. Expression under the control of the tac promoter, which is induced by the lactose analog isopropyl B-D thiogalactoside (IPTG). All pGEX-

5X-3 vectors are also engineered with an internal lacI^q gene. The lacI^q gene products is a repressor protein that binds to the operator region of the tac promoter, preventing expression until induction by IPTG, thus maintaining tight control over expression of insert.

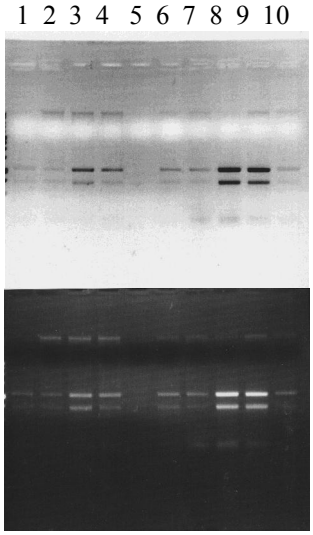


Fig. (1): Total RNA of *Erwinia uredovora*, Lanes 1,2,3,4,5,7,8,9,10 and 11 contains 2µl of R

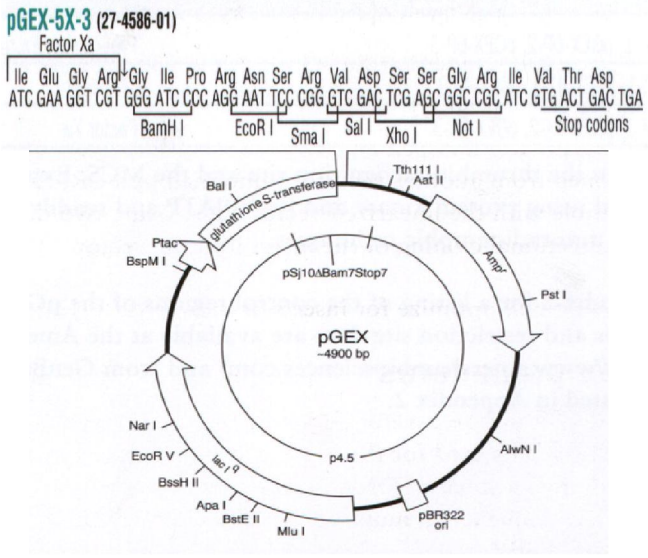


Fig. (2): Map of the glutathione sepharose transferas fusion vectors showing the open reading frames of the main feature **(Anonymous a, 2002).**

One Step Reverse Transcriptase –Polymerase Chain Reaction (RT-PCR)

One Step RT-PCR Amplification assay has been employed to transcript the carotenogenic genes, c*rt*E, c*rt*B, c*rt*I, and *crt*Y of *Erwinia uredovora*. For this purpose, primers for carotene biosynthetic gene (forward and reverse) were designed from published genomic DNA sequences of the *crt*E, c*rt*B, c*rt*I, and c*rt*Y **(Misawa *et al.* 1990)**; GenBank accession number D90087. Primers were designed to flank the sequences of the targeted genes (open reading frames). Each primer to be used for PCR had an added restriction enzyme digest site (the flanking restriction enzyme site) on the 5`of the primer *Erwinia uredovora*... [gi: 22474502]. The expected fragment length of each RT-PCR product is shown in Figs. (82A, 83A, 84A and 85A) showed the bands of c*rtE* gene (GGPP synthase), c*rt*B (phytoene synthase), c*rt*I (lycopene synthase), c*rt*Y (lycopene cyclase) and its expected size (909, 930, 1479, and 1149 bp respectively) under U.V lamp after loaded on a 1% agarose gel and stained with ethidium bromide.

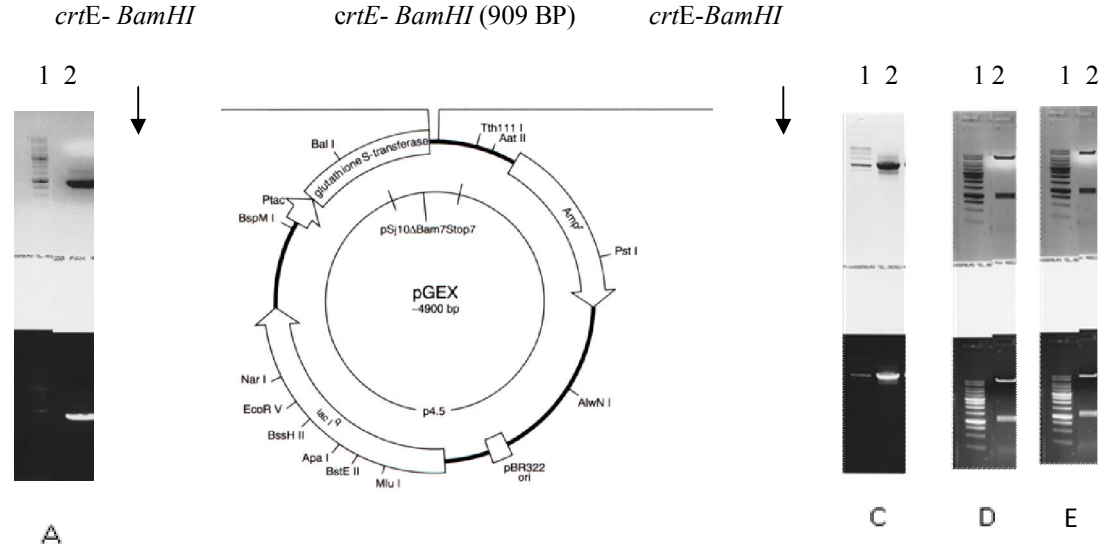
Generation and verification of pGEX-5X-3 plasmids:

The PCR products of each gene were purified from the One Step RT-PCR reaction. A pGEX5X-3-cloning was then performed on each separate PCR as pGEX5X-3-c*rt*E, pGEX5X-3-c*rt*B, pGEX5X-3-c*rt*I, *and* pGEX5X-3-c*rt*Y. Figs.(3, 4, 5 and 6) showed that the four genes (*crt*E, *crt*B, *crt*I and *crt*Y respectively) were inserted and ligated into the pGEX5X-3. The ligated plasmids were transformed individually into chemically–component of *E. coli* TOP10 cells and plated onto LB ampicilline plates. Some of the colonies were picked and minipreparations were performed. Figs (3C, 4C, 5C and 6C) showed the PCR product of each gene obtaineed from the colony that resulted from the transformation of *crt*E, c*rt*B, c*rt*I and c*rt*Y into *E.coli* respectively. The restriction enzymes digests were performed to verify that the expected PCR products had been incroporated into pGEX5X-3. The BamHI digest was performed on the plasmid of pGEX5X-3-c*rt*E Fig. (3D). Similary EcoRI digest was performed on the plasmid of pGEX5X-3-c*rt*B Fig. (4D); SalI digest was performed on the plasmid of pGEX5X-3-c*rt*I, Fig. (5D) and XhoI digest was performed on the plasmid of pGEX5X-3-c*rt*Y*;* Fig.(6D) in order to check the size of the insert. Minipreparations were then performed on all samples that appeared correct after the three verification digests. Minipreparation samples that appeared correct were then sequenced Figs.(82E, 83E, 84E; and 85E) showed the sequences of *crt*E, c*rt*B, c*rt*I and c*rt*Y and respectively by the Agowa company (Germany) and verified by comparing them to the provided sequence of **(Misawa *et al.* 1990)**; GenBank accession number (D90087). *Erwinia uredovora*[gi:22474502].

Introducing of expression vectors into E. coli BL21

In order to introduce the expression vectors into *E. coli* BL21. The purified plasmids, which were

obtained by minipreparation from *E. coli* Top 10F´, were digested with the suitable restriction enzymes. The purified fragments were then ligated in only one pGEX5X-3 begining with c*rt*E, c*rt*B, c*rt*I, and c*rt*Y. The ligated plasmid which contains the four genes were transformed into *E. coli* BL21 and grown on LB plates containing ampicilline 50µg/ml. The growing colonies were then picked and minipreparations were performed. The plasmid was verified by four restriction enzymes used. Plasmids that produced the expected fragments in all of the minipreparation verification digests were then isolated on a larger scale via the minipreparation protocol. Four restriction enzymes digests, including the droupout digest as shown in Fig. (7).



F

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CATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTCCAAAATCGG
ATCTGATCGAAGGTCGTGGGATCCGCGATGACGGTCTGCGCAAAAAAACACGTTCATCTC
ACTCGCGATGCTGCGGAGCAGTTACTGGCTGATATTGATCGACGCCTTGATCAGTTATTG
CCCGTGGAGGGAGAACGGGATGTTGTGGGTGCCGCGATGCGTGAAGGTGCGCTGGCACCG
GGAAAACGTATTCGCCCCATGTTGCTGTTGCTGACCGCCCGCGATCTGGGTTGCGCTGTC
AGCCATGACGGATTACTGGATTTGGCCTGTGCGGTGGAAATGGTCCACGCGGCTTCGCTG
ATCCTTGACGATATGCCCTGCATGGACGATGCGAAGCTGCGGCGCGGACGCCCTACCATT
CATTCTCATTACGGAGAGCATGTGGCAATACTGGCGGCGGTTGCCTTGCTGAGTAAAGCC
TTTGGCGTAATTGCCGATGCAGATGGCCTCACGCCGCTGGCAAAAAATCGGGCGGTTTCT
GAACTGTCAAACGCCATCGGCATGCAAGGATTGGTTCAGGGTCAGTTCAAGGATCTGTCT
GAAGGGGATAAGCCGCGCAGCGCTGAAGCTATTTTGATGACGAATCACTTTAAAACCAGC
ACGCTGTTTTGTGCCTCCATGCAGATGGCCTCGATTGTTGCGAATGCCTCCAGCGAAGCG
CGTGATTGCCTGCATCGTTTTTCACTTGATCTT
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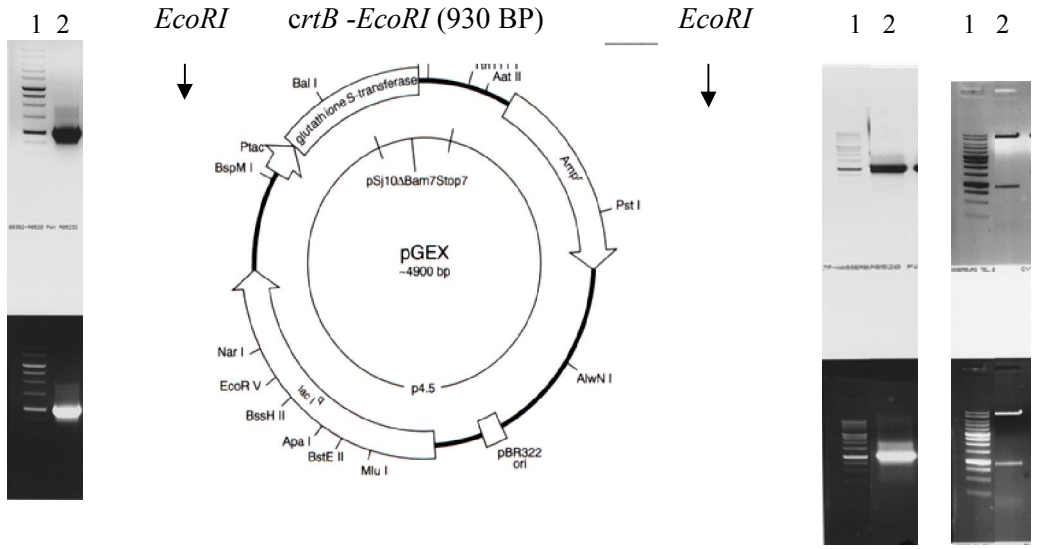
Fig. (3): A, lane1: contain the 1kb laddere and lane 2 contains the *crt*E on step pcr product that created using primers of *crt*E-*BamHI* down and *crt*E-*BamHI* up.(B), pGEX-5x-3 vector + *crt*E inserted cloning.(C), lane1 contains 1k ladder and lane2 pcr product by using GST primer and *crt*E AS.(D), lane1 contains 1kb ladder and lane 2 contains *BamHI* digest of pGEX-5x-3-*crt*E.(E), was the part of *crt*E-GST gene that was done by Agowa company for verifying the correct insert.

Expression in E. coli BL21

Four expression vectores were created that individually expressed the c*rt*E, c*rt*B, c*rt*I, c*rt*Y genes. The expression vectors were then transformed into *E. coli* TOP 10F´ then into BL21 and iduced by isoprpyl ß-D thiogalactoside (IPTG) to express their respective genes.

For expression the protein of each carotenoid gene in *E. coli* BL21 under conditions minimize inclusion body formation. Fig.(8A) showed the *crtE* protein and its molecular weight of 32.5 kDa was detected linked with GST protein 26 kDa, (lane: 4). Fig. (8B) showed the *crtB* protein and its molecular weight of 34 kDa was detected linked with GST protein 26 kDa, (lane: 4). Fig.(8C) showed the *crtI* protein and its molecular weight of 55 kDa was detected linked with GST protein 26 kDa, (lane: 4). Fig.(8D) showed the *crtY* protein and its molecular weight of 43 kDa was detected linked with GST protein 26 kDa, (lane: 4) after purification by glutathione sepharose 4B medium and eluted by elution buffer (50mM Tris-HCl, 10 mM reduced glutathione, pH 8.0).



crtB-EcoRI-GST

E CAGTATTAGCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGC
GA
CCATCCTCCAAAATCGGATCTGATCGAAGGTCGTGGGATCCCCAGGAATT
CGCGATGAATAATCCGTCGTTACTCAATCATGCGGTCGAAACGATGGCAG
TTGGCTCGAAAAGTTTTGCGACAGCCTCAAAGTTATTTGATGCAAAAACC
CGGCGCAGCGTACTGATGCTCTACGCCTGGTGCCGCCATTGTGACGATGT
TATTGACGATCAGACGCTGGGCTTTCAGGCCCGGCAGCCTGCCTTACAAA
CGCCCGAACAACGTCTGATGCAACTTGAGATGAAAACGCGCCAGGCCTA
TGCAGGATCGCAGATGCACGAACCGGCGTTTGCGGCTTTTCAGGAAGTGG
CTATGGCTCATGATATCGCCCCGGCTTACGCGTTTGATCATCTGGAAGGC
TTCGCCATGGATGTACGCGAAGCGCAATACAGCCAACTGGATGATACGC

Fig. (4): A, lane1: contain the 1kb laddere and lane 2 contains the *crtB* one step pcr product that created using primers of *crt*B-*EcoRI* down and *crt*B-*EcoRI* up.(B), pGEX-5x-3 vector + *crt*B inserted by cloning.(C), lane1 contains 1k ladder and lane2 pcr product by using GST primer and *crt*B AS.(D), lane1 contains 1kb ladder and lane 2 contains *EcoRI* digest ofpGEX-5x-3-*crt*B.(E), was the part of *crt*B-GST gene that was done by Agowa company for verifying the correct insertion.

Sequence analysis and plasmid construction

The nucleotide sequence of the fragment 4467 bp in length was determined. linked i.e., the stop codon of each ORF overlapped the start codon of the following ORF. The designation that we have assigned these ORFs to the counterpart genes of *Erwinia uredovora* i.e., *crt*E, *crt*B, *crt*I, and *crt*Y. Four open reading frames (ORFs) with the same orientation (Fig. 9). They are closely physically linked i.e., the stop codon of each ORF overlapped the start codon of the following ORF. The designation that we have assigned these ORFs to the counterpart genes of *Erwinia uredovora* i.e., *crt*E, *crt*B, *crt*I, and *crt*Y.

Escherishia coli as a heterologous host for carotenoid biosynthesis:

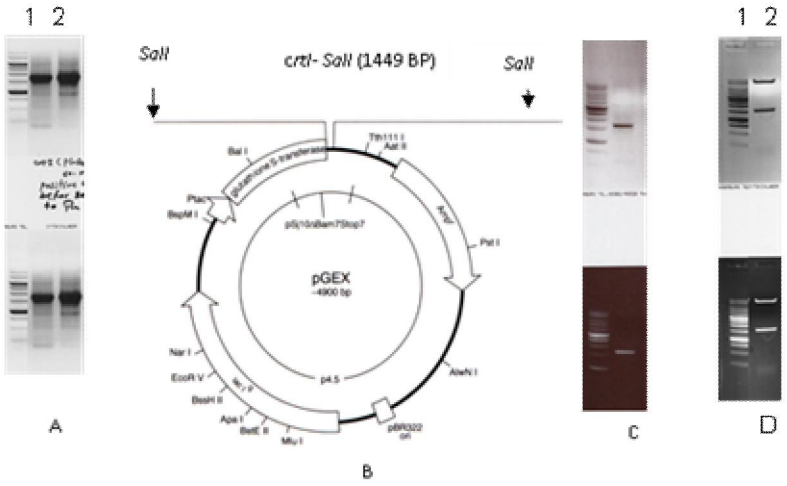
E. coli was the first heterologus host in which carotenoids were produced. In the course of cloning of a gene cluster from *Erwinia uredovora* yellow pigmentation *of E. coli* cells was observed (**Perry *et al.* 1986).**

E. coli is a very convenient host for heterologous carotenoid production. Most of the carotenogenic genes from bacteria, fungi, and higher plants can be functionally expressed in this bacterium. Furthermore, plasmids belonging to different incompability groups with different antibiotic resistance markers are available. They can all be introduced simultaneously in *E. coli* for carotenoid synthesis allowing combinations of ndividual genes. The potential of E. coli as carotenoid production system has been reviewed recently, **(Sandmann, 1999)**

Production of ß-carotene in E. coli BL21

Bacteria such as *E. coli* contain isoprenoid compounds such as dolichols (sugar carrier lipid) and the respiratory quinones ubiquinone and menaquinone **(Sherman *et al.* 1989*)*.** FPP is the common precursos of the bacterial isoprenoids.

It is thus feasible to direct the carbon flux for the biosynthesis of this isoprenoid compounds partially to the pathway for carotenoid production by the introduction of carotenogenic genes starting with the Erwinia *crt*E gene. The *crt* genes derived from *Erwinia uredovora* were successfully used for the de novo biosynthesis of ß-carotene in *E. coli* and other *bacteria* **(Misawa *et al.* 1990; Ausich *et al.* 1991).**



*crt*I-SalI-GST

CAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTAAACCAAC
TACGATAAATGGTGCAGGCTTCGGTGGCCTGGCACTGGCAATTCGTCTACAAGCTGCGGGGATCCCCG
TCTTACTGCTTGAACAACGTGATAAACCCGGCGGTCGGGCTTATGTCTACGAGGATCAGGGGTTTACC
E TTTGATGCAGGCCCGACGGTTATCACCGATCCCAGTGCCATTGAAGAACTGTTTGCACTGGCAGGAAA
ACAGTTAAAAGAGTATGTCGAACTGCTGCCGGTTACGCCGTTTTACCGCCTGTGTTGGGAGTCAGGGA
AGGTCTTTAATTACGATAACGATCAAACCCGGCTCGAAGCGCAGATTCAGCAGTTTAATCCCCGCGAT
GTCGAAGGTTATCGTCAGTTTCTGGACTATTC

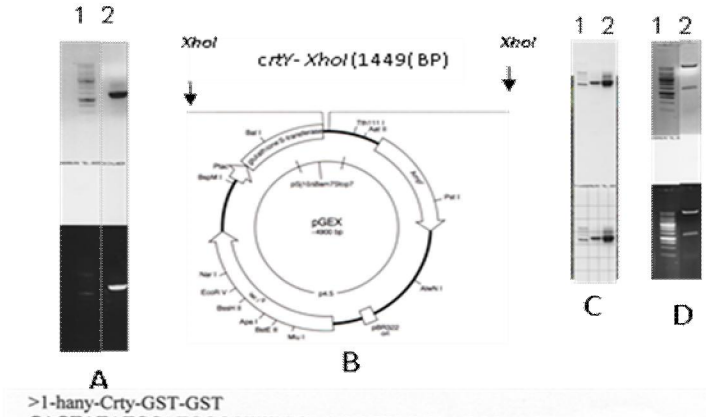
Fig.(5): A, lane1: contain the 1kb laddere and lane 2 contains the *crt*I one step product that created using primers of *crt*I-*SalI* down and *crt*I-SalI up.(B), pGEX- 5x-3 vector + *crt*I inserted by cloning.(C), lane1 contains 1k ladde and lane2 pcr product by using GST primer and *crt*I AS.(D), lane1 contains 1kb ladder and lane 2 contains SalI digest of pGEX-5x-3-*crt*I.(E), was the part of *crt*I-GSTgene that was done by Agowa company for verifying the correct insertion.

Fig. (10) showed carotenoid accumulation in *E. coli BL21* colonies after transformation with *Erwinia uredovora* gene cluster confering ß-carotene biosyntheis. The pigmentation of colonies converted to the yellow colour after incubation the plates in a dark place for about 15 days.

Crt*E:* when plasmid pGEX-5X-3 carrying the *Erwinia uredovora crt*E gene was introduced into *E. coli* transformant accumulating GGPP. The predicted 33-kDa *crt*E protein of *Erwinia uredovora.*

***Crt*B**: when plasmid pGEX-5X-3 carrying the *Erwinia uredovora crt*B gene was introduced into *E. coli* transformant accumulating GGPP, as a result of the presence of plasmid pGEX-5X-3, the transformant synthesized phytoene (15,15`-cis). It is therefore evident that the *crt*B gene encodes phytoene synthase, which catalyzes the condensation reaction of two molecules of GGPP to produce 15, 15`-cis-phytoene. This function of phytoene synthase seems to be conserved among various species. The predicted 34-kDa *crt*B protein of *Erwinia uredovora* shows significant amino acid similarity to the phytoene synthase from any carotenogenic organisms.

***Crt*I**: when plasmid pGEX-5X-3 carrying the *Erwinia uredovora crt*I gene was introduce into *E. coli* transformant, as a result of the presence of plasmid pGEX-5X-3 -*EB*; the transformant produced lycopene (all trans). It is therefore clear that the *crtI* gene codes for phytoene desaturase, which is responsible for the conversion of 15, 15´-cis-phytoene to all-trans-lycopene. The pedicted 55 kDa *crt*I protein of *Erwinia uredovora.*



>1-hany-Crty-GST-GST
CAGTATATGCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTC
CAAATCGGATCTGATCGAAGGTCGTGGGATCCCCAGGAATTCCCGGGTCGACTCGAGGCG
ATGCAACCGCATTATGATCTGATTCTCGTGGGGGCTGGACTCGCGAATGGCCTTATCGCC
CTGCGTCTTCAGCAGCAGCAACCTGATATGCGTATTTTGCTTATCGACGCCGCACCCCAG
GCGGGCGGGAATCATACGTGGTCATTTCACCACGATGATTTGACTGAGAGCCAACATCGT
TGGATAGCTCCGCCGGTGGTTCATCACTGGCCCGACTATCAGGTACGCTTTCCCACACGC
CGTCGTAAGCTGAACAGCGGCTACTTTTGTATTACTTCTCAGCGTTTCGCTGAGGTTTTA
CAGCGACAGTTTGGCCCGCACTTGTGGATGGATACCGCGGTCGCAGAGGTTAATGCGGAA
TCTGTTCGGTTGAAAAAGGG

Fig.(6): A, lane1: contain the 1kb laddere and lane 2 contains the *crt*Y one ste product that created using primers of crtY-*XhoI* down and *crt*Y-*XhoI* up.(B), pGEX-5x-3 vector + *crt*Y inserted by cloning. (C), lane1 contains 1k ladder and lane2 pcr product by using GST primer and *crt*Y AS.(D), lane1 contains 1kb ladder and lane 2 contains *XhoI* digest of pGEX-5x-3-*crt*Y.(E), was the part of *crt*Y-GST gene that was done by Agowa company for assure the correct insertion

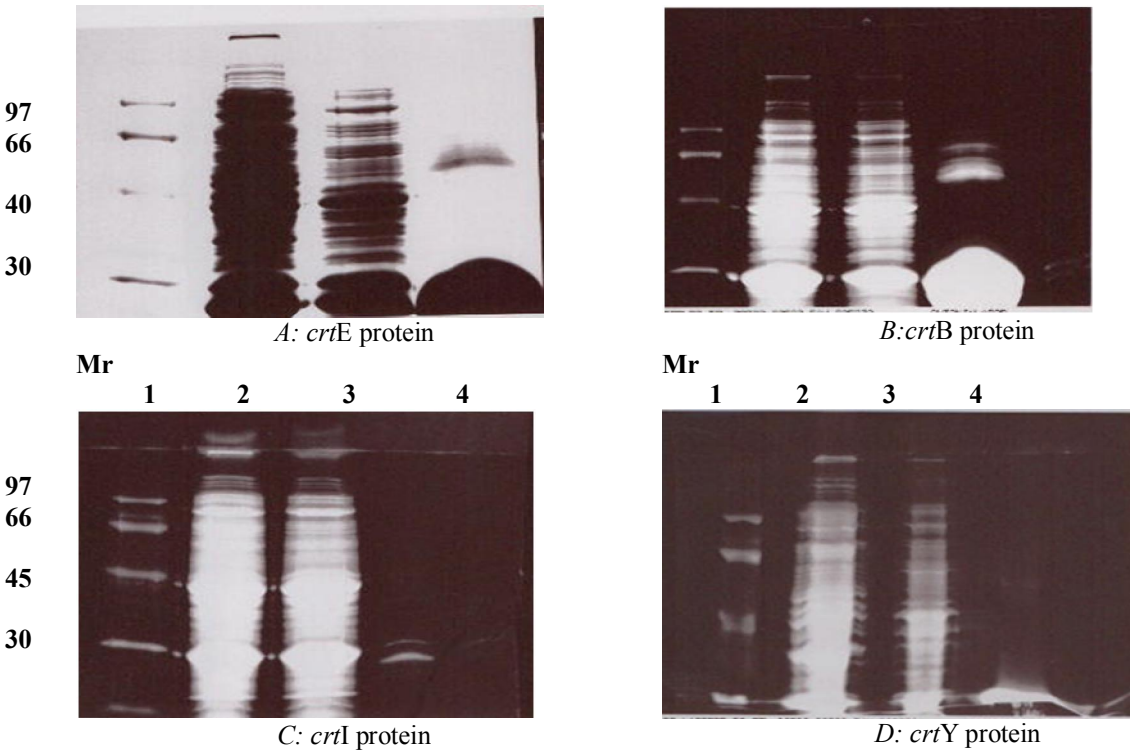


Fig. (8): Lane 1: contains low molecular weight markers Lanes 2,3,4,were the protein expression into *E. coli BL21* for every gene.

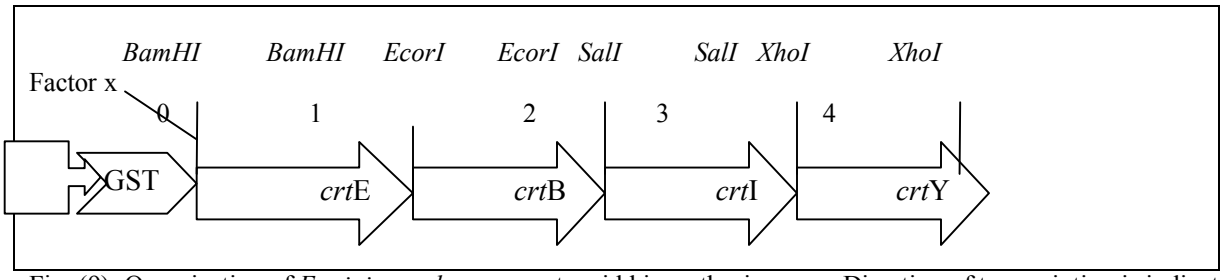


Fig. (9): Organization of *Erwinia uredovora* carotenoid biosynthesis genes. Direction of transcription is indicated by arrows. Numbers above of the genes show the putative nucleotide positions of translation starts and Stopps. The nucleotide position and numbered from the 5′ of cloning site.pGEX-5X-3 represent hybrid plasmids, in which the 1-4467 fragment carrying ORF-*crt*E, ORF-*crt*B, ORF-*crt*I, and ORF-*crt*Y are inserted into *E. coli* BL21 vector pGEX-5X-3, since all four ORFs were proven to be essential for production of the same carotenoids of *Erwinia uredovora.*

Fig.(10): ß-carotene accumulation in *E. coli* BL21 transformed with the *Erwinia ured ovora* gene cluster conferring ß-carotene biosynthesis.

***Crt*Y**: when plasmid pGEX-5X-3 carrying the *Erwinia uredovora crtY* gene was introduced into an *E. coli* transformant accumulating all-trans-lycopene as a result of pGEX-5X-3, the transformant synthesized ß-carotene (all trans). It is therefore evident that the *crt*Y gene encodes lycopene cyclase, which catalyzes the terminal cyclization reaction from all trans lycopene cyclase to all trans ß-carotene. The predicted 43 kDa *crtY* protein of *Erwinia uredovora.*

The functional analysis of the gene products expressed expressed in *E. coli* transformants, have elucidated the pathway for the biosynthesis of general carotenoids as ß-carotene. This study hasrevealed that the biosynthetic pathway clarified.

This study reports successful application of metabolic engineering to the production of carotenoid pigment as ß-carotene in non carotenogenic bacteria such as *E. coli.*

The major importance of *E. coli as* a carotenoid production system is to provide a broad selection of carotenoid structures. The structurally diverse carotenoids may be useful to study the relationship between structural features an antioxidative activity as well as pharmaceutical proporties. Metabolic engineering of the *E. coli* terpenoid biosynthetic reactions led to a considerable increase carotenoid production **(Sandmann, 1994).**

Conclusion:

It can be concluded from the brevious results that the transferring genes of carotenoid biosynthesis from bacteria *Erwinia uredovora* to *Esherichia coli* can be accumulate different carotenoid product which provided by a variety of genes and employed as tools for a new strategy of heterologous expression in different host organisms. These may lead to the production of one or more of carotenoid pigments in *E. coli* which lack the ability to produce carotenoids. Anyhow these points needs further studies and it may be also used for improving the productivity of carotenoids in plants deficient in carotenes.

Acknowlegments

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