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Molecular Cloning and Anti-Cancer Activity of Carotenoid Pigments Isolated from *Micrococcus spp*. and *Rhodotorula spp*.

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Abstract

Biological sources of pigments receive major attention nowadays because of the stringent rules and regulations applied to chemically synthesized pigments. The aims of this study were isolating carotenoids producing *Micrococcus spp. and Rhodotorula spp.* from soil sources, optimizing the culture conditions for biomass and carotenoids production, molecular cloning of Crt gene, characterizing yielded pigment, and investigating their anti-cancer activities on human breast cancer cells. Carotenoid producing strains, *M. luteus* and *R. mucilaginosa,* were isolated from the soil and sediment samples in Kerman Province, Iran; they were identified using 16srDNA analysis. Optimum conditions for biomass and carotenoids production were determined. Further, Crt gene cloned into pTG19 vector and the effect of carotenoid pigments on MCF-7 breast cancer cell line were evaluated. The optimum growth and carotenoids production by strains were 25°C and pH 7.0 for *M. luteus*, and 25°C and pH 6.5 for *R. mucilaginosa*. FTIR and TLC analysis showed high similarity of extracted pigments with carotenoids. The expression levels of Crt genes mRNA found to be approximately 5-fold higher in transformed *E. coli* in comparison with *M. luteus* and *R. mucilaginosa* ($p<0.001$). MCF-7 cells viability decreased in a concentration and time dependent manner ($p<0.05$). The doses IC_{50} against MCF-7 cells for yellow and pink pigments were calculated to be 1426.69 µg/ml ($r^2=0.95$) and 1412.1 µg/ml ($r^2=0.92$), respectively. Microorganisms presented in this study can be used as potential sources of commercial carotenoids production and antitumor metabolites.

Keywords: Carotenoids; *Micrococcus*; *Rhodotorula;* Antitumor metabolites.

Introduction

Carotenoids are terpenoid and yellow to red colored pigments found in bacteria, plants, algae, and fungi. It has been shown that carotenoids play a vital role in mitigating oxidative damage in microorganisms as well

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as their significance in the production of beta carotene, the precursor of vitamin A. Furthermore, recent surveys have demonstrated a number of additional functions including antitumor properties, antioxidant effects, immunity boosting, and gene expression regulation. Studies revealed that beta carotene may trigger redox-sensitive regulatory pathways and act as a powerful quencher of singlet oxygen and a scavenger of free radicals which prevent cells and tissue from oxidative damage and tumor promotion. In addition, antioxidant activities of the isolated carotenoid pigments of *micrococcus* spp. were determined using DPPH radical scavenging assay [1, 2].

Numerous studies have reported that an increased consumption of a diet rich in carotenoids, including kales, tomatoes, spinach, carrots etc. is correlated with diminished risk of various types of diseases specially cancers. However bacterial and yeast carotenoid pigments offer promising avenue for various applications because of their competence to use wide range of nitrogen and carbon sources, safety, predictable yield and pigment can be easily extracted from the cell mass. Presently, microalgae are the main organic sources of industrial carotenoid pigments production, however with rapidly rising demand for these pigments, there has been renewed interest in identifying novel and valid alternatives for carotenoid producing microorganisms [1-4].

It has been revealed that of the six hundred known carotenoids, only small proportion can be produced in viable quantities by their hosts. Several groups of yeasts, belonging to the genera *Rhodotorula* sp. *Sporobolomyces* sp. and *Phaffia* sp., and Gram-positive bacteria, including species from the genera *Flavobacterium, Corynebacterium,* and *Micrococcus* has the potential to produce large amount of pigments such as beta carotene, astaxanthin, etc. [4].

The genus *Micrococci* are Gram-positive cocci, non-motile, non-sporulating, obligate aerobes and rich in carotenoid pigments with bioactive and radioprotective properties. *Rhodotorula* genus is pigmented and ubiquitous saprophytic basidiomycetous yeasts that can be recovered from common environmental sources. *Micrococcus* and *Rhodotorula* produced carotenoids are useful for food, pharmaceutical and cosmetic industries, the percentage of which depends on cultivation conditions. Previously major carotenoids synthetized by *m. luteus* NCTC 2665 had been characterized and identified as sarcinaxanthin by Netzer [1]. Benjamin et al, inexpensively isolated and characterized the carotenoid pigments produced by M. *luteus* BAA2 [6]. In addition, it had been established that various strains of *Rhodotorula* present significant properties such as production of large amounts of carotenoids [7, 8].

Production of natural carotenoids using bacteria and yeasts has been favored owing to its highly efficient, low batch-to-batch variations and simple manipulation in processing schemes. Carotenoid pigments production by *Micrococcus spp*. and *Rhodotorula spp*. can become more economically feasible by optimizing fermentation process [8]. The aims of the present research were isolating carotenoids producing *Micrococcus spp*. and *Rhodotorula spp*. from soil sources, optimization the culture conditions for carotenoids production, molecular cloning of crt gene, characterizing yielded pigment and investigating their anti-cancer activities on human breast cancer cells (MCF-7).

Materials and Methods

Microorganisms isolation

A variety of environmental soil and water samples were collected from different sites in Kerman Province, Iran. Samples were collected and placed in a sterile bag with an ice bag and transported to the laboratory within 6 hours and then processed immediately to isolate yeasts. For bacterial and yeast isolation, samples were cultivated in nutrient agar (NA) and potato dextrose agar (PDA), respectively. The pure cultures of the pigmented bacterial colonies were grown overnight at 35° in nutrient broth (NB) and stored at 4° for further studies. The isolated yeast strains were cultivated in NA (containing 45% glucose, 13 µg/ml Penicillin G and 40 µg/ml Streptomycin) and tested for morphological, physiological and biochemical characteristics according to the methods of Barnett et al. [9]. Differentiation of pigmented *Rhodotorula* yeast species based on carbohydrate fermentation was performed according to the standard procedures [10].

PCR amplification for identification of Carotenogenic microorganisms

For identification of Carotenogenic microorganisms, isolates that exhibited more colored pigments were analyzed for the presence of Crt gene cluster sequences in the genome. Yeast and bacterial DNA extraction had been carried out using QIAamp Minikit (QIAGEN, Germany) following the manufacturer procedure. The oligonucleotide concentration was determined spectrophotometrically by O.D. reading at wavelengths of 260 nm and 280 nm (NanoDrop, Thermo Fisher Scientific, USA). The DNA purity was determined using both O.D. ratio; 260/280 nm (1.5-2) which indicates that the DNA solutions is well purified. The conserve sequence of the Crt gene cluster, including *crtE*, *crtI*, $crtY_g$, $crtY_H$ and *crtEb genes*, from

micrococcus and *Rhodotorula* was identified from NCBI (National Center for Biotechnology Information, USA) and primers were designed using the identified sequence. The target sequences were amplified using PCR method as described previously by Stafsnes et al [2] and Netzer et al. [5]. The PCR conditions were as follows: 95 ◦C for 3 min, followed by 35 cycles, 95 ◦C for 30 min, 56 ◦C for 1 min, 72 ◦C for 1 min, and final synthesis at 72 ◦C for 5 min. The DNA fragments were separated by 2% agarose gel electrophoresis and sequenced. Isolated strains were identified by 16srRNA sequence analysis and the phylogenic tree was constructed using the neighbor-joining method [4]. Then, two strains of *micrococcus luteus* (*m*. *luteus*) and *R. Mucilagenosa* that had Crt gene and exhibited the good coloration were sub-cultured. These strains alongside three control strains, which were mentioned above, were selected for carotenoid pigment production examinations. Additionally, *Micrococcus luteus* NCTC 2665, *Micrococcus roseus* PTCC 1411 and *Rhodotorula mucilagenosa* PTCC 5257 were purchased from Pasteur Institute Laboratory of The Biology Department and used as a positive control.

Optimization of growth parameters for production carotenoids pigments

Since production of pigments is directly associated with the microorganism growth rates and biomass yield, optimum conditions for growth and carotenoids production have to be analyzed [12]. As mentioned above, the ability to produce biomass and carotenoid pigments by the two finally selected *M. luteus* and *Rhodotorula spp*. was further assessed alongside control strain. To evaluate the effects of carbon, nitrogen, temperature and pH on the growth and carotenoids production of microorganisms, all compounds and other factors were kept constant, and only the level of testing factor was considered variable. The growth of the microorganisms was observed spectrophotometrically. Optimization of carotenoid production and biomass growth in *Rhodotorula* and *M. luteus* were determined using YPD and GPY mediums, respectively. The YPD growth medium containing 4% NaCl inoculated and incubated at 16°,25°,30°,37° and 40°C and were shaken at 120 rpm for 4 days. Carotenoid pigments concentration and dry cell mass in media were examined every 24 hrs. Bacterial strains were incubated at 16,25,30,37 and 40°C and biomass production were noted at every 6 hrs intervals. Both bacterial and yeast cultures were grown at different initial pH (6, 6.5, 7, 7.5, and 8), carbon and nitrogen percent (0.1,1,1.5,2 and 2.5%) to determine best condition for optimum growth and pigment production.

Extraction of carotenoid pigment

The microorganism's cells were harvested by centrifugation at 10,000 rpm and 4°C for 20 min, followed by washing with distilled water. The biomass of microorganisms was quantified through drying at 60°C for *M. luteus* and 105 °C for *Rhodotorula* spp. until a constant mass was obtained.

The extraction of carotenoid pigments from *Rhodotorula spp.* cell mass was performed using techniques by Zhao el al [13]. Briefly, a tube of the mixture of 0.1 g dry weight biomass, 5mL acetone and 6 mL DMSO was subjected to 5 ultrasonic cycles at 40kHz for 10 min, vortexed and kept standing for 15 min. Biomass was removed from carotenoid containing supernatant using centrifugation at 5000 g for 10 min and re-suspended in acetone and DMSO for additional examinations. Then, the supernatant was stored for further studies.

The isolation of pigments in *M. luteus* was carried out as follow: The *M. luteus* isolates were inoculated in LB broth and incubated at 37°C using a rotating incubator at 120rpm for 72 hours. Then, the cultured media was centrifuged at 8000 rpm for 18min, the supernatant was discarded and pellets were extracted using acetone (1:5 ratio). Once needed, several extraction cycles were done to remove all visible colors from the cell pellets.

Extracted carotenoid pigments of microorganisms were covered with aluminum foil to protect the sample from light, which was stored in 4°C for further examinations.

Characterization purification of the carotenoid pigments

The absorption spectra of extracted pigments were measured at wavelengths between 300 and 600 nm using the UV-1202 spectrophotometer (Shimadzu, Germany) that revealed that the maximum absorbance occurred at 480 nm and 471 nm for *M. luteus* and *Rhodotorula* spp., respectively. The total carotenoid pigments dissolved in petroleum ether and samples concentration were assessed using the formula described by Davis et al [3]. Fourier transform infrared spectroscopy (FTIR) analysis of extracted carotenoids was performed as described by Song [4]. The carotenoid extracts were concentrated and pelleted with potassium bromide. FTIR analysis was performed using Thermo Scientific Nicolet iS5 FT-IR Spectrometer (Thermo Fisher Scientific, USA). Thin-Layer Chromatography (TLC) analysis of the extracted pigments were performed following Basker et al [16] procedure and repeated three times. TLC runs were performed in Silica

gel G plates on 10 different solvent mixtures with different ratios: A and B: toluene/methanol (7:3) and (5:5), C,D and E: chloroform/methanol/water (6:16:1), (16:6:1) and (65:25:4), F and G: water/acetone (3:5), (1:9), H and I: toluene/isopropanol (7:3),(5:2), J: acetone/petroleum ether (2:8).

Gene Cloning

To clone the Crt gene from *M. luteus* and *Rhodotorula,* the conserve sequence of *crtYg*, *crtYH* and *crt I* genes was identified from NCBI conserved domain site. Cloning was performed by TA cloning vector kit (Invitrogen, USA) following manufactures instructions. Total DNA was extracted using DNA extraction kit (QIAGEN, USA) and PCR was carried out as follows: initial denaturation at 94 °C for 7 min, then 35 cycles of denaturation at 94 °C for 1 min, followed by annealing at 62 °C for 40 sec, extension at 72 °C for 120 sec, and final extension at 72 °C for 10 min in a thermal cycler. PCR products were analyzed by electrophoresis in 0.8% agarose, purified using a QIAEXII Gel extraction kit (QIAGEN, USA) and ligated into the pTG19 vector. The ligated plasmid (pTG-crt) was transformed to the competent bacteria, *Escherichia coli* XL-1 blue strain following the method introduced by Rahimi et al [17], and incubated in Luria-Bertani (LB) broth medium free antibiotic for 55 min, at 35 °C. The transformed bacteria were cultivated on the LB agar medium (peptone 1% (w/v), yeast extract 0.5% (w/v), agar 1.2% (w/v) , NaCl 1% (w/v)) containing IPTG 200 mg/ml, ampicillin 100 mg/ml, X-Gal 20 mg/ml and incubated at 37 °C for 24h. Gene cloning was confirmed by a screening of blue (non-recombinant) and white (recombinant and effective ligation) colonies. The recombinant plasmid DNA was extracted from positive colonies incubated in the liquid LB medium containing ampicillin (100µg/ml) for 24 hrs using plasmid extraction kit (QIAprep Spin Miniprep Kit, QIAGEN, Germany). The products were analyzed by electrophoresis on 0.6% agarose gel, their size was compared with a 1kb DNA ladder (Fermentase, USA), and the recombination was verified by sequencing.

Quantitative analysis of Crt mRNA expression

Non-recombinant and successful ligated *E. coli* XL-1 colonies were collected from the media and washed twice with PBS separately to evaluate the Crt gene expression. Total RNA was extracted using RNA isolation kit (QIAGEN, Germany) and was stored under -80°C; cDNA was synthesized using Thermoscript reverse transcriptase (Invitrogen, USA) and the real-time PCR was performed using Real-time PCR kit (QIAGEN, Germany) according to the manufacturer's protocol.

Real-time PCR was performed on a StepOnePlus System (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master MIX (Applied Biosystems). A relative quantification of gene expression was performed using the 16SrRNA gene as a reference. The specificity of the PCR reaction was checked with a melting curve analysis following the final step of the PCR.

MTT cell viability assays for carotenoid cytotoxicity

The MTT-assay was used to assess the carotenoid pigments effect on MCF-7 cell viability, and the results were expressed as viable cell percentages with respect to the control (MCF-7 cell that were not exposed to pigments) according to the method described by Csepanyi et al (18). The human breast cancer cells were purchased from Pasteur Institute and were grown in an RPMI 1640 medium (Gibco, Invitrogen) supplemented with 3mM L-glutamine (Sigma), 10 % (v/v) fetal bovine serum (Gibco, Invitrogen), 1.0 mM sodium pyruvate (Sigma, USA) at 37°C. The cells were grown for 24 hrs. at RPMI medium and transferred into 96 well plates and treated with extracted pigments for 48 hr. Approximately, 10000 cells/well were seeded onto a 96-well plate and allowed to adhere for 24 hr. Three replicates of each plate were incubated with different concentrations of carotenoid extracts (62.5, 125, 250, 500, 1000 µg/ mL), and viability was recorded at 48 h. Then, the medium removed and 20 µl of 10 mg/ml solution of MTT (Sigma, USA) in PBS was added to each well. Next, following a 2-hr incubation period at 37°C, the medium was removed and 250 µl of the dimethyl sulfoxide was added to each well. Dye absorbance was measured at 560 nm and the doses inducing 50% cell growth inhibition IC_{50} were determined. The MTT test was set up in triplicate and data were analyzed using a three-way fixed effect ANOVA in SPSS (ver.15).

Results and Discussion

In this study, 40 bacterial and 61 yeast strains were isolated from different soil and water samples. Of the totally 104 isolated strains, 5 strains found to be carotenoid pigments producers. Based on the biochemical tests, morphological examinations and molecular analysis data, 70% of bacterial strains isolated from soil were *M. luteus* and 30% were *Micrococcus roseus*. Among *Rhodotorula* spp. isolated yeasts, 95% belonged to *R. glutinis* or *R. mucilagenosa* species. *M.luteus* and *Rhodotorula* spp. isolates on NA and PDA plates produced yellowish and pink-red

shaded smooth colonies, respectively. The isolated yeasts that exhibited more colored pigments could grow in liquid medium containing NaCl >10% and produced acid from Mannitol, Fructose, Mannose, sorbitol, sucrose and soluble starch which can be attributed to *R. mucilagenosa* according to Barnett et al [5]. Analysis of 16SrRNA gene sequences of isolated strains to determine phylogenetic relationship are shown in Figure 1.

Among isolated and identified pigment producing strains, seven microorganism had high pigment producing and rapid growth ability, and only the *M. luteus* and *R. mucilagenosa* presented *crtE, crtI, crtYg, crtYH* and *crtEb* genes in their genome that were selected for further studies. Several bacterial and yeast species produce carotenoids as previously mentioned. The production of pigments, in particular, carotenoids in numerous isolates of genera *Micrococcus* and *Rhodotorula* such as have been studied by others. Zhao et al and Muthezhilan et al isolated yeasts belonging to *Rhodotorula* sp. which can produce carotenoid red pigment [6, 7]. Surekha et al purified the water insoluble and extracellular yellow colored carotenoid pigment secreted by *M. luteus* strain BAA2 [8] and similar study was performed by Al-Wandawi et al [9]. According to previous studies, *M. luteus* and *Rhodotorula mucilaginosa* are in the list of most promising bacteria and yeasts for the commercial production of pigments. The effect of various parameters on biomass and carotenoids production was studied.

Effect of various parameters on biomass and carotenoids production

The incubation time of growth is an important growth parameter affecting carotenoids production and biomass of *M. luteus* and *R. mucilagenosa* (Fig. 2). Maximum biomass and carotenoids production rate reached after 72 hrs of incubation time. Yield of biomass in optimum condition was 10.5±0.34 and 11.5±0.028 g/L by *M. luteus* and *R. mucilagenosa*, respectively. Further increasing the incubation time over 72 hrs resulted in the reduction of both carotenoids production and biomass. Elsanhoty et al showed the effects of incubation time on carotenoids production in *Rhodotorula mucilaginosa*. The maximum increases for biomass and pigment production were observed after 3 days of cultivation [10]. Taskin et al studied the effects of incubation time of pigments production in extract from waste loquat kernel as substrate and showed that the maximum increase for carotenoids production was observed in 96th hour [11].

As shown in Figure 3, pH value, temperature, carbon and nitrogen concentrations have considerable effect on the biomass and carotenoids production. Microorganisms grown at 18 to 38 °C but *M. luteus* exhibited the optimal condition for biomass and pigment production in 25°C and pH:7.0, whereas *Rhodotorula* at 25°C and pH 6.5. The cell biomass increased notably with increasing the temperature with up to 25°C and lessened sharply at 36°C. With increasing the incubation time and pH of growth

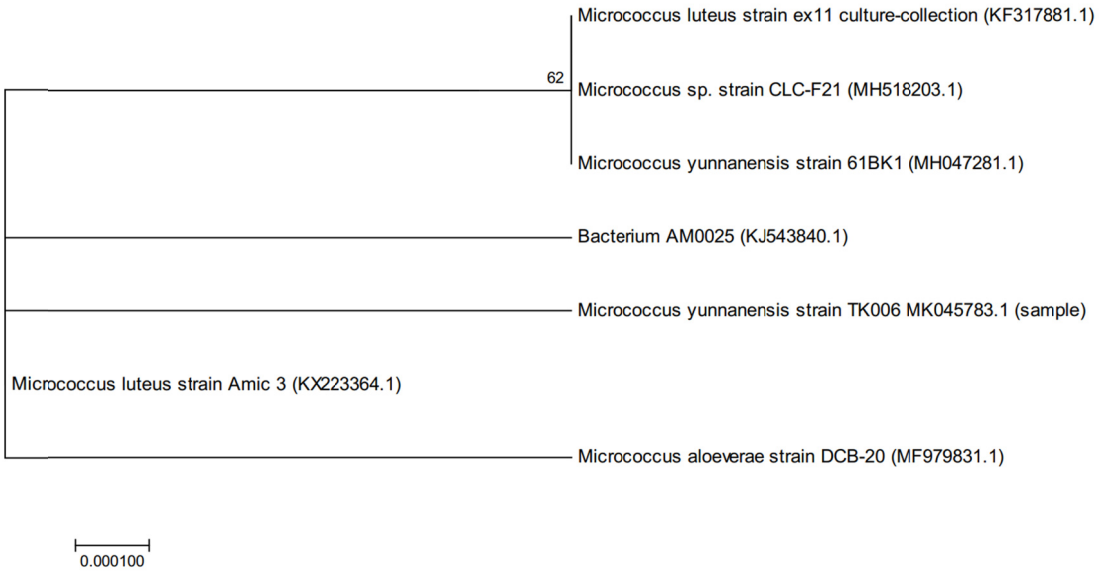

Figure 1. Molecular phylogenetic trees of 16SrRNA based on the neighbor-joining method. Numbers at nodes indicate levels of bootstrap support based on 1000 replicated datasets.

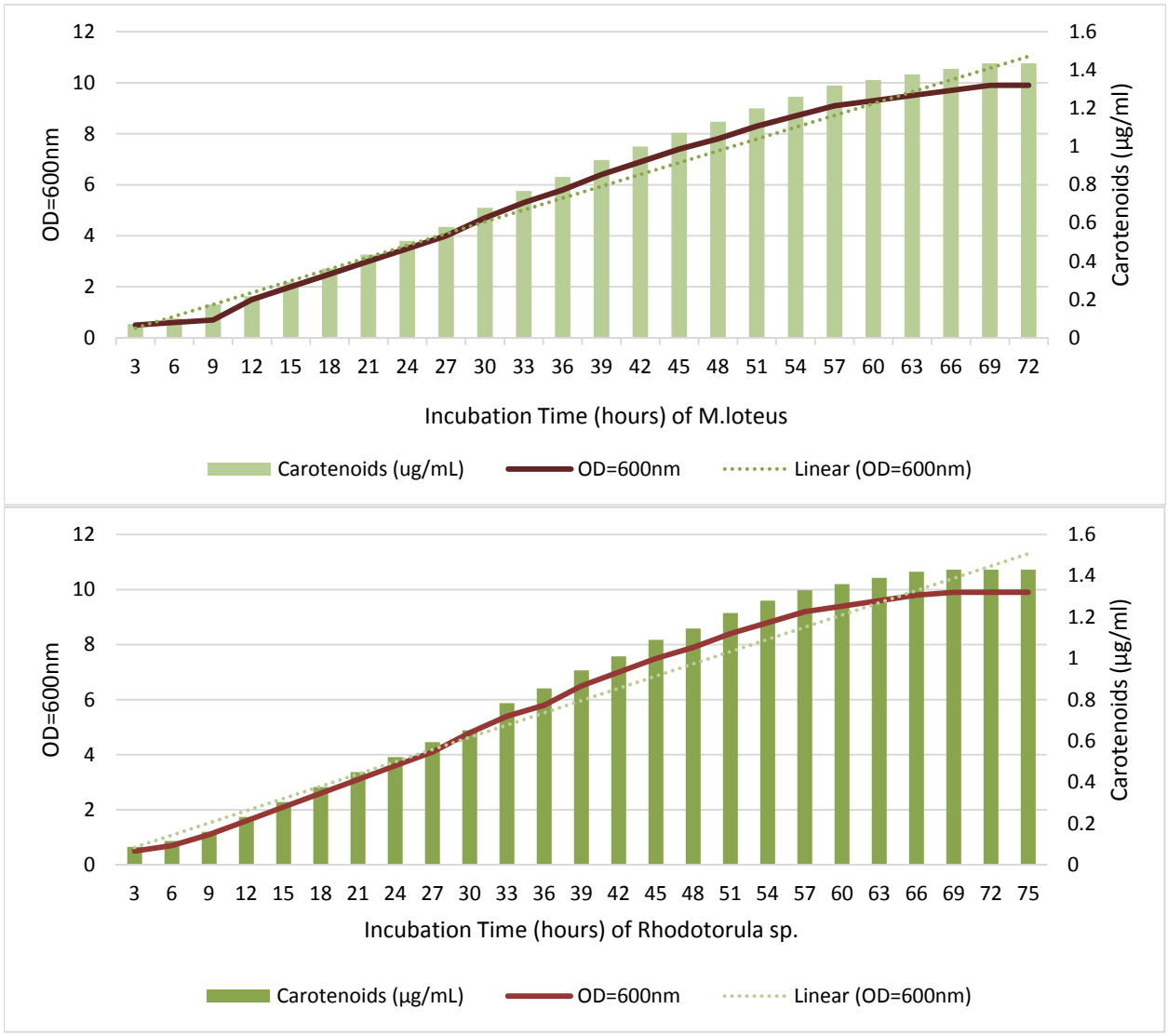

Figure 2. Effect of incubation time on growth (OD=600nm) and Carotenoids production by *M. luteus* and *R. mucilagenosa*.

medium biomass, productivity and carotenoid production increased and reached to a maximum value (Figures 2 and 3). According to the reports by Elsanhoty et al and Malisorn et al, the optimum pH for production of carotenoids was 6.5 and 6, respectively [10, 12]. The growth medium carbon and nitrogen percentage in basal medium investigated using glucose and yeast extract as proved suitable nitrogen and carbon source [6, 13], respectively. As shown in Figure 3, the maximum cell mass was reached in mediums with 1% carbon and 2% nitrogen.

Carotenoids isolation and characterization

The yellow and pink pigments produced by *M. luteus* and *R. mucilagenosa* strains were extracted and characterized by measuring absorption spectra by UV-Vis spectrophotometry (to determine the λ_{max} of the pigment), FTIR spectroscopy and TLC method. FT-IR determined the functional groups of the sample, and different functional groups absorb characteristic frequencies of IR radiations differently [8]. Extracted pigments showed the maximum absorbance of 440 and 470 nm in UVA region, respectively. In the absorption spectrum of both pigments, one major and two minor peaks were seen. FTIR absorption spectra of both pigments showed broad and strong peaks (Fig. 4). FTIR analysis showed high similarity of these pigments with chromatogram given by beta carotene. TLC of the yellow pigments produced two dots using H solvent system with a R_f value of 0.310 in UV light and R_f=0.19

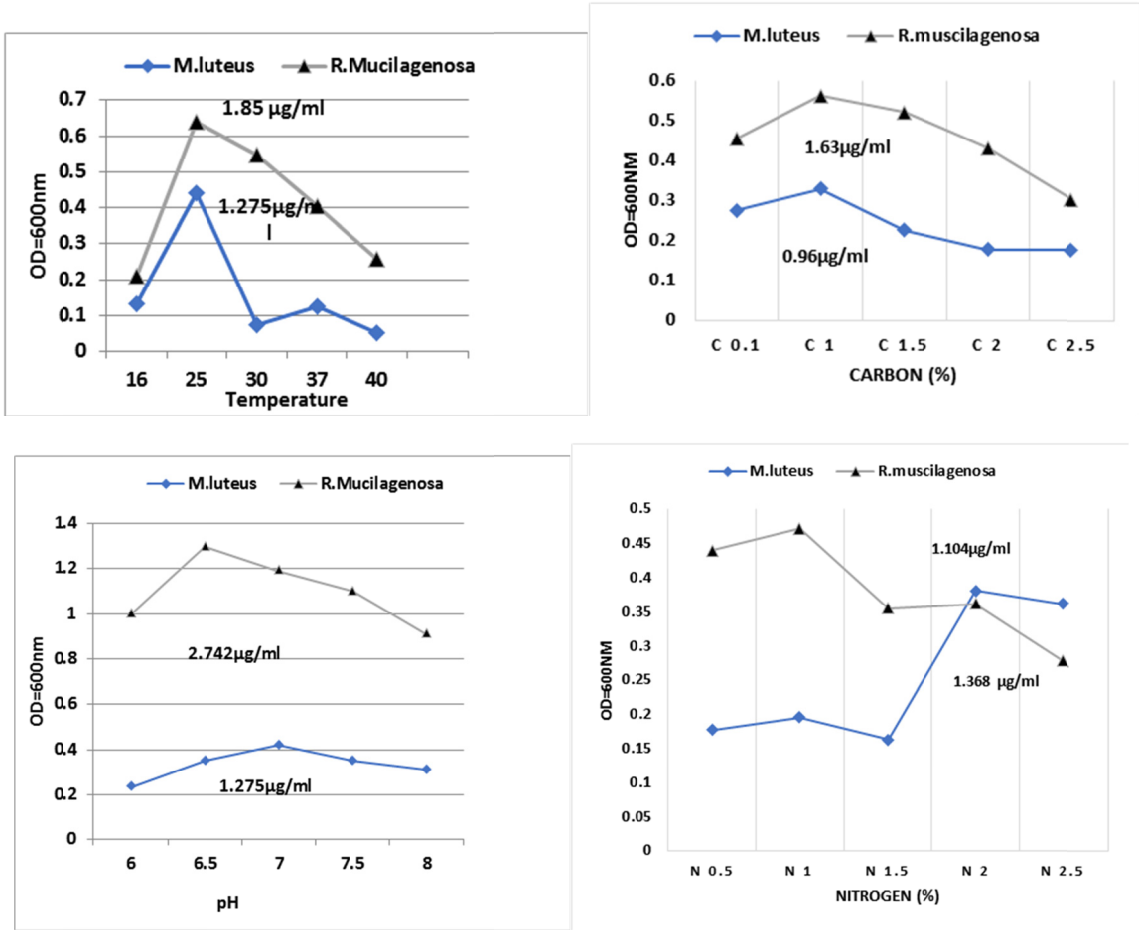

Figure 3. Effect of Temperature, pH, carbon and nitrogen percent of growth medium on biomass (OD=600nm) and carotenoids production by *M. luteus* and *R. mucilagenosa*.

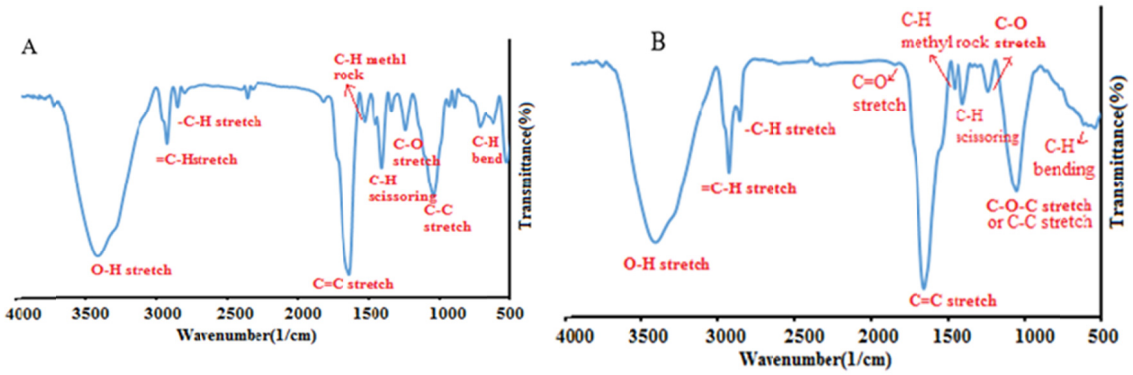

Figure 4. FTIR profiling of red carotenoid pigment produced by *micrococcus luteus* (A) and pink carotenoid pigment produced by *R. mucilagenosa* (B).

in 366nm. Two spots with R_f value of 0.240 in UV light and R_f=0.110 in 366 nm were obtained after developing TLC plates of pink pigments with I solvent system.

Cloning and gene expression data

Cloning Crt gene in pTG19 vector was confirmed by evaluating white and blue colonies. The pTG-crt was successfully constructed using TA cloning method, and

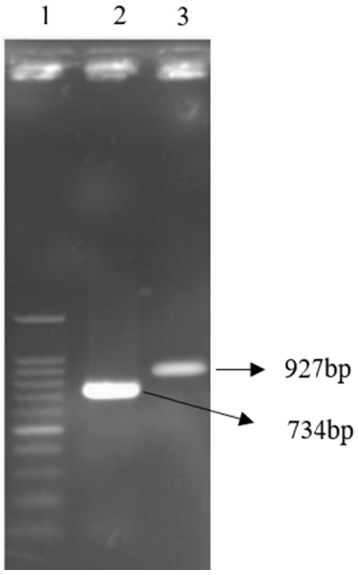

Figure 5. Gel electrophoresis analysis on PCR product. Lane 1, 100 bp DNA ladder, lane 2; pTG19T vector without insert; lane 3; pTG19T vector containing Crt gene.

sequenced (Fig. 5). The sequenced Crt gene showed 99% homology of the original gene in NCBI GenBank. The expression levels of Crt genes mRNA found to be approximately 5-fold higher in transformed *E. coli* in comparison with *M. luteus* and *R. mucilaginosa* ($p<0.001$).

Inhibition of MCF-7 cell viability

It was interesting to look for the effect of these pigments on the cancer cell viability. The results of the study of the effect of carotenoid pigments on MCF-7 breast cancer cell line during 48 hrs of treatment are detailed in Figure 6. The results showed that carotenoid pigments decreased cell viability of MCF-7 cells in a concentration and time dependent manner ($p<0.05$). The doses IC_{50} against MCF-7 human breast cancer cells for yellow and pink pigments were calculated to be 1426.69 µg/ml ($r^2=0.95$) and 1412.1 µg/ml ($r^2=0.92$), respectively. These findings are in accordance with the significant decrease in cell viability due to carotenoids extract treatment observed by Abbes et al [14]. Shree et al have reported that the beta carotene induces apoptosis and downregulates cell survival in MCF7 [15]. Vijay et al also showed that low-dose doxorubicin with carotenoids selectively upregulates oxidative stress-mediated apoptosis in MCF7 cell line [16].

Conclusion

In this study, the high carotenoid pigments producer isolates were identified as *M. luteus* and *R. mucilaginosa*. The growth and pigment production were dependent on incubation time, temperature, pH, and carbon and nitrogen percentage. Microorganisms presented in this study can be used as potential sources of commercial carotenoids production and antitumor metabolites.

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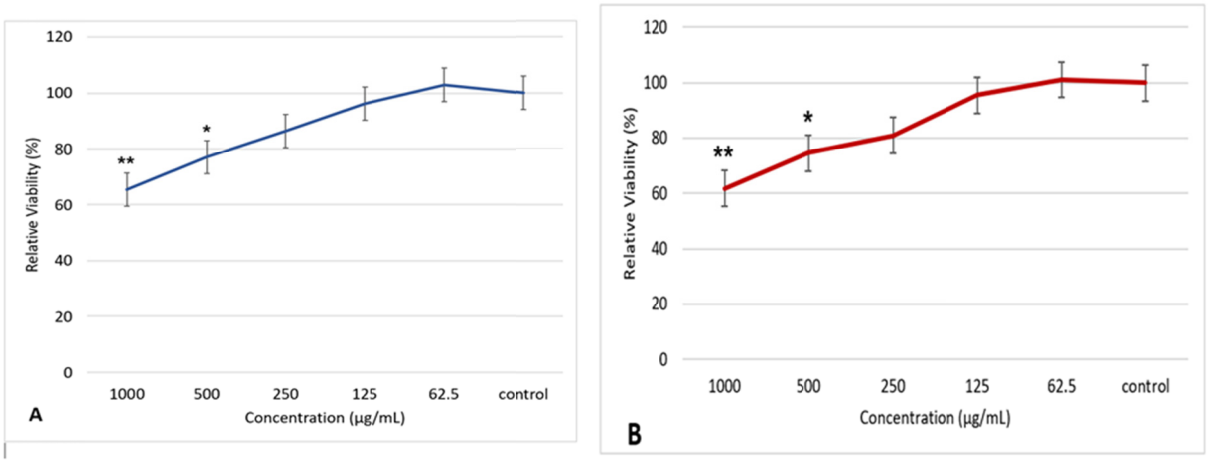

Figure 6. Impact of different carotenoids pigment concentrations on MCF-7 cell proliferation after 48h of exposure using MTT assay absorbance (570nm). The representative experiment is presented as mean± SE and statistically significant difference between yellow (A) and pink (B) pigments concentration and control cells. *$p< 0.05$, **$p<0.001$.

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