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Original article

Antioxidant and antibacterial activity of β-carotene pigment extracted from *Paracoccus homiensis* strain BKA7 isolated from air of Basra, Iraq

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Abstract
carotenoids are widely used in natural and have different colors as yellow , orange , red and pink. Carotenoids having many applications as antioxidant ,antibacterial and food additives. In this study an orange pigment produced from *Paracoccus homiensis* strain BKA7 was isolated from air in Basra city, Iraq. Identified this strain by the 16SrNA gene analysis and recorded in Gene Bank as new strain with accession number LC495622.1. The optimum growth and pigment production by this strain were by Nutrient broth supplement with 0.5%yeast extract and 2% glycerol, incubation temperature 30 C$^{\circ}$, initial pH 9, 1% inoculum, 150 rpm as culture shacking and 72 hours as period of incubation. The carotenoid was extracted by methanol solvent and its analyzed by spectrophotometric within 400-600nm and wave length maximum was found at 460nm three peak spectra. The extract was separated using TLC, and three spots were found at Rf 0.98 which revealed the present of β-carotene while Rf 0.81 and Rf 0.47 were revealed to the present of xanthophyll. The β-carotene was purified by silica column chromatochraphy and confirmed by HPLC with a concentration of 574.00 μg/gm. The β-carotene have strong antioxidant activity, the maximum antioxidant was 72% at concentration 10mg/ml comparing with BHT 96% and Ascorbic acid 88% using DPPH method. The β-carotene showed high antibacterial activity with concentrations ranged 1200μg/ml against some pathogens strains. It was observed that β-carotene have a significantly stronger impact on Gram-positive than Gram-negative bacteria.

Key words: carotenoids, β-carotene pigment, *Paracoccus homiensis* strain BKA7, antioxidant, antibacterial

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Introduction

Color is part of humans culture and life and is one of the visible characteristics and an important characteristic of the increase acceptance the consumer of the food product and its rule sometime on its quality (Samyukth and Manajan,2016).The color of the foodstuff is also important to indicate its safety and freshness, and it is also an indication that it is of aesthetic and sensory value (Malik *et al*. 2012). FAD is defines the colored substance is dyes, pigment or other substance that produce or extract from plants ,animals and minerals which added to food , medicine products and cosmetics that gives them suitable color (Nigam and Luke,2016). Pigments and food colorants are classification to two groups: natural pigments and synthetic dyes. Natural pigments were produce from two sources are microorganisms such as bacteria and plant (Kumar *et al*.,2015).Bacteria which produce pigments are called chromogenic

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bacteria, these bacteria produce types of natural pigments such as carotenoids, chlorophyll, Flavin, Quinine and prodigiosin, these pigments are important in various commercial filed such as food, medicine animal feed and cosmetic (Monika and Archana ,2017). Carotenoids are widely used in natural, they cannot be synthesized inside humans body so they found in plant, animal and microorganism. There are 700 types spread wide in natural (Kirti *et al*.,2014). Carotenoids have different colors as yellow, orange, red and pink, this different due to the presence of many double bonds in their polyene chain structure (Meddeb-Mouelhi *et al*., 2016; Rao and Rao, 2007). Carotenoids classification two types, carotenes don't have oxygen molecular and xanthophyll have oxygen molecular (Botella -Pava and Concepcion ,2006 ; Berman *et al*.,2015).The production of microbial carotenoids pigments depends on some requirements that are essential and necessary for the growth of microorganisms such as type of strain, the culture medium and the conditions of development pH, temperature, light, fermentation method, inoculum volume (Joshi *et al*.,2003).Carotenoids are very important as they involved in reducing on set of many diseases which are producing due to free radical damage and it used as antibacterial (Sen *et al*.,2019). *paracoccus* spp a carotenoids producing with commercial value as a food additive and feed supplement (Venil *et al*.,2013). The aim of the study was to investigate carotenoids pigment produced by *Paracoccus homiensis* strain BKA7 was isolated from air environmental. This was achieved in three sub-objectives, viz.: (1) Study the appropriate conditions related to the production of carotenoids pigment of submerged state fermentation by *Paracoccus homiensis* strain BKA7 (2) Study the separation and purification the β-carotene producing by *Paracoccus homiensis* strain BKA7 by TLC methods and its identification by HPLC (3) Study the using the β-carotene producing by *Paracoccus homiensis* strain BKA7 as antioxidant and antibacterial activity.

Material and Methods

Isolation of identification of bacteria

Petri dishes containing a culture medium R2A agar were exposed to the air in the laboratory near the window for 4 hours, after that they incubated at 30 C$^{\circ}$ for 48 hours in air conditions.

Purification of orange pigment produce bacteria

Petri dishes with discrete orange pigments colonies were screening and the study morphological and characteristics this colonies. These isolates were purified by streak on R2A agar plate by pure culture methods. These isolates strains were characteristic by orange pigment colonies and biochemical characteristics to confirm the identity of the culture such as cell shape, Grams staining, motility, catalase (Harley and Prescott,2002). oxidase, gelatinase (Mcfadden,2000) and hemolysis test (Chen *et al*., 2017).

Molecular characterization of bacteria strain

1. Preparation of template DNA

The bacterial strain were identified by 16SrRNA gene sequence analysis. The bacterial isolated were grown 48 hours in R2A agar .Genomic DNA from *Paracoccus homiensis* strain BKA7 was extracted using the genomic DNA purification Kit (G-Spin/Korai) according to the manufacture procedure. Determinates concentration was extracted DNA and its pure by Nano drop spectrophotometer at 260-280nm for getting DNA concentration 1.8-2nm (Sambrook *et al*., 2001).

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2.Purification of PCR products

Nearly full-length 16SrRNA gene sequences were amplified from genomic DNA by polymerase chain reaction (PCR) using universal primers (Srinivasan *et al*.,2015), Forward primer (5'- AGA GTT TGATCCTGGCTCAG- 3') and reverse primer (5'- GGTT ACC TTG TT ACGACTT-3').PCR fragment was purified using intron/PCR pre mix Kit).PCR was performed in a master cycle using an initial denaturation at 94 C° for 3 min , followed by 33 cycle at 94 C° for 4 5 sec ,56 C° for 45 sec , at 45 min for 45 sec and a final extension step at 72 C° for 7 min.

3.Sequencing and Sequence Alignment

The PCR products were separated on a 2% agarose gel electrophoresis and visualized by exposure to ultra violate light 302nm after ethidium bromide or Red Stain staining. Sequencing of gene was performed by national instrumentation center for environmental management (NICEM) online (http://nicem.snu.ac.kr/main/en_skin=index.html), biotechnology lab in Korai, the machine is DNA sequencer 3730XL, Applied Biosystem), Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http:// www. ncbi. nlm. Nih .gov) and Bio Edit program. Multiple alignments were generated by the CLUSTAL W program and the phylogenetic tree was constructed by a neighbor-joining algorithm using MEGA 6 software.

Optimization of culture conditions for enhancement of carotenoids

Optimization of Culture media

Using four of culture media (Nutrient broth supplement with 0.5% Yeast extract, Luria broth , Tryptic soy broth and (Nutrient broth supplement with 0.5%yeast extract and 2% glycerol) were incubation in shaking rotary for 150 rpm at 30 C° for 72 hours, after incubation growth and pigment production was determinate separately to know optimum medium for pigment production.

Incubation period

Paracoccus homiensis strain BKA7, has been cultivated in 250 ml conical flask containing 50 ml of Nutrient broth supplement with 0.5% yeast extract and 2% glycerol, each flask was inoculated with 2% v/v of bacterial inoculum after that incubation at 30 C° for 24, 48, 72 and 96 hours. During incubation, samples were withdraw every 24 hours for determinate of dry cell biomass g/l and cellular carotenoids µg/g dry bacteria.

Incubation temperature

The growth medium (Nutrient broth supplement with 0.5%yeast extract and 2% glycerol) was inoculated and incubation at different temperature (25, 27, 30,33 and 36 C̊) on shaking rotary for 150 rpm for 72 hours, the media were tested for dry cell mass and carotenoids concentration every temperature degree.

Optimization of pH

The growth medium (Nutrient broth supplement with 0.5%yeast extract and 2% glycerol)) was inoculated and incubation at different pH (6, 7, 8, 9 and 10) on shaking rotary for 150 rpm at 30 C° for 72 hours, the media were tested for dry cell mass and carotenoids concentration every pH.

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Inoculum volume

The growth medium (Nutrient broth supplement with 0.5%yeast extract and 2% glycerol) was inoculated at different inoculum volumes (0.5, 1, 1.5, 2 and 2%) and incubation on shaking rotary for 150 rpm at 30 C° for 72 hours. the media were tested for dry cell mass and carotenoids concentration every inoculum volumes.

Optimization of rpm

The growth medium (Nutrient broth supplement with 0.5%Yeast extract and 2% Glycerol) was inoculated and incubation at different for rpm (130, 140, 150,160,170) on shaking rotary at 30 C° for 72 hours, the media were tested for dry cell mass and carotenoids concentration every rpm.

Extraction of carotenoids pigment

Preparation of bacterial inoculum

Preparation of bacterial inoculum by Boontosaenget *et al*. (2016) method by taking Loop full from bacterial culture and tranformate to test tube containing a sterile 50ml of NB medium incubating the tubes incubator shaking at 150 rpm and incubation at 30 C° for 48 hours, after that, 1 ml of the bacterial culture was diluted 10^{-1}–10^{-6} in solution peptone to measure growth in terms of optical density (OD) at 660 nm using a spectrophotometer for getting dilution suitable for compere with standard McFarland solution (2.0) show that the number was 6 x 10^{8} / ml.

Extraction method of carotenoid

1ml of inoculum volume was transferred to 250 ml Erlenmeyer flask containing 49 ml of NB at 30 C° on a rotary shaker at 150 rpm for 72 hours, transferred the culture media to the sterile tube (50ml) and centrifuged at 6000 rpm for 10 min and the supernatant was discarded, cell pellets were washed twice with deionized water, followed by centrifugation 6000 rpm for 10 min to recover the cell by discharging the supernatant again, the 25 ml of methanol (99.7%) was added to the cell for extraction of bacterial pigment and the mixture was vortexed, extracted twice with the same solvent, the mixture of the cells and methanol was treated by ultra-sonication (59KHz,35-40 C° ,90 min) and keep overnight in light protects, pigment extracts were separated by centrifugation at 6000 rpm for 10 min and filtrated by 0.45µm filter.

Characterization of carotenoids pigment

UV-Vis spectra absorption

The pigment extract was analyzed by scanning the absorbance in the wavelength region of 400-600 nm using a spectrophotometer, the solvent was evaporated to dryness in the evaporator at 40 C° and crud pigment was collected in the tub and keep light protects at -20 C°. The total carotenoids were calculated by below equations (Rodriguez-Amaya and Kimura, 2004)

$$\text{Total carotenoids} = \text{ABS} \times \text{Extract volume (ml)} \times 10^{-4} / E^{1\%}_{1cm} \times \text{Sample weight (g)}.$$

ABS = absorbance , $E^{1\%}_{1cm}$ = coefficient specific of absorption of mixtures (2500)
Sample weight (g) = (Dry cell weight)

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Pigment separation by TLC

Silica gels coated on TLC sheet 20×20 cm was used as stationary phase, few drops from each phase were spotted on baseline of TLC sheet and then plate was placed inside prostrated TLC chamber containing mobile phase (7:3 hexane: ethyl acetate), the solvent was allowed to run till it reach up of the plate, the chromatography was analysis visually for pattern and spot were markered relative Rf value were calculated (Benjamin *et al*., 2016) .

Purification by column chromatography

β-carotene pigment was produced by *Paracoccus homiensis* strain BKA7 and its purified by column chromatography using silica gel (60-120 mesh size) and eluted initially with n- hexane with flow rate 1min/min. the polarity of the solvent was increased subsequently by adding ethyl acetate (7:3 hexane: ethyl acetate), after that the yellow- orange fractions were collected from the column.(Benjamin *et al*.,2016).

HPLC analysis of β-carotene

The β-carotene pigment, extracted from *Paracoccus homiensis* strain *BKA7*, (HPLC) (Dionix-USA) was using assessed and diagnosed, and this method was used for quantitative and qualitative estimation according to the conditions mentioned. Wang *et al*. (2012), the separation was carried out on a C18 separator column (Xselect CSH, Ireland) the dimensions (4.6 x 100 mm).The mobile phase was composed of Acetonitrile -Tetrahydrofuran (60:40 v/v),the injection volume was (10) µl, with a flaw rate (0.8) mL / min and a pressure of 158 - 166 bar, the column temperature at 25 C° and the UV detector at wavelength of 450 nm.

The standard β-carotene pigment (Sigma-USA) was dissolved in methanol with of a concentration of 6000 µg / ml. The separation process resulted in drawing a peak curve (Peak) for each sample with its own holding time, calculating the beta-carotene pigment concentration by comparing the results of the quantification of the β-carotene pigment, retention time and the curve area of the known standard beta-carotene pigment according to the following equation (Kupiec, 2004).

Application of beta-carotene pigment

1. Antioxidant

The free radical scavenging activity of the methanol extract of β-carotene (Haddad *et al*.,2016). A carotenoids family compound was measured by DPPH, solution of DPPH (0.1mM) in methanol was prepared, 1ml solution was added to 1 ml of the pigment in methanol at different concentrations(2-10mg/ml). Next 1 ml of the standard solution of each (BHT and ascorbic acid) and 1ml of DPPH, the mixture was shaken vigorously and allowed to stand at room temperature in dark for 30 min, then the absorbance was measured at 517 nm by using a UV– visible spectrophotometer .The percentage DPPH scavenging effect was calculated using the following equation :

DPPH scavenging effect(%)= A0-A1/A0×100

2. Antibacterial activity

antibacterial activity performed by Disc diffusion method, the assay was employed to determine antibacterial activity of β-carotene pigment of *Paracoccus homiensis* strain BKA7 against pathogens isolates of *staphylococcus aureus*, *E.coli, pseudomonas aeruginosa* and *Bacillus cereus* on Muller Hinton agar media and was prepared in the plate with sterilized paper disc

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(0.6cm), following the procedure of Karpinski and Adamczak. (2019).The extracted pigment was dissolved in DMSO of different concentration of β-carotene pigment (100-1200μg/ml) and using DMSO as a negative control. Sterile filter papers soaked in different concentration of β-carotene pigment was were transferred on to plate, after that the plate incubation at 37 C° for 24 hours bacterial suspension containing 10^8 CFU/ml of test bacteria.

Statistical analysis

Statistical analysis using one - way ANOVA ,The results were obtained from a minimum of three independent experiments and averaged. Data were analyzed by the analysis of variance ($p \leq 0.05$)to estimate the differences between values of tested compounds using SPSS program.

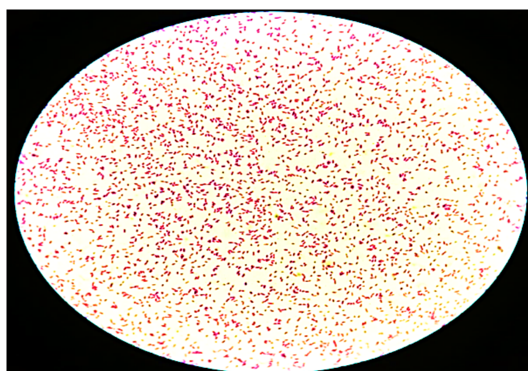
Results and Discussion

Isolation and identification of bacteria strain

In this study, *Paracoccus homiensis* strain BKA7 strain isolated from air and purified was described on R2A medium at 30 C° for 48 hours. Based on morphological characterization the colony of isolate was an orange pigmented bacterium and was mucoid surface in plate, shape cells were short rod and Gram negative. The biochemical characterization of the *Paracoccus homiensis* strain BKA7 was discussed in table(1). The strain was negative for gelatinase, hemolysis ,and motility, also was positive for oxidase and catalase. Detailed biochemical and morphological of strain in comparison with closely related reference and Berge's manual systematic Bacteriolgy,2005 (Spanning *et al*.,2005).

Table (1) Morphological and biochemical of *Paracoccus homiensis* BKA7

parameters	Observation	Biochemical test	results
isolate	*Paracoccus homiensis* BKA7	Oxidase	+
Source of isolation	Air	catalase	+
Medium of isolation	R2A	Gelatinase	-
Color of colony	Orange	Hemolysis	-
surface of colony	Mucoid	motility	-
Shape of cell	short rod	Gram stain	-
Optimum temperature	30 C°		
Optimum PH	9		



Paracoccus homiensis BKA7

Figure (1) microscopic view Gram stain and growth on R2A slant

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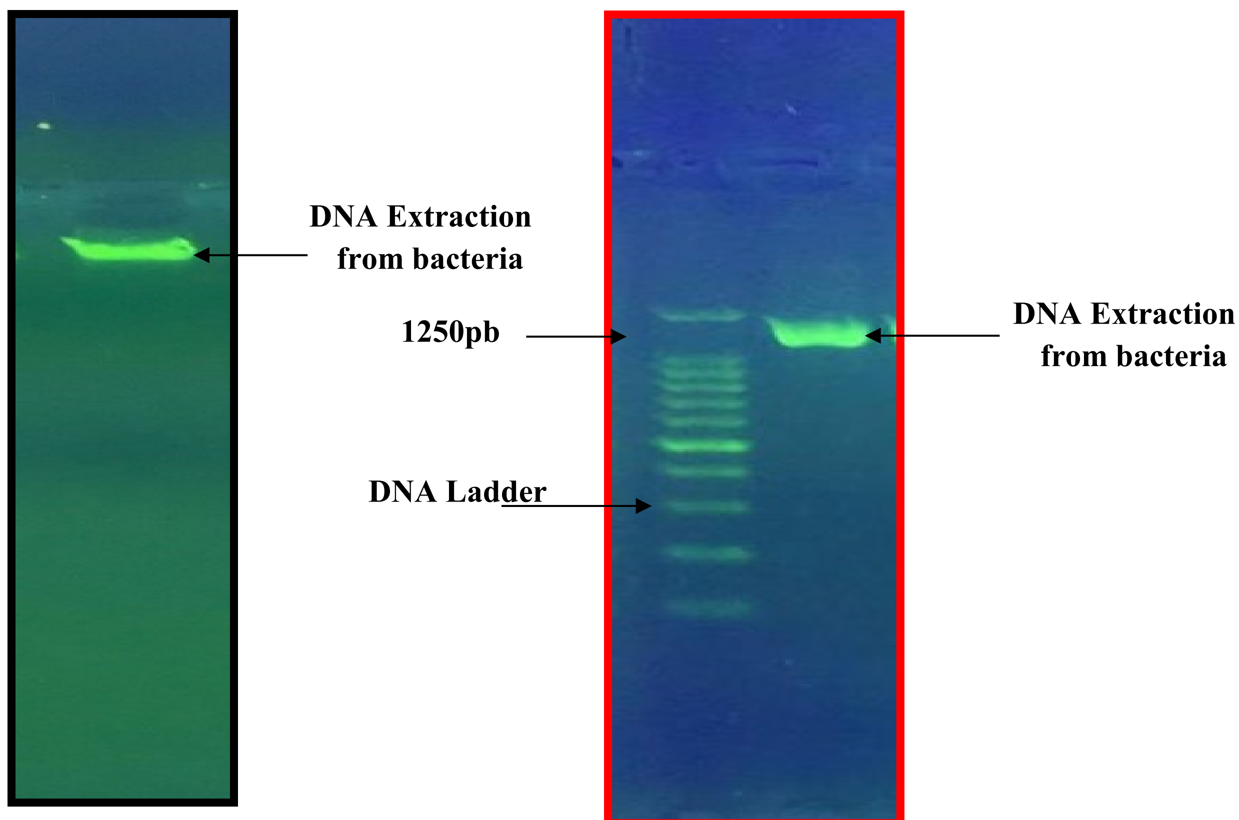
Molecular characterization and Phylogenetic tree

The genomic DNA was isolated from *Paracoccus homiensis* strain BKA7 and subjected to PCR amplification. A consensus sequence of 759 bp of 16S rRNA gene was generated to carry out BLAST with the non-redundant database of NCBI Gen Bank. Based on the maximum identity score, first sequence was selected and aligned using multiple alignment software program clustal omega.



figure(1) Gel electrophoresis of Genome DNA extraction from bacteria,1% agarose gel at 5vol/cm

figure(2) PCR product the band size 1250 bp .the product on was electrophoresis on 1.5% agarose at 5 volt/cm^2

Comparison of the 16SrRNA gene sequences of the *Paracoccus homiensis* strain BKA7 with other strain peaks by clustal omegas, *Paracoccus homiensis* strain BKA7 is closely related (similarity 98.29%) with strain *Paracoccus homiensis* TK029 (GenBank accession number: MK045803.1) was isolated by Tang in 2018 in china. a mutation in which the several nitrogen basics was removed and replaced with other at different sites Figure(3)

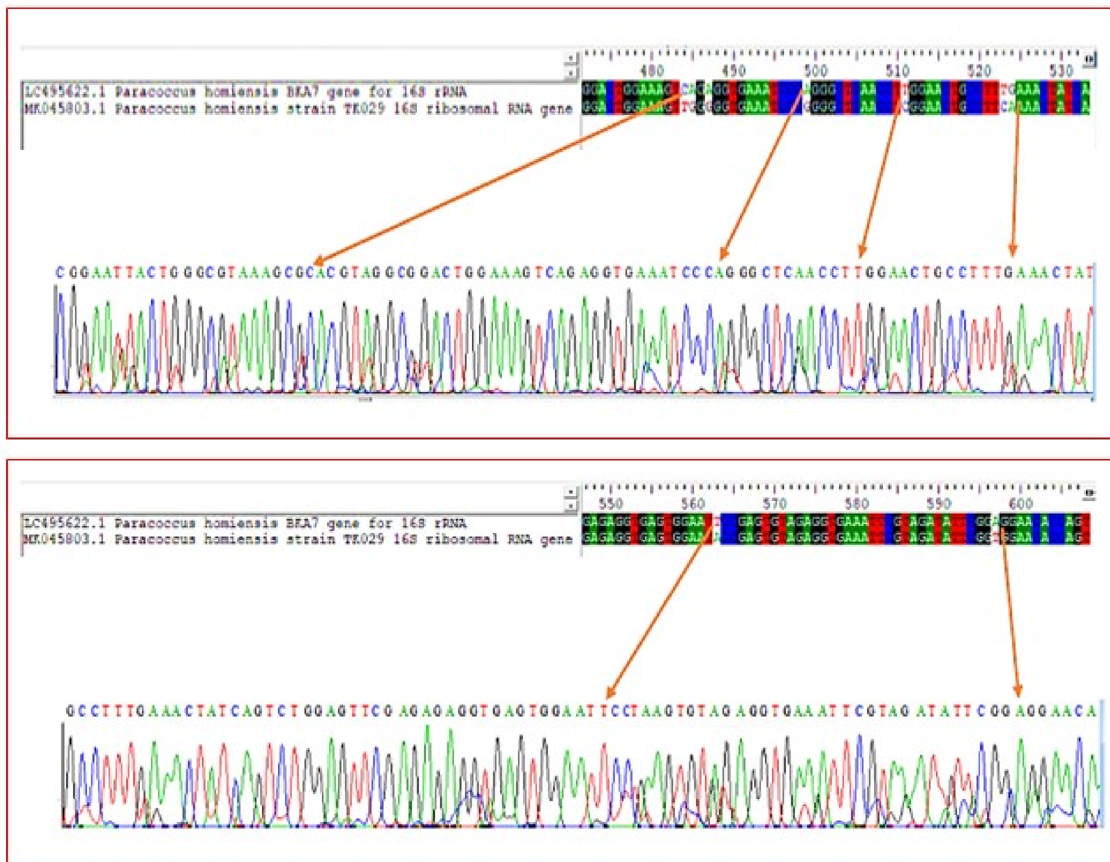


figure (3) Comparison of the 16SrRNA gene sequences of the *Paracoccus homiensis* strain BKA7 with other strain peaks of *Paracoccus homiensis* TK029 (MK045803.1). in a mutation in which the several nitrogen basics was removed and replaced with other at different sites.

the phylogenetic tree analysis was constructed using tree view by MEGA software, *Paracoccus homiensis* strain BKA7 was showed similarity with all the sixteen accessions analyzed in NCBI database (Figure 4).

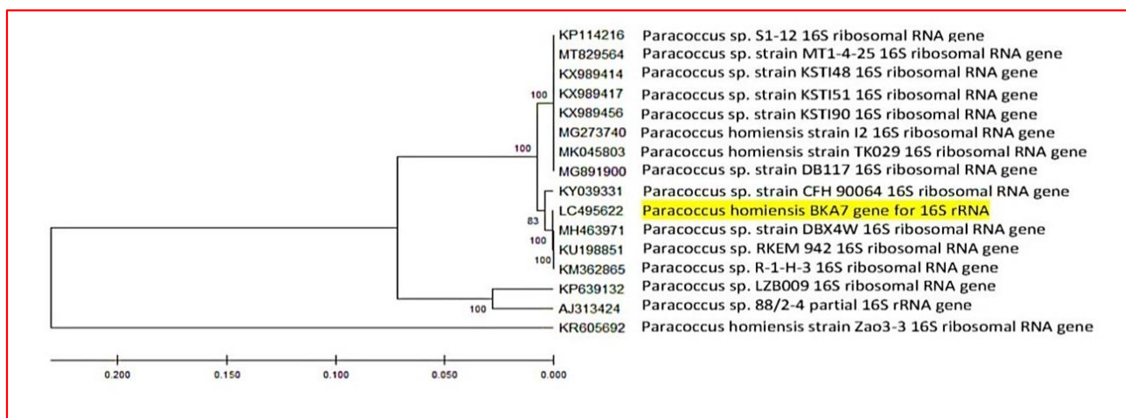


Figure (4) phylogenetic tree of *Paracoccus homiensis* BKA7

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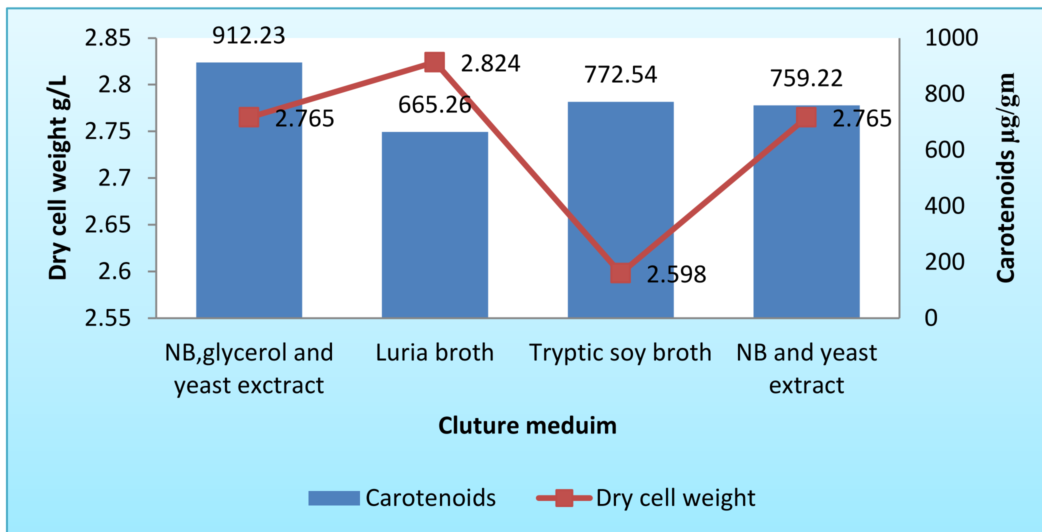
Optimization of growth and carotenoids production Culture media

Several factors that affected the growth and carotenoids production by *Paracoccus homiensis* strain BKA7 were indicated significant differences ($p<0.05$) in use the media different investigated. based on results shown in figure(5) which the Carotenoids and growth (dry cell weight) was 912.23 µg/gm and 2.765 g/l when strain growth on (nutrient broth supplement with 2% glycerol and 0.5% extract yeast) medium, then when growth in Luria broth, Tryptic soy broth and nutrient broth, Carotenoids was 665.26, 772.54, 759.22 µg/gm and growth was 2.824, 2.598, 2.765 g/l respectively. The microbial growth and accumulation of carotenoid in the cells requires complex media(Joshi *et al.*,2011).

Dhere and Dharma hiker (2015)found that carotenoids content less in growth *paracoccus beibuensis* culture media as such nutrient broth, Luria broth ,and MacConkey broth compered to its growth in peptone at 30 C°.



LSD (media × carotenoid contain µg/gm$_{0.05}$)=0.0325

LSD (media × Dry cell weight g/l $_{0.05}$)= 0.0035

Figure (5) Effect of culture media on growth and carotenoids production

Effects of pH

The effect of pH of culture medium on growth and carotenoid production by *Paracoccus homiensis* strain BKA7 was also investigated**.** based on results shown in Figure (6), It was noticed that the bacterial isolate exhibited growth and carotenoids production were indicated a significant differences ($p<0.05$) in use within pH range 6-10, however, maximum carotenoids production was 912.23 µg/gm was obtained at pH 9, while maximum growth equivalent to 2.382 g/l was recorded at pH 10. carotenoids production were 647.26, 768.32, 793.63 µg/gm , and bacterial growth were 2.302, 2.374, 2.326 g/l obtained at pH 6, 7, 8 respectively. The obtained results were in agreement with Wairimu**,** (2009) who was reported the maximum carotenoids and bacterial growth for *paracoccus beibuensis* occurs at pH 9.0, while was less at pH 10 and 10.5.

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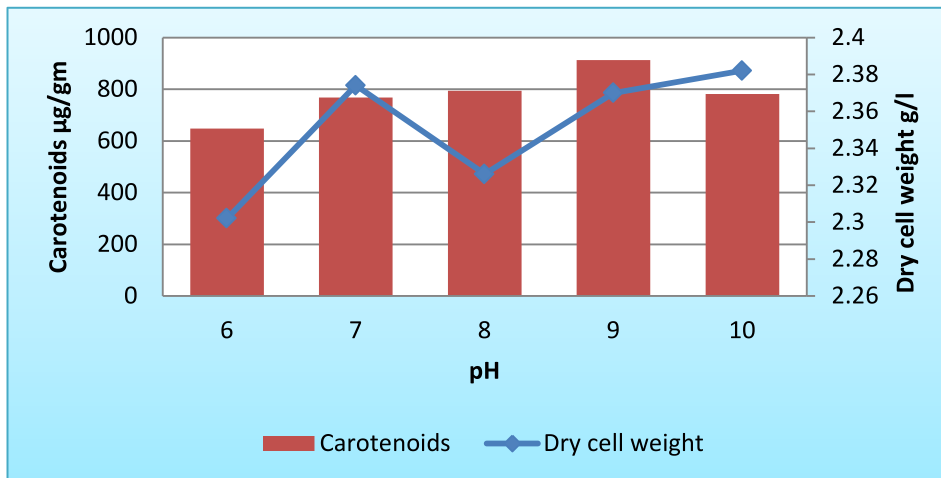
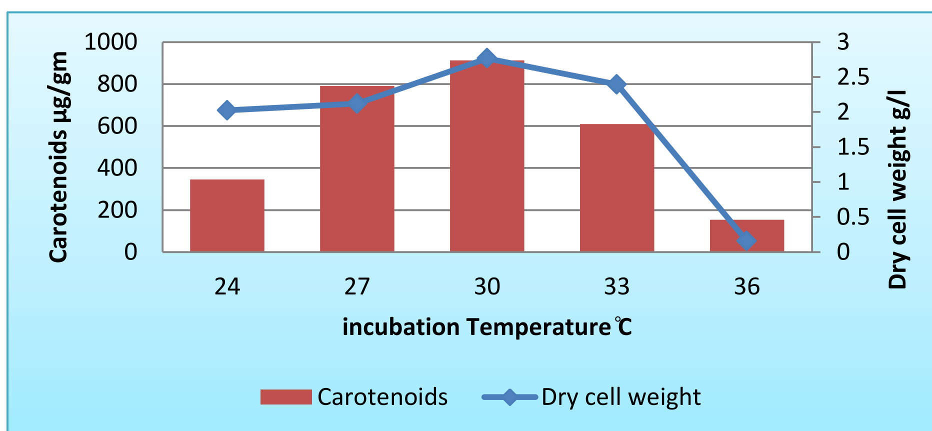


LSD (pH × carotenoid contain µg/gm$_{0.05}$)=0.006
LSD (pH × Dry cell weight g/l $_{0.05}$)= 0.006

Figure(6) effect of pH of culture medium on growth and carotenoids production

Incubation Temperature

The temperature is one of most important environmental factors effecting the growth of microorganisms and its causes change in many biosynthesis pathways such as carotenoids pathway (khodaiyan *et al.*,2008). Figure(7) estimation of growth and carotenoids production by *Paracoccus homiensis* strain BKA7 shows significant differences ($p<0.05$) in use incubation at different incubation temperature (24, 27, 30, 33, 36 C°), maximum carotenoids production were 790.47 and 912.23 µg/gm and maximum growth was 2.118 and 2.765 g/l at 27C and 30 C° respectively, while lower results were obtained 346.19 and 153.8 µg/gm and growth was 2.022 and 0.156 g/l at 24 C° and 36 C° respectively, These results are in agreement with others Wairimu, (2009), who demonstrated that the maximum carotenoids and bacterial growth for *paracoccus beibuensis* occurs at 30 C° and also he found that increase temperature lead to increase biomass but pigment production was reducing.



LSD (Incubation Temperature × carotenoid contain µg/gm$_{0.05}$)=0.0725
LSD (Incubation Temperature × Dry cell weight g/l $_{0.05}$)= 0.003

Figure(7) effect of Incubation Temperature of culture medium on growth and carotenoids production

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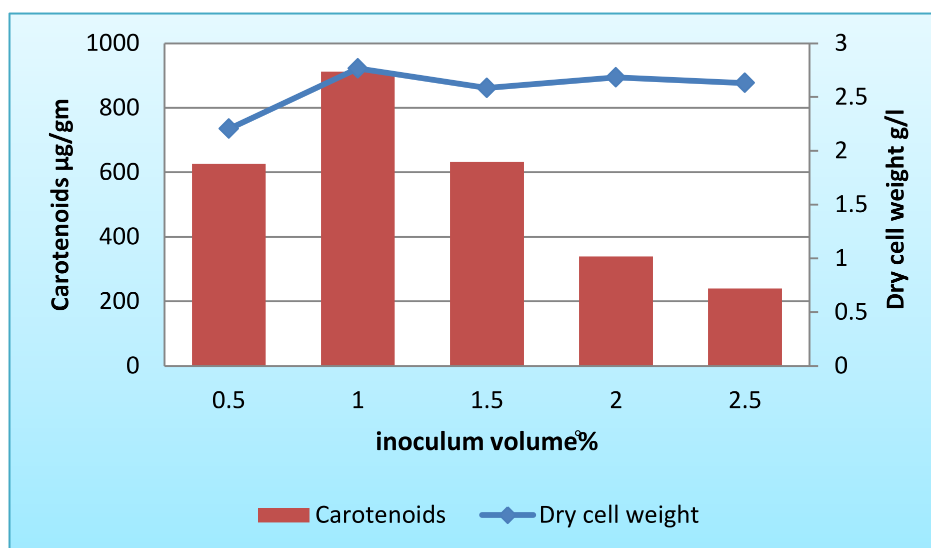
Inoculum volume

The volume of the bacteria l inoculate that effects on the content of carotenoids and bacterial growth (biomass). Figure(8) showed The influence of inoculum volume was tested were indicated a significant differences ($p<0.05$) in use volumes different, it was noticed that 1% was inoculum volume conducive to maximum carotenoids production by *Paracoccus homiensis* strain BKA7 was 912.23 µg/gm and maximum growth was 2.765 g/l , while minimum carotenoids production was 239.54 µg/gm and maximum growth was 2.630 g/l when flasks were fortified with 2.5% inoculum volume. This is in agreement with previous studies, Ji *et al*. (2012) which confirmed that high inoculum volume may be increase biomass but decreases pigment production, the inhibition of pigment formation was due to the lack of some substances in culture medium which were consumed by the high bacterial biomass.



LSD (inoculum volume × carotenoid contain $\mu g/gm_{0.05}$)=0.0875

LSD (inoculum volume × Dry cell weight $g/l_{0.05}$)= 0.007

Figure(8) effect of inoculum volume of culture medium on growth and carotenoids production

Incubation period

The incubation period is an effected on growth and carotenoids production by *Paracoccus homiensis* strain BKA7 were indicated significant differences ($p<0.05$) in use different Incubation periods are displayed in figure(9).The maximum of carotenoids production was 912.32 µg/gm and growth was 2.765 g/l at in incubation time 72 hours, while decline in both carotenoids production equivalent 328.52, 563.27, 230.33 µg/gm and growth were 2.156, 2.244, 2.136 g/l at incubation time 24, 48, 96 hours respectively. this results similar to study Dhere and Dharmadhiker (2015) who reported the maximum carotenoids and bacterial growth from *paracoccus beibuensis* was observed after 72 hours incubation.

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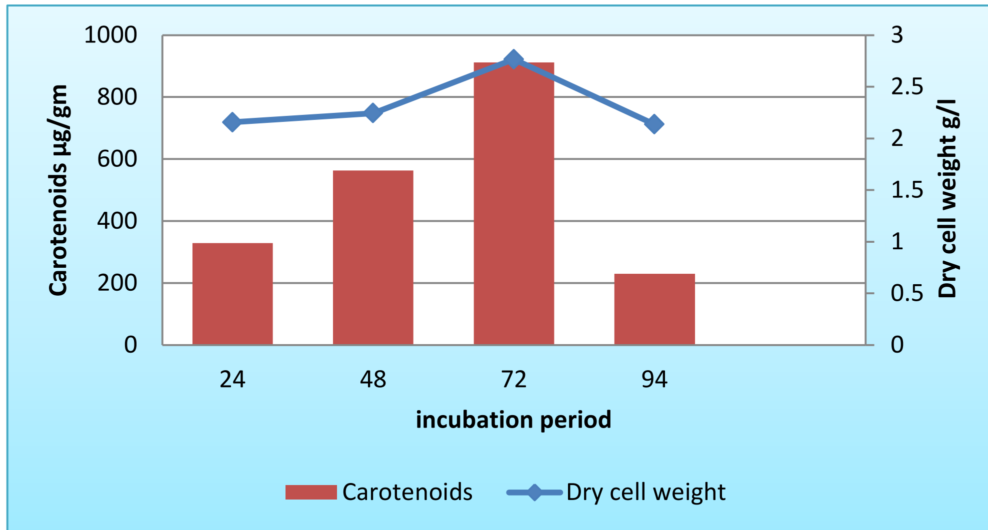
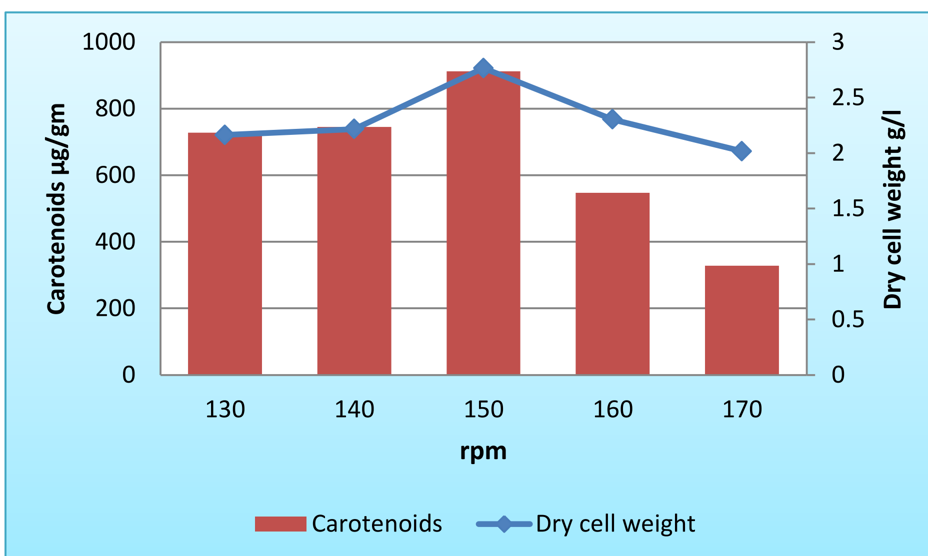


LSD (incubation period × carotenoid contain µg/gm$_{0.05}$)=0.1165
LSD (incubation period × Dry cell weight g/l $_{0.05}$)= 0.01

Figure(9) effect of incubation period of culture medium on growth and carotenoids production

Rpm

The fermentation methods using shacking and static condition are affected carotenoids production and growth. the carotenoids production by *Paracoccus homiensis* strain BKA7 and it growth were indicated a significant differences ($p<0.05$) an increase in shacking condition at different agitation rate (130-170) rpm, the carotenoids production equivalent to 728.02 and 745.48 µg/gm, the growth was 2.162 and 2.216 g/l at an agitation rate 130 and 140 rpm respectively.



LSD (rpm × carotenoid contain µg/gm$_{0.05}$)=0.1895
LSD (rpm × Dry cell weight g/l $_{0.05}$)= 0.0125

Figure(10) effect rpm of sacking culture on growth and carotenoids production

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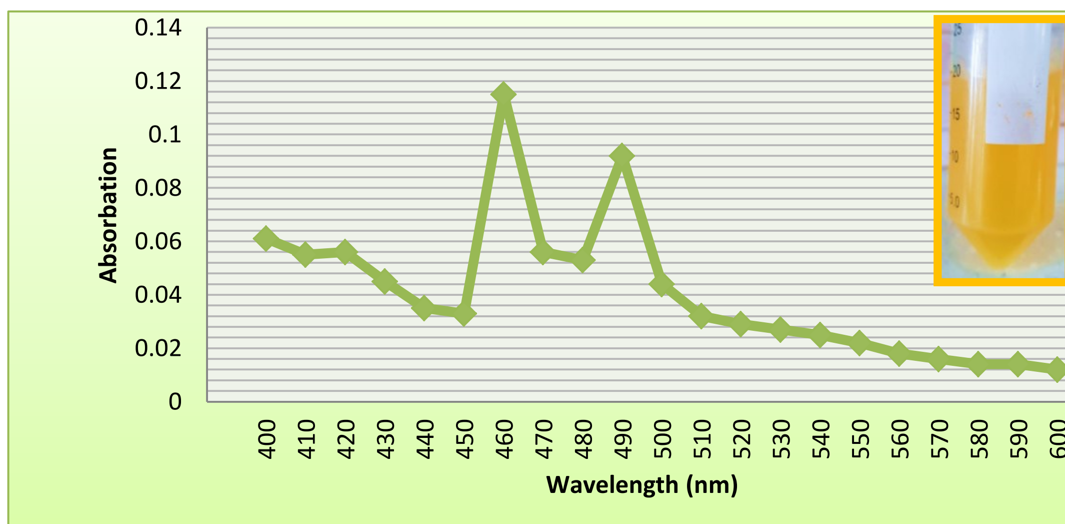
The carotenoids production and growth an increase at agitation rate 150 rpm equivalent to 912.23 µg/gm and 2.756 g/l , while minimum the carotenoids production and growth were 547.35 and 328.37 µg/gm and 2.303 and 2.016 g/l at agitation rate 160 and 170 rpm respectively. based on results shown in figure(10), this could be attributed to the aeration which was beneficial to the growth and performance of microbial cell by improving the transfer of substrates and oxygen (Valduga *et al*.,2009). This is also in agreement with previous studies Shatila *et al*. (2013) which reported that the maximum carotenoids and bacterial growth by *Exiguobacteruim aurantiacum* FH occurs at agitation rate 150 rpm.

Characterization of carotenoids pigment

Identification by spectrophotometric

The methanolic extract of carotenoid pigment was produced from *Paracoccus homiensis* strain BKA7 was analyzed by spectrophotometer by scanning the absorbance light within a wave length region 400-600nm. The extracted pigment demonstrate the presence of high region with maximum absorbance at 460 and with three peak figure (12) which was a typical characteristic of the absorption spectrum of carotenoid. This is in agreement with previous studies,Varsha and Aspana (2013) which demonstrate that the carotenoids produced from *Planococcus maritimus* AHJ_2 absorb light in the visible region 466nm. Also, Bridoux (2008) who mentioned that most carotenoid absorb light maximally at three wave length region 400-600nm thus in three peak spectra.



Figure(12) Spectrum scan(λmax460nm) of the carotenoids extracted pigment

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Thin layer chromatography(TLC)

Thin layer chromatography(TLC) was used for analyzing, identifying or separating mixtures of the crud carotenoids pigment were extracted from *Paracoccus homiensis* strain BKA7. Figure(13) showed three spots first is yellow with Rf 0.47 and second also yellow Rf 0.81 these indicating of xanthophyll because of mono hydroxylated compounds migrate to an intermediate distance while di hydroxylated compounds remain close to the baseline of the chromatography sheet, while third spot is orange with Rf 0.98 these indicating of β-carotene because of carotenes aren't retained and they migrate with the solvent
front (Mishra and Singh ,2010).

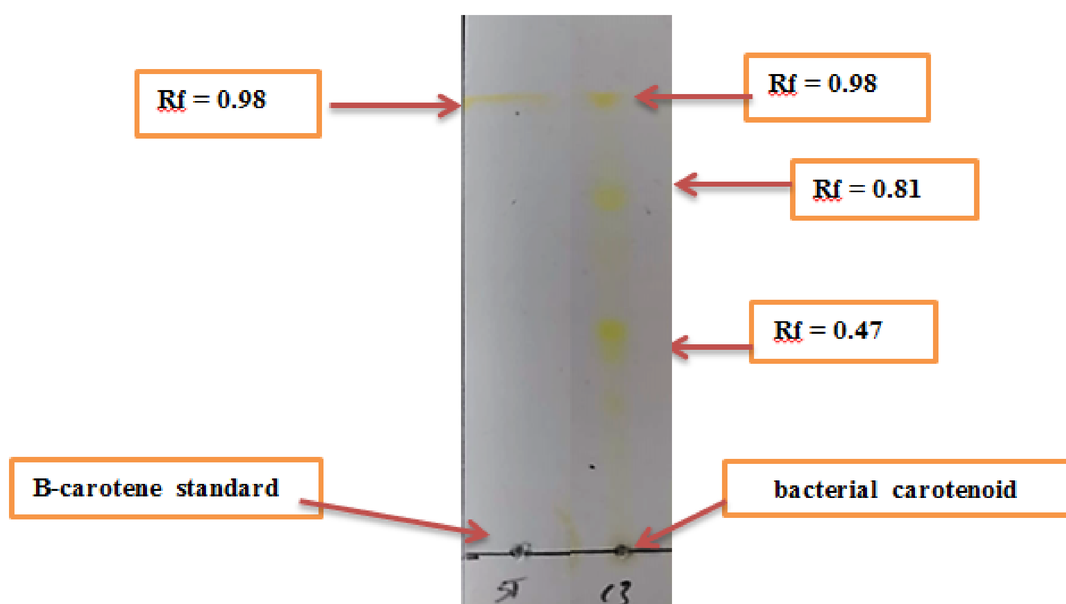
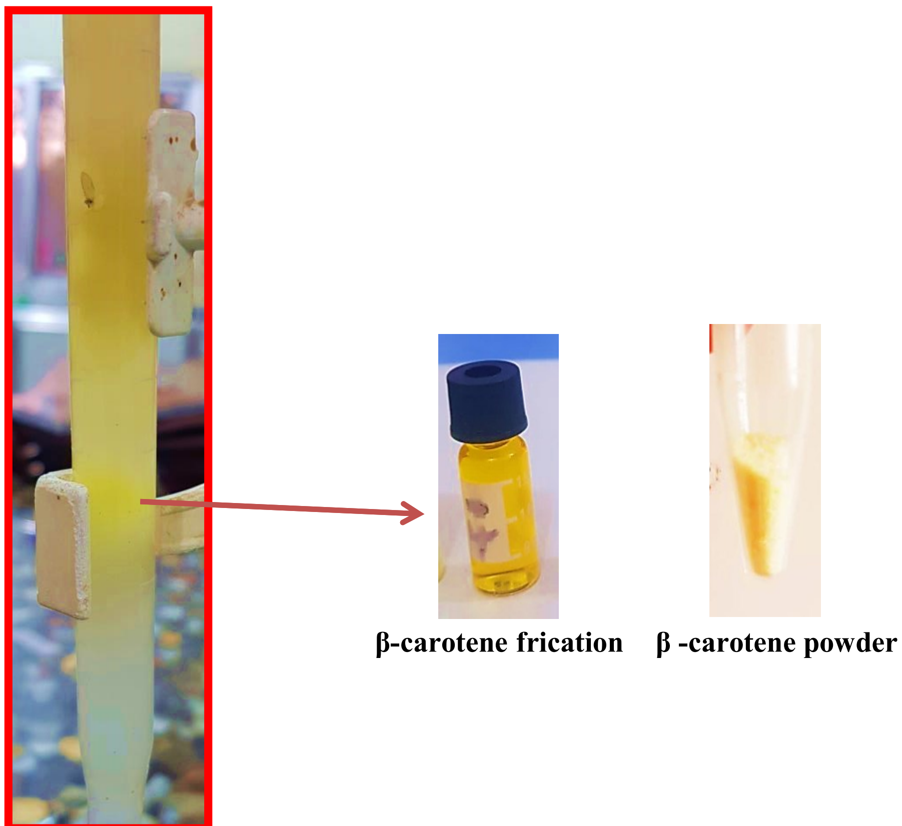


Figure (13) TLC profile of the carotenoid pigment

Extraction and purification of carotenoids pigment

The crude carotenoids extracted(methanol extract) from *Paracoccus homiensis* strain BKA7 were Purification by using silica gel 60 (0.063 - 0.200 mm) and (70-230 mesh), used the solvent system for column chromatography. The solvent system of hexane was first used in the washing process of the separation column and then the separation solution of hexane and ethyl acetate was used at a ratio of (7: 3) to increase the polarity by 50 ml and at a flow rate of 1 ml / min. The major fractions of β-carotene pigments, were separation and collected, then which concentrated, dried and preserved at -20 ° C to carry out the following analyzes. figure(14).



Figure(14) purification of of β-carotene from *Paracoccus homiensis* strain BKA7 by column chromatography

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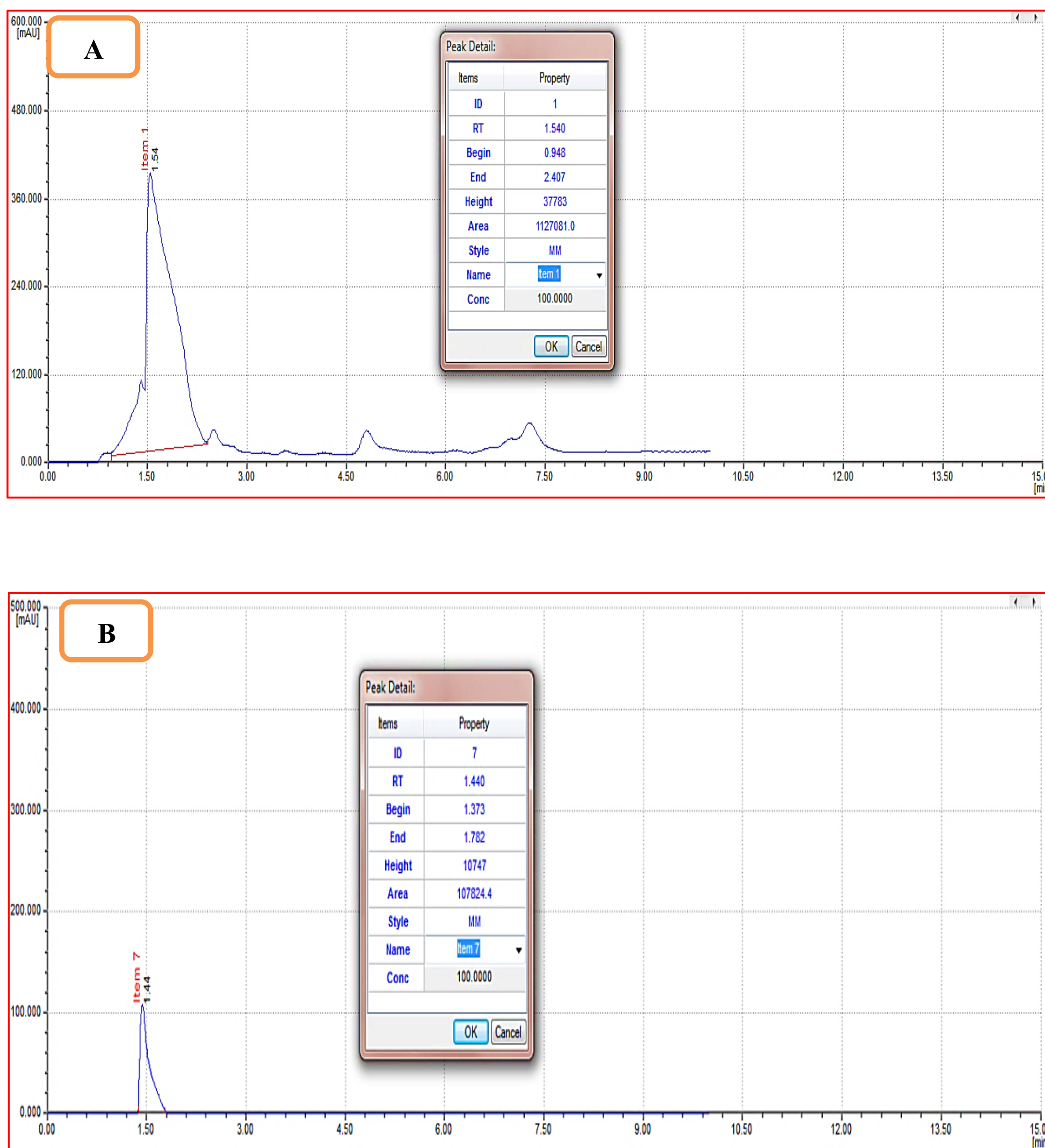
HPLC analysis of β-carotene

Identification of β-carotene was based on retention times and comparison with a pure standard by HPLC .Quantitative analysis was determined by comparing the peak area and retention time of the sample as (RT 1.440 and area 107824.4) with retention time of the β-carotene stranded as (RT 1.540 and area 1127081.0) which shown in figure(15).the result showed that bacterial β-carotene had concentration 574.00 µg/gm .



Figure(15) chromatograms of HPLC anaylsis A: β-carotene stranded B: β-carotene extracted from *Paracoccus homiensis* strain BKA7

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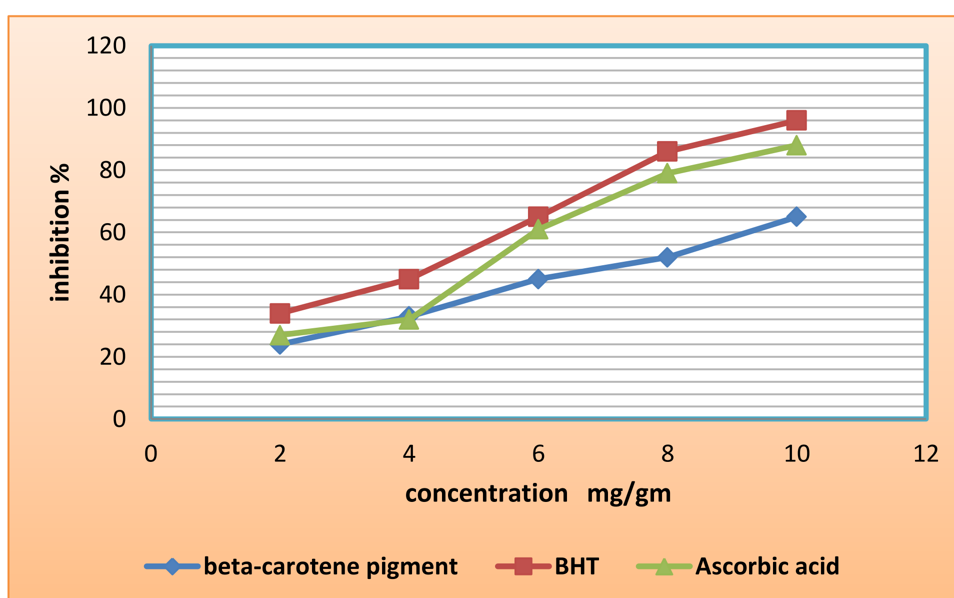
Evolution of in vitro Antioxidant activity of β-carotene pigment

DPPH is a stable radical and is frequently used for evaluating the antioxidant activity of natural colorant products. In The present study, the β-carotene pigment was produced from *Paracoccus homiensis* strain BKA7 showed had the antioxidant was evaluated and examined by DPPH method with compering to synthetic antioxidant (BHT). figure(16) showed significant differences ($p<0.05$) the antioxidant activity of pigment concentrations compare with BHT and Ascorbic acid, the IC50 value of β-carotene pigment were found to be 8mg/ml and the resulted were compared with BHT and Ascorbic acid it IC50 value were 4.6 mg/ml and 5.6 mg/ml respectively. This is in agreement with a study by Mohana *et al*.,(2013) who reported that the carotenoid pigments had antioxidant properties of *Micrococcus luteus* was reported at IC50 of 4.5mg/ml, also the IC50 value of carotenoid producing from *Rhodococcus rhodochrous* was found to be 8mg/ml (Haddad *et al*.,2017).



LSD (carotenoid concentration mg/gm × PPPH inhibition % $_{0.05}$)=1.33
LSD (BHT concentration mg/gm × PPPH inhibition % $_{0.05}$)=1.277
LSD (Ascorbic acid concentration mg/gm × PPPH inhibition % $_{0.05}$)=1.277

Figure(16)PPPH assay for determining the antioxidant activity of β-carotene pigment extracted form *Paracoccus homiensis* strain BKA7

Evolution of in vitro Antibacterial activity of β-carotene pigment

Many microorganisms produce antibacterial compounds, microbial pigments that have properties is highly advantageous .

Table(2) and Figure(17) results were showed in antibacterial activity assay, the β-carotene pigment produced from *Paracoccus homiensis* strain BKA7, them pigments concentrations were measured was showed significant differences ($p<0.05$) inhibitory activity against the pathogens bacteria compare to negative control (DMSO), According to the Disc-diffusion method, high inhibition clear zone was (ZOI 22)mm against *staphylococcus aureus*, using concentration 1200 µg/ml, also inhibition zone was (ZOI 7,17,18) mm giants *pseudomonas aeruginosa* , *Bacillus cereus* and *E.coli,* respectively , except concentration (100-600 µg/ml)

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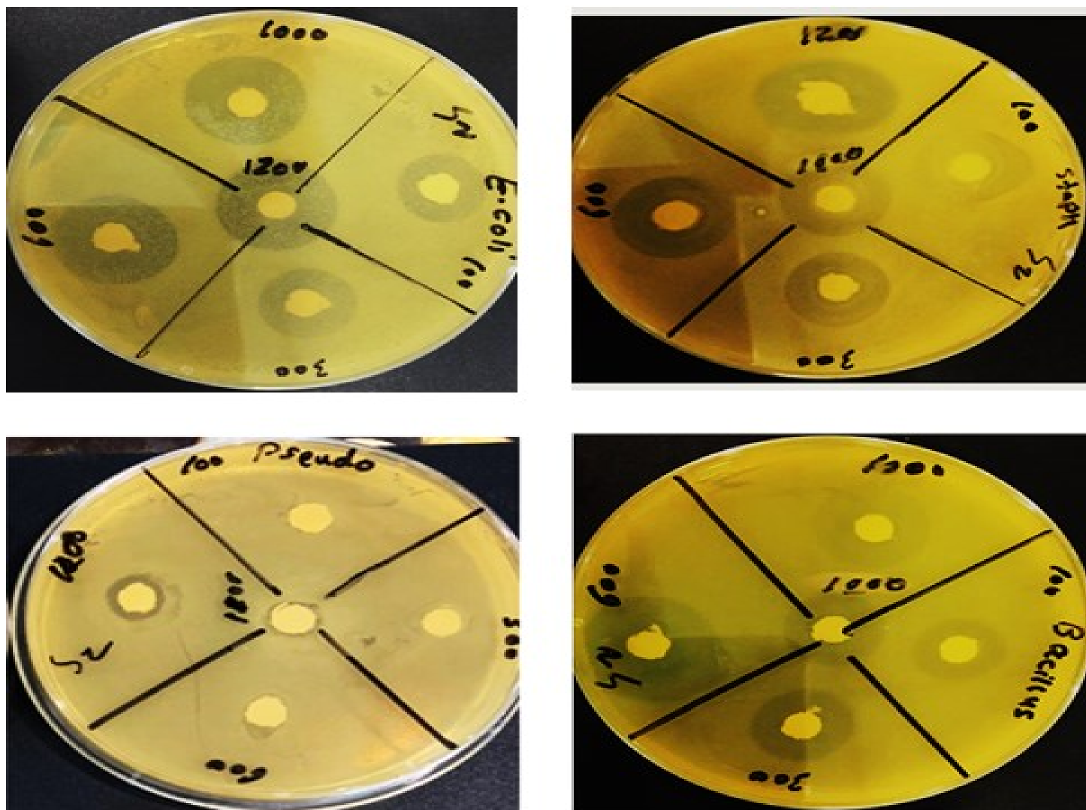
showed didn't any inhibitory effect against *pseudomonas aeruginosa*, the reason may be due to this concentration was insufficient to appropriate the desired effect, or it may be due to the low of active components in this concentration or this bacteria has resistance for antibiotic. this results similar to study Mohana *et al*. (2013) who reported that the carotenoids pigments produced by *M. roseus* and *M. luteus* had antibacterial activity against *Streptococcus feacalis* with inhibition zone of(ZOI 6.5-15) mm. Similar results were observed by several researchers such as Rostami *al et*.(2016) which that bacterial carotenoids had more antibacterial effects against gram positive bacteria than gram negative bacteria, this fact due to the presence of lipopolysaccharide in the cell wall of gram negative bacteria that prevent passes the active compounds to enter the bacterial cell . In our research and the works of other authors such as Karpinski and Adamczak (2019), the stronger effect of carotenoids pigment against Gram-positive than Gram-negative bacteria shows that biological activity depends on the differences in the cell wall structure and composition of both types of bacteria



Figure(17) inhibition zone of β-carotene pigment producing by *Paracoccus homiensis* strain BKA7 against pathogens bacteria *Pseudomonace earogenosa*، *Bacillus cereus*، *E.coli*، *Staphylococcus aureus*

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Table(2) antibacterial activities of β-carotene pigments isolated from *Paracoccus homiensis* strain BKA7 (mm)

concentration µg/ml	*Staphylococcus aureus*	*E.coli*	*Bacillus cereus*	*Pseudomonace earogenosa*
100	14	12	12	-
300	15	13	14	-
600	17	17	21	-
1000	17	17	15	5
1200	22	18	17	7
DMSO	-	-	-	-

Conclusion

In this research, it was showed that efficient productivity of carotenoid by *Paracoccus homiensis* strain BKA7 depends on optimization of the condition of both growth rate and cellular carotenoid production. The highest production of biomass and carotenoids by this strain was obtained after 150 hours at 30 °C with an initial pH of 9.0, for a period of 72 hours. Carotenoid biosynthesis in bacteria is involved in stress response mechanism, hence shacking stress is considered a major component influences pigment production in *Paracoccus*. The data obtained suggested *Paracoccus homiensis* strain BKA7 new strain of bacteria were recorded in Gene Bank as new strain which will be a new addition to list of carotenoid producing from other paracoccus strains described so far and it consider as a promising microorganism for commercial production of carotenoid from air and it's have biological functions such as antioxidant and antibacterial.

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