JOURNAL OF PURE & APPLIED MICROBIOLOGY, Nov. 2014. Vol. **8**(Spl. Edn. 2), p. 543-548

Production of Lycopene from *E. coli* as a Non-Carotenogenic Bacterium and Evaluation of Antioxidant Effect of Lycopene

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(Received: 10 August 2014; accepted: 21 October 2014)

Lycopene is a bright red carotene and carotenoid pigment and phytochemical found in tomatoes and other red fruits and vegetables. The aim of this study was production of lycopene from *E. coli* as a non-carotenogenic bacteria and evaluation of antioxidant effect of lycopene. Tansformation PTLYCm4 & PSSNSN in *E.coli* DH5α, then measurement of dry cell weight and determination of lycopene content and extraction of lycopene from both transformed bacteria to compare the amount of lycopene production and finally treatment with H_2O_2 was performed for both transformed bacteria and also non-transformed bacteria as a control strain. The study was carried out in National Institute of Genetic Engineering and Biotechnology in Tehran over six months from April 2012 to September 2012. Three types of culture media including LB broth, LB agar medium and selective LB agar medium containing ampicillin & chloramphenicol. Preparation of competent cell was done by ESB buffer for cultivation of *E.coli* DH5α. Foreign genes were transferred into competent cells by heat shock method. Lyophilization was done in order to measurement of dry cell weight. Characterization of lycopene was done by spectrophotometer. Finally a buffer phosphate 0.2 M was prepared for treatment of bacteria with hydrogen peroxide. Dose of hydrogen peroxide was from 0 to 900mM in order to extract lycopene, a solution of methanol/chloroform was used. Firstly *E.coli* DH5α was transformed by PTLYCm4, and then transformation was performed by both PTLYCm4 and PSSNSN. PTLYCm4 contains the gene coding for geranylgeranyl diphosphate synthase (crtE), phytoene synthase (crtB) and phytoenedesaturase (crtI) of lycopene production and gene coding for isopentenyl diphosphate isomerase (IDI), while the PSSNSN contains six genes of *Streptococcus pneumoniae* and Ralstoniaeutropha in a synthetic operon coding for a mevalonate pathway. Bacteria contain lycopene (*TLYCm4* & *TLYCm4-mev*) has a remarkable resistance to non-transformed bacteria (*E.coli* DH5α) against hydrogen peroxide. DH5α had tolerated only 100 mM of H_2O_2 while *TLYCm4* had tolerated 500 Mm and *TLYCm4-mev* had tolerated 800 Mm. After extraction of lycopene amount of lycopene of *TLYCm4* was determined 52mg/L and for *TLYCm4-mev* was 89 mg/L in similar DCW (Dry Cell Weight). Remarkable difference of tolerance of transformed bacteria to oxidative agent was due to lycopene. The bacterium that produce more lycopene from both mevalonate and non-mevalonate pathway (TLYCm4-mev) has more resistance against H_2O_2. This evidence suggest that lycopene has a strong antioxidant property.

Key words: *E.coli*, Lycopene, Antioxidant properties, Transformation.

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Carotenoids are natural lipid -soluble isoprenoid pigments that biosynthesized by plants, algae, fungi and bacteria. They are a class of naturally occurring pigments, usually red, orange or yellow in color[1, 2]

The carotenoid pigments play an important role in photosynthetic systems and have protective functions against various type of reaction oxygen species (ROS) like hydrogen peroxide (H_2O_2), hydroxyl radical and superoxide anions[3,4]

Lycopene is an unsaturated open chain carotenoid which gives tomato, apricots, watermelon their red colour. It is used for carotenoid production as a biosynthetic precursor, and most of the carotenoids like beta-carotene and astaxanthin are derived from[5-8]. Lycopene is a powerful antioxidant that has been shown to reduce risk of sun damage, human cancer, atherosclerosis and muscular diseases[9]. Lycopene is made of 7 IPP (isopentenyldiphosphate) and its isomer, dimethylallyl phosphate (DMAPP) which is essential building blocks to isopernoids. There are two different procedure to IPP and DMAPP biosynthesis, including the mevalonate (MVA) and non-mevalonate (MEP) pathways[10,11]

In the non-mevalonate pathway, IPP and DMAPP are synthesized independently through the late step of the pathway, that is, IPP isomerase seems to be non-essential for non mevalonate organisms[12].

Some gram positive bacteria and archaea bacteria have the MVA pathway in contrast plant plastids. Some gram positive bacteria and most gram negative bacteria contain the MEP pathway. All eukaryotes use the mevalonate pathway in the Cytoplasm[13,14]. All higher plants seem to have both the mevalonate\ and non-mevalonate pathways[15-18]. Currently, only a few carotenoids can be produced commercially by chemical synthesis, fermentation or isolation from a few abundant natural sources[19, 20].

The increasing demand of natural colors has led to increase carotenoid production in carotenogenic and even non carotenogenic sources such as *E. coli* by metabolic engineering[21].

Escherichia coli is a non-carotenogenic bacterium that makes isoprenoids using non-mevalonate pathway. The mevalonate pathway genes areatoB, mvaA, mvaK$_1$, mvaS, mvaK$_2$ and mvaD along with the genes responsible for lycopene productions are crtE, crtB, crtI plus IDI. Recently, carotenoids have been successfully synthesized in non-carotenogenic bacteria such as *E.coli* using recombinant gene techniques[22-25].

MATERIALS AND METHODS

Bacterial strains, Plasmids, and Cultivating conditions

E.coli DH5α strains were cultivated on Luria-Bertani agar for 24h at 37°C. Single colonies of *E.coli* DH5α were grown into 3ml LB medium while shaking (180 rpm) at 37°C for preparation selective LB agar medium, antibiotics were added to LB medium at final concentration of ampicillin, 50 µg/ml and chloramphenicol, 2 µg/ml.

The plasmids used in our study were PTLYCM4 and PSSNSN. PTLYCM4 contain the gene coding for geranylgeranyl diphosphate synthase (crtE), phytoene synthase (crtB) and phytoenedesaturase (crtL) of lycopene production and gene coding for isopentenyl diphosphate isomerase (IDI)[26]. The PSSNSN carry six genes of *Streptococcus pneumoniae* and *Ralstonia eutropha* in a synthetic operon coding for a mevalonate pathway[27].

Transformation procedure

The PTLYCM4 and PSSNSN plasmids, were transformed into *E. coli* DH5α cells using heat-shock method, according to the protocol as described in previous studies[28]. Consequently three strains of *E. coli* DH5α were provided, The non carotenogenic *E. coli* as control, *E.coli* harboring PTLYCM4 plasmids (*E.coli* Tlycm4) together with *E.coli* carrying PTLYCM4 and PSSNSN plasmids (*E. coli* Tlycm4-mev).

Measurement of dry cell weight

The transformed strains (*E. coli* DH5α Tlycm4-mev) were cultured in 10 cc LB broth. Several culture media with different OD (600 nm) were removed, and then centrifuged at 4ºC and 5000 rpm for 15 min. Lyophilization process was carried out on precipitates, after that dry cell weight was measured. Eventually density chart was plotted versus cell dry weight[29]

Quantification of lycopene

The both Tlycm4 and Tlycm4-mev strains were cultured in 10cc LB broth to reach OD=3(600 nm), then lycopene contents were quantified as

explained by 1984, briefly 1ml of culture were centrifuged at 5000 rpm for 5 min, the harvested cells were washed with distilled water then resuspended in 400 µl of methanol/chloroform (70:30 v/v) by vortexing for 5 min. The approach resulted in extraction of lycopene by the solvent mixture, after centrifugation at 5000 rpm for 5 min, resulting pellets were extracted once more by 400 µl of fresh methanol/chloroform mixture. The two lycopene extracts were pooled together in a clean microfuge tube, as consequence total volume was elevated up to 1 ml by the addition of fresh solvent mixture.

Lycopene production was quantified using absorbance at 474 nm .Measurement attained from extracts were compared to standard curve generated by commercial lycopene (sigma)[30]

Preparation of bacterial suspension to treatment with H_2O_2

Before treatment, bacterial cells were grown in LB broth at 37°C and 110 rpm to reach the mid-log phase (OD=0.6), following the bacterial suspensions were centrifuged at 7400g at 4ºC for 10 min the supernatant was discarded and pellet washed with phosphate buffer (67mM, pH: 6.8). Cell washing was repeated several times. Finally, the cell sediment became completely uniform in volume 30-40 ml of buffer.

Treatment with H_2O_2

Different concentrations of Hydrogen peroxide (0-800Mm) were added to 5 ml of cells in phosphate buffer (67nm, pH: 6.8), shook at 37°C for 1h.

In order to remove the H_2O_2 and preventing damages in the later stages, cells were washed twice in phosphate buffer (0.2 M, pH: 6.8) by centrifugation. The prepared dilutions from each tube were cultured on selective LB agar medium overnight at 37°C[31].

RESULTS

Transformation

In this study, *E.coli* DH5α was used as a host for the recombinant plasmid construction and lycopene production. The colored-antibiotic resistant colonies were selected as transformed bacteria. Consequently 2 recombinant red bacterial strains were achieved, *E.coli* Tlycm4 and *E.coli* Tlycm4-mev.

Measurement of dry cell weight

In several different OD (600nm), dry cell weight was measured according to the method and the results were provided in fig 3.1

Quantification of Lycopene content

By Commercial lycopene, a graph optical density versus amount of bacterial lycopene generation was plotted (Fig 3.2). Using this diagram, the amount of lycopene produced can be calculated from the amount of optical density (at 474nm) and then applying the Fig 3, lycopene produced per gram dry weight of the cell can be appraised.

Table 3 displays that dried cell weights achieved from both bacteria in OD=3 were 2.4g/l. the pigment content obtained 52mg/l and 89mg/l at the defined lycopene OD for Tlycm4 and Tlycm4-

Table 1. Description of Strains

Strains	Description	
E.coli DH5á	[F´ /endA1 hsdR17 (rKmK) glnV44 thi-1 recA1 gyrA (Nalr) relA1 "(lacIZYA-argF)U169 deoR 5(ö80dlac"(lacZ)M15)]	(Gibco BRL)
E.coli Tlycm4	*E.coli DH5á* harboring *PTLYCm4*	In this study
E.coli Tlycm4-mev	*E.coli DH5á* harboring *PTLYCm4* and *PSSNSN*	In this study

Table 2. Extraction of Lycopene

Amountof LYCOPENE (mg/L)	O.D2 (LYCOPENE) (474nm)	DCW(g/L)	repetition	O.D1 (CULTURE) (600nm)	Amount of culture	
52mg/L	0.7	2.4g/L	3	3	1OCC	TLYcm4
89mg/L	1.2	2.4g/L	3	3	10CC	TLYcm4-MEV

mev respectively.

Table 3.1 shows that dried cell weights obtained from both bacteria in OD=3 were 2.4g/l. the pigment content achieved 52 mg/l and 89 mg/l at defined lycopene for Tlycm4 and Tlycm4-mev respectively.

H_2O_2 sensitivity assay

Experiment was conducted to evaluate the antioxidant properties of lycopene and resistance of three strains of bacteria (DH5α, Tlycm4, and Tlycm4-mev) against H_2O_2. The observation indicated that the bacteria contained lycopene (Tlycm4, Tlycm4-mev) had a remarkable resistance compared to non-transformed bacterium (DH5α).

DISCUSSION

Carotenoids are fat-soluble molecules that are abundant in nature. These compounds cause the colors green, yellow, red and orange leaves of plants, fruits, flowers. Carotenoid pigments are usually in plants, algae and photosynthetic bacteria, due to their crucial role in photosynthetic processes are found .these pigments also in some types of non-photosynthetic processes are found.

Lycopene is a carotenoid pigment that found in tomatoes, red carrots, red bell peppers, watermelons and paper in plants, algae and other photosynthetic organism, lycopene is an important intermediate in the biosynthesis of many carotenoids including beta carotene. Like all carotenoids, lycopene is a polyunsaturated hydrocarbon. Structurally, it is a tetraterpene assembled from eight isoprene units, composed entirely of carbon and hydrogen and is insoluble in water. Lycopene's eleven conjugated double bonds give it its deep red color and are responsible for its antioxidant activity. Due to its strong color and non-toxicity, lycopene is a useful food coloring. Considering the benefits of lycopene, we decided that produce this matter through genetic engineering in *E.coli* from both mevalonate and non-mevalonate pathways.

E.coli is a very convenient host for heterologous carotenoid[32-35]. Most of the carotenogenic genes from bacteria, fungi and higher plants can be functionally expressed in this bacterium[36]. *E.coli* is a non carotenogenic bacterium that makes isoprenoids using non-mevalonatepathway[37].The growth of *E.coli* DH5α Tlycm4 &Tlycm4-mev in medium containing the appropriate antibiotics, and also color differences between bacteria colonies, demonstrate that transformation was successful conversely non-

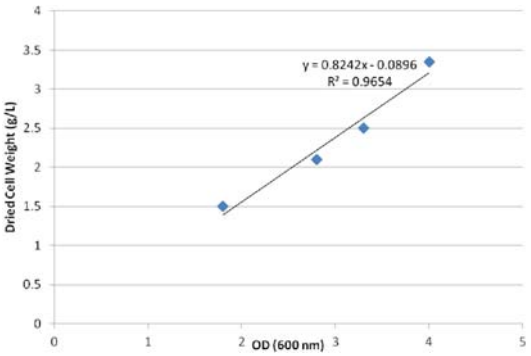

Fig. 1. Graph of Dried Cell Weight/OD

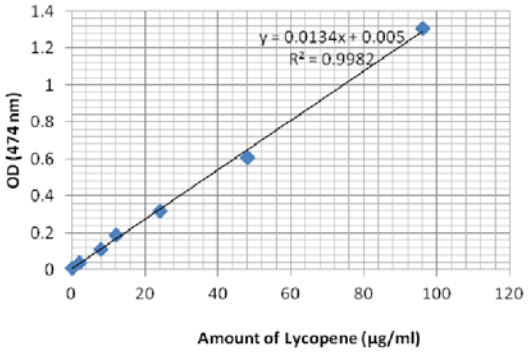

Fig. 2. Graph of Amount of Lycopene/OD

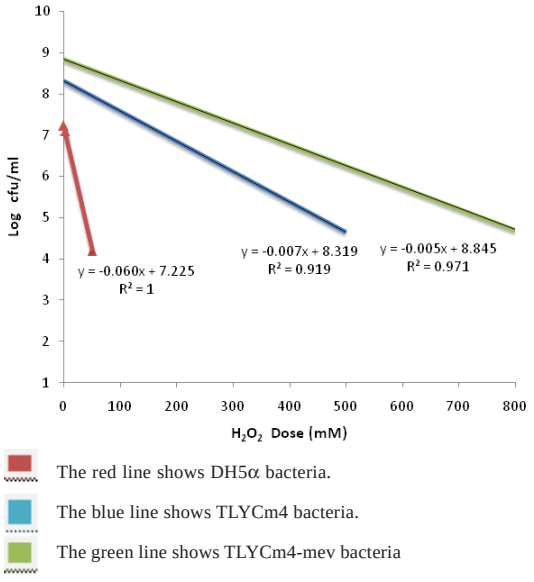

Fig. 3. Graph Treatments with H_2O_2

transformed bacteria (DH5α) did not grow in these conditions. The Tlycm4 bacteria had produced lycopene only from non-mevalonate pathway, while the TlyCm4-mev bacteria that contained both plasmids, had produced lycopene from both mevalonate and non-mevalonateproceedings. The extracts showed maximum absorbance at 474 nm, this indicate the material was produced by transformed strains had been lycopene pigment.

Lethal effects of Hydrogen peroxide are related to produce free radicals that are seriously harmful for critical structure within the cytoplasm of the cell wall or to macromolecules such as protein and DNA. Carotenoids are efficient free-radical scavengers[38]. Lycopene may be the most powerful carotenoid quencher of singlet oxygen[39]. The permeability of the wall and membrane structure in these 3 bacterial strains is identical therefore differences in bacterial resistance against oxidative agent, due to differences in the production of lycopene and amount of lycopene.Carotenogenic strains (Tlycm4, Tlycm4-mev) had a remarkable resistance compare to non-transformed bacterium (*E.coli* DH5α) opposed to H_2O_2. Altogether our work confirmed that the *E.coli* Tlycm4-mev had more resistant to H_2O_2 due to the more lycopene production.

Before this study also genes of lycopene's production was transferred in *E.coli* by genetic engineering and lycopene was produced by E.coli but The advantage of our study compared with previous studies is that transformation of genes of both MVA and non-MVA pathways simultaneously in *E.coli* (TLYCm4-mev). Also the cell resistance of this bacterium against oxidative agents was evaluated. At the end this bacterium was compared with two other bacteriaI items of resistance to oxidative agents H_2O_2 that *E.coli* DH5α lack the genes for lycopene production and *E.coli* Tlycm4 produce lycopene from only non-mevalonate pathway.

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