Antibacterial activities of C-phycocyanin from *Spirulina laxa*

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Abstract
Spirulina is a phylum of cyanobacteria, which are known for their distinctive photosynthetic capacity. These filamentous, gram-negative cyanobacteria or blue-green algae are one of the sources of natural bioactive compounds with therapeutic qualities like C-phycocyanin (C-PC). In this study, the C-phycocyanin (CPC/β) gene was amplified from local isolate of Spirulina laxa. The CPC/β gene (1119bp) was digested by EcoRI and BamHI, then ligated with the expression vector pGEM®-3Zf (+) to form recombinant DNA, and then was transformed to an expression host E. coli BL21(DE3). For the production of the CPC protein, we induced by 1mM IPTG. The expressed protein was analyzed by 12% SDS-PAGE. The molecular weight around 37 kDa. The antibacterial assay revealed that spirulina phycocyanin produced inhibition zones against four pathogenic bacteria, both Streptococcus agalactiae and Staphylococcus aureus, as well as Escherichia coli and Klebsiella pneumonia.
Keywords: *Spirulina laxa*, C-phycocyanin, Antibacterial activity, cloning.

1. Introduction

Prokaryotic and eukaryotic forms of the extremely diverse group of organisms known as microalgae. Microalgae are photosynthetic microorganisms that produce algal biomass from sunlight, water, and CO2. A portion of the biomass from microalgae is a rich source of certain nutrients, including proteins, minerals, carbohydrates, and other crucial components. Fatty acids, carotenoids, polysaccharides, and colored proteins with a variety of biological functions are just a few of the substances that are developing from cyanobacteria (Safari et al., 2020) Numerous studies have been conducted on cyanobacterial proteins and peptides, and they have revealed potential biological applications (Hassan et al., 2022).

A common species that is commercially grown in many nations is spirulina, a photosynthetic blue-green microalga with a spiral-shaped filament. One of the most nutrient-dense microalgae food sources on the market is this one. Spirulina's chemical make-up consists of proteins (55–70%), carbohydrates (15–25%), essential fatty acids (18%), vitamins, minerals, and pigments including carotenes, chlorophyll-a, and phycobiliproteins (phycocyanin, phycoerythrin, and allophycocyanin) (Xalxo et al., 2013). Phycocyanin (C-PC), a photosynthetic pigment of the phycobiliprotein family, is found in numerous cyanobacteria and some red algae. It is blue in color, water soluble, luminous, and located in the cytoplasm membrane's photosynthetic lamella. It is generally known that microbial pigments have a significant role in the positive effects on health (Gabr et al., 2020). This study aimed production of C-PC protein from S. laxa in E. coli BL21(DE3) and assessment its activity against pathogenic bacteria.

2. Materials and Methods

Spirulina culture and Genomic DNA Extraction

Spirulina laxa were collected from the hot water springs in Kubaisa district - Anbar Governorate, weast of Iraq. The alga was cultivated in a 1000 ml conical flask containing 500 ml of BG11 medium with pH 7.0 under sterile condition. BG11consists of NaNO3 (1.5 g), K2HPO4 • 3H2O (0.04 g), MgSO4 . 7H2O (0.075 g), CaCl2. 2H2O (0.036g), Citric acid (0.006g), Ferric ammonium citrate (0.006 g), EDTA (Na2Mg salt) 0.001g, Trace metal solution (0.02 g). The algal culture was growth at room temperature at 29±2 °C under 12/12 hour light- dark cycles for 16 days. Manual shaking of cultures was done 3 times daily. After 16 days, the biomass was harvested and its morphological structure examined under a microscope (Prabakaran and Ravindran, 2013) and the genomic DNA was extracted according to the methodology of the Geneaid Presto Mini gDNA Bacteria kit (Geneaid Kit Taiwan), the purified DNA was stored at -20 C°.

Strain of bacteria, vector and reagents

In order to clone the phycocyanin beta subunit (CPC/β) gene, the T7 expression vector pGEM®-3Zf (+) was used. The expression host for the cpcβ was E. coli BL21 (DE3) (NEB, USA).Restrictions digestions were carried out using the restriction enzymes EcoRI and BamHI from Promega, USA. Korean company Bioneer's taq DNA polymerase was employed from Promega, USA T4 DNA ligase was purchased. Geneaid (Taiwan) sold us a kit for PCR purification.

Construction and Molecular cloning of the CPC/β gene and vector

By utilizing the gene-specific primers CPC/β F1 and

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R1 (Table1) (Saker et al., 2007).The CPC/β gene was amplified by PCR at annealing temperature of 58 °C (Shoja et al., 2015). The forward and reverse primers, respectively, contained restriction sites for EcoRI and BamHI. Once the restriction enzymes EcoRI and BamHI have completed their digestion, The pGEM®-3Zf (+) expression vector which had previously been digested by the same enzymes was used to ligate the PCR amplicons.

Table 1: Oligonucleotides used in this study

Target	Primer	Primer Sequence 5' '3	Product size (bp)
CPC/β	F1	GGGCGAATTCGGAGATAAGTCCATGTTTG - 3'	
	R1	CGCGGATCCCATGCTTAGGGCGTTGATCGC - 3'	1119

Expression analysis of the constructs

The constructs were transformed using the Mandel and Higa method into the expression host E. coli BL21 (DE3) to produce the recombinant protein CPC/β(Mandel and Higa, 1970). The transformants were grown in Luria-Bertani medium (10% tryptone, 5% yeast extract, and 10% NaCl), The media was supplemented with 100µg/ml of ampicillin. the culture reached an O. D600 by vigorously shaking the cells at 200 rpm and 37 °C. The target protein's expression was boosted by the addition of 1mM IPTG. After the induction period had been in place for 3 hours, both induced and uninduced cultures reached O. D600. To evaluate the constructs' expression profiles, 12% SDS-PAGE (Solar Bio, China) was employed, and the gels were stained with Coomassie brilliant blue R-250.

3. Biological activity of CPC/β

Antibacterial assay

Bacterial test organisms used in present study obtained from Ramadi General Hospital. Antibacterial assay was carried out according to (Bauer, 1966). The pure cultures of bacterial strains were sub-cultured in Mueller Hinton broth (MHB) and incubated at 37 ˚C (Staphylococcus aureus, Streptococcus agalactiae, E. coli and Klebsiella pneumonia) on shaker incubator at 200 rpm for 24 h. Wells of 6-mm diameter were made on Mueller–Hinton Agar using gel puncture. Aliquots (50 µl) of recombinant protein CPC/β were transferred onto each well. After incubation at 37 ˚C for 24 h, the different resulting inhibition zones diameter were measured using a transparent ruler and the diameter was recorded in mm. Treatments using uninduced cells as negative control.

4. Results

Genomic DNA Extraction and Construction of the Expression Vector

In this study, Genomic DNA from S. laxa cells was extracted, the 1119 bp gene that encodes the phycocyanin protein was amplified using gene-specific primers (Figure 1). Following their purification, the amplicons were subjected to restriction digestion using the EcoRI and BamHI restriction enzymes. The expression vector, pGEM®-3Zf (+), was simultaneously digested with EcoRI and BamHI enzymes (Figure 2) to prepare it for ligation, Using T4 DNA ligase.

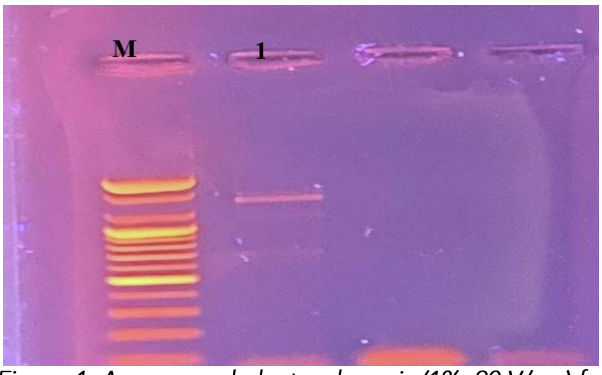


Figure 1: Agarose gel electrophoresis (1%, 90 V/cm) for PCR product of CPC/β gene. M: 100bp DNA Ladder; lane 1-PCR product at Annealing temperature 58 °C

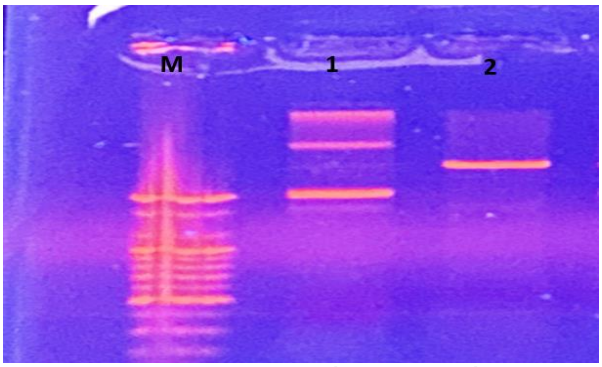


Figure 2: Double Digestion of pGEM®-3Zf (+), M: 1kb DNA Ladder; lane 1: double digestion of pGEM®-3Zf (+) by EcoRI and BamHI enzyme; lane 2: pGEM®-3Zf (+) without digestion (control).

Cloning and SDS PAGE analysis of CpC/β

The ligation products were then transformed into E. coli BL21 (DE3). After that, IPTG was used to promote the constructs' expression in E. coli BL21 (DE3). The expression profile of the CPC/β gene (37 kDa) in E.coli BL21 (DE3) was overexpressed when run on 12% SDS-PAGE as in figure (3).

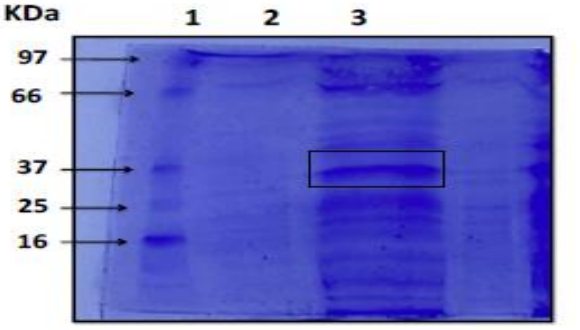


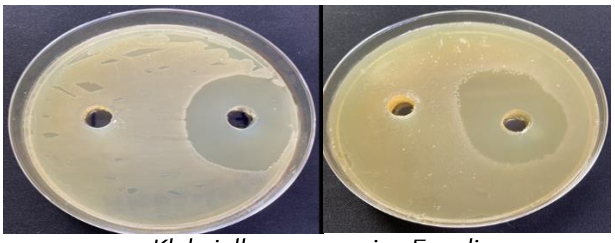
Figure 3: Expression analysis of the CpC/β protein in E. coli BL21 (DE3). The cell lysate samples were analyzed on 12% SDS–PAGE and stained with Coomassie brilliant blue G-250.

The activity of the recombinant protein CPC/β

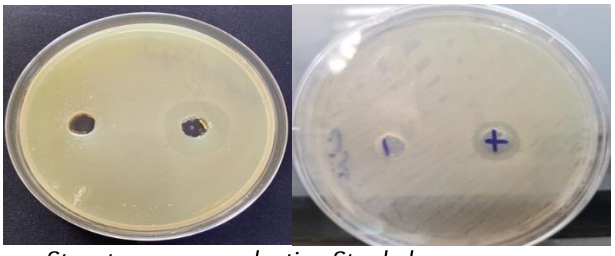
Agar well-diffusion assay. The antibacterial activity of phycocyanin was assayed against four bacterial pathogenic strains (Staphylococcus aureus, Streptococcus agalactiae as gram+ bacteria as well as E. coli and Klebsiella pneumonia as gram nagtive bacteria) by measuring the area of the inhibition zones. Spirulina phycocyanin generally prevented the growth of all of the aforementioned microorganisms. The highest inhibition zone was obtained on Klebsiella pneumonia (35 mm clear zone) (Table 2 and Figure 4). The fact that Spirulina phycocyanin exhibited about same levels of inhibitory effect on both Gram-positive and Gram-negative bacteria is noteworthy, even though the action against gram-negative bacteria was significantly stronger.

Table 2: Agar well diffusion assay of the antibacterial action of *Spirulina* phycocyanin against two gram+ (*Strepto. agalactiae*, *Staph. aureus*) and two gram- (*K. pneumonia and E. coli*) bacteria. Inhibition zone diameter (mm)

Spirulina phycocyanin	Control	Strain
13	0	*Staphylococcus aureus*
30	0	*E. coli*
35	0	*Klebsiella pneumonia*
11	0	*Streptococcus agalactiae*



Klebsiella pneumoniae E. coli



Streptococcus agalactiae Staphylococcus aureus

Figure 4: Inhibition zones in two gram+ (Strepto. agalactiae, Staph. aureus) and two gram- (K. pneumonia and E. coli) bacteria induced by Spirulina phycocyanin and uninduced cells of Spirulina phycocyanin as negative control.

5. Discussion

Beyond its application as a food coloring, phycocyanin is the blue pigment has anticancer, antioxidant, antiviral, and anti-inflammatory properties. Additionally, phycocyanin is an effective immune system booster for both humans and animals, protecting against a wide range of diseases (Ślusarczyk et al., 2021). Escherichia coli (E. coli) expression systems have been used for recombinant protein engineering for about 40 years, and E. coli is still the most frequently employed host organism today. Recombinant protein engineering is a highly effective method that applies to numerous fields. It enables the manufacture of large yields of specifically tailored proteins at a reasonable cost and with customizable, straightforward steps. Baseline expression and the stability of recombinant proteins are influenced by the vector, E. coli cell strain, peptide tag additions, and growth conditions that are used (McCormick et al., 2014).

For instance, Wang et al.(2007) used the pGEX-2T vector with the Tac promoter to biosynthesize the phycocyanin beta component from Anabaena. Recombinant CPC/β was discovered to have anti-cancer qualities (Wang et al., 2007).

Two speciesof Gram- negative bacteria, Klebsiella pneumoniae, Escherichia coli, and Gram- positive bacteria, Staphylococcus aureus, and Streptococcus agalactiae, was inhibited in the current study by C-phycocyanin from Spirulina laxa.

In the study carried out by (Sitohy et al., 2015) phycocyanin from Anabaena sp . have shown antibacterial activity against two Gram-positive and two Gram-negative bacteria. (Muthulakshmi et al., 2012) studied the inhibitory effects of phycocyanin from Spirulina on E. coli, Streptococcus sp., Pseudomonas sp., Bacillus sp., and S. aureus. The overall results of Sitohy et al. (2015) and Muthulakshmi et al. (2012) confirm the current study. This study's suggests that Spirulina phycocyanin may primarily target bacterial cell walls and membranes.

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7. References

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