

Expression in *Saccharomyces boulardii* of recombinant Toxin-coregulated pilus A subunit (TcpA) of *Vibrio cholerae* O1

Suadad Awad^{1*} ORCID: <https://orcid.org/0000-0002-4346-306X>

Saife D. Al-Ahmer² ORCID: <https://orcid.org/0000-0001-5552-9452>

Shahla'a M. Salih³ ORCID: <https://orcid.org/0000-0002-2882-9676>

¹ Ministry of Science and Technology, Baghdad, Iraq.

² Institute of Genetic Engineering and Biotechnology for Post Graduate study, Baghdad University, Iraq.

³ College of Biotechnology, Al-Nahrain University, Baghdad, Iraq.

email: suadad.awad@gmail.com; sosomicro22@gmail.com

Cholera is endemic in over 50 countries with an estimated mortality of 100,000-120,000. Vaccination is considered the complementary key to prevent and control cholera; therefore, alternative vaccine preparations are needed. Toxin Co-regulated Pilus is part of the *toxR* virulence regulon, which is necessary for colonization in the intestinal mucosa. In order to express *Vibrio cholerae* TcpA protein in *Saccharomyces boulardii*, the expression plasmid pYES2 was constructed by inserting *tcpA* gene isolated from local *Vibrio cholerae* Eltor Inaba isolates. The new construct was transferred into *Saccharomyces boulardii* cells and the expression of *tcpA* gene was induced from the GAL1 promoter by adding galactose to the medium. The SDS-PAGE and Western blot analysis showed the presence of TcpA in yeast. These results showed that *Saccharomyces boulardii* is a promising host to express *Vibrio cholerae* toxin TcpA as the first step in attempt to produce an oral *Vibrio cholerae* vaccine.

Palabras clave: *Saccharomyces boulardii*; *Vibrio cholerae* O1; cholera; cloning; DNA; *Escherichia coli*.

Introduction

Vibrio cholerae is a globally important pathogen, causing an estimated of 1.3 million to 4.0 million cases of cholera and approximately 21,000 to 143,000 deaths worldwide every year.⁽¹⁾ Although infection is treatable with rehydration therapy and the use of antibiotic, the explosive nature of outbreaks makes it difficult to treat infected patients quickly and efficiently. Effective control measures rely on prevention and preparedness.

In Iraq, cholera became an endemic disease, strikes in epidemic form nearly every ten years; with irregular outbreaks, since first appear in 1820. Periodic outbreaks have been recorded since 1966 until 2015. The disease still have high fatality rate especially in the poor districts and the refugee camps, according to the Humanitarian Needs Overview (UNOCHA) in 2018. At least 1.89 million people are currently displaced throughout Iraq, concentrated in camps, informal settlements, host communities and newly reclaimed areas; these crowded living conditions and restricted access to safe water and sanitation put people at risk of exposure and transmission

of communicable diseases, including cholera. Although the infection rate in the current years (2015-2020) is considered relatively low, Iraq is vulnerable to an epidemic at any time due to previously mentioned bad conditions.⁽²⁾

Vaccines are a necessary component in preventing cholera. Many cholera vaccine iterations have been explored throughout the last 125 years. In the 1960s, a parenteral cellular killed cholera vaccine proved to be effective against the disease in adults, but resulted in a short protection period and caused reactogenic effects including fever and swelling.⁽³⁾ Presently there are different kinds of oral vaccines: Dukoral (killed whole cell monovalent (O1) recombinant B subunit of cholera toxin), Shanchol™, Euvichol, mORCVAX™ (killed modified whole cell bivalent O1 and O139 vaccines), and the last developed vaccine known as Vaxchora™, that is approved in the US. All these types give imperfect protection with a limited time in adults and limited and minimal immune response in children under 5 years, pregnant women and immunodeficiency patients who vaccinated through oral administration.⁽⁴⁾

* PhD Biotechnology student and Scientific Researcher.

S. boulardii is an exceptional probiotic. It can grow at 37°C as an optimal temperature, remains alive in gastric acidity, does not impede by antibiotics and does not impact the normal microflora.⁽⁵⁾ When using up *S. boulardii*, it preserves persistent levels, does not constantly settle the colon and does not proceed easily out of the intestinal tract;⁽⁶⁾ all these features have nominated it as a perfect probiotic agent and is listed in generally recognized as safe (GRAS) by the Food and Drug Administration. It is widely used in pharmaceutical and food industries, being possible to manufacture it in large quantity with minimum cost. Since its uncovering nearly 100 years ago until now, it is used as a probiotic microorganism. It has several medicinal effects such as the treatment of infectious diarrhea and inflammatory bowel diseases.⁽⁷⁾ Also, it has the ability to eliminate the enteric pathogens, such as *V. cholerae*, *Shigella*, *Clostridium difficile* and *Salmonella*.⁽⁸⁾

Nowadays, *S. boulardii* is used as prophylactic and vehicle for delivering drugs. It gives the advantage to use low drug doses, additional interactions with the mucosal immune system and low cost compared with other delivery methods, such as nanoparticles and liposomes. Also, as a drug delivery vehicle is able to express intricate, glycosylated antigens. In addition, it reveals high impedance to high temperatures and low pH, which is a chance to survive transit through the intestine. All these features allow the successful transformation of DNA and production of recombinant protein in *S. boulardii*.⁽⁹⁾ DNA transformation and production of recombinant protein in *S. boulardii* have been successfully reported by several studies.⁽¹⁰⁾

Toxin Co-regulated Pilus (TCP) is a type IV pilus and consists of 20.5-kDa homopolymer pilin subunit called TcpA. It exists in environmental and pathogenic strains in all serotypes and biotypes, represents an essential virulence factor of *V. cholerae*, plays a very critical role in *V. cholerae* colonization in intestine, protects bacterial cells from antimicrobial agents, is the bacterial receptor for CTX phage and enhances biofilm formation on chitinaceous surfaces. This role was detected from studies when mutation or completely deletion of *tcpA* lead to loss pathogenicity and the colonization ability from *V. cholerae*.⁽¹¹⁾ This microorganism is considered a public health risk. To control and prevent spreading of this bacteria, preparation of safe and cheap alternative vaccines that provide high immunity for all ages for a long time is required. In order to achieve this goal, the present study was designed to produce a candidate

subunit vaccine by expressing recombinant TcpA protein of *V. cholerae* in *Saccharomyces boulardii*.

Materials and Methods

V. cholerae

The *V. cholerae* strains used in this study, as a source of chromosomal DNA gene (*tcpA*), were obtained from Iraqi hospitals laboratories where they were isolated from patients primarily diagnosed with cholera. Identification of these strains was confirmed by biochemical test using BIOMÉRIEUX VITEK® 2 SYSTEM (France). All strains were re-grown on Thiosulfate citrate ball salt sucrose agar (Himedia, India) plate to have pure colonies. Single colony was transferred and streaked on Nutrient agar (Oxoid, UK) plate, and incubated at 37°C for 24 h. Then, a sufficient number of colonies of a pure culture was transferred by sterile swab and suspended in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a test tube.

The turbidity was adjusted to 0.5 by the turbidity meter DensiChek™; the suspension was loaded to a reagent card (have 64 an individual test) and was incubated for 6 h, to read the results. Biotyping test was determined depending on polymyxin B susceptibility, hemolysin test, and Voges proskauer test.⁽¹²⁾ The serologic affirmation test was carried out by utilizing polyvalent (O1) and monovalent Ogawa/Inaba antisera (Biotic lab, England).⁽¹³⁾ Molecular diagnosis for *V. cholerae* was achieved by sequence technique (IDT, Singapore) using two types of primers (16sRNA and EUB338).

All *V. cholerae* isolates in this study, that contained *tcpA* gene, were confirmed by two ways. First, by observing the autoagglutination between *V. cholerae* cells. For this, an inoculation in 20 mL of Brain heart infusion broth (Oxoid, UK) and incubation in shaking incubator (250 rpm) at 37°C for 18 h were carried out. Overnight cultures were watched for clumping and clotting of the bacterial growth with the appearance of autoagglutination phenomenon, which is correlated with the increase of hydrophobicity when TCP is produced by the bacteria.⁽¹⁴⁾ Second, by PCR technique using *tcpA* forward primer (AGGGATCCATGAA CCGGTCAAGAGGGTATGA) and reverse primer (AACTCGA GCT TC CTG GTGCAATGGACTT) manufactured by IDT, Singapore. The PCR thermocycling conditions were: initial denaturation at 94°C for 3 min, 1 s for denaturation at 94°C, annealing at 85°C for 1 min, extension at 72°C for 1 min, and final

extension at 72°C for 5 min. The PCR products were analyzed by agarose gel electrophoresis.

S. boulardii

S. boulardii strain was obtained from re-growing the yeast capsules (Dietary supplement/Piping rock health products, USA). The yeast capsules were opened and poured in yeast extract peptone dextrose (YPD) broth, incubated at 37°C with shaking for 1 day, then transferred to YPD plate to collect single colony and after that, were sent for sequencing by IDT to confirm the species.

Mutagenesis

A single colony from *S. boulardii* was grown on YPD broth (1% yeast extract, 2% peptone, 2% dextrose in distilled water) for 20 h. Cells were precipitated by centrifugation (TomyKogyo, Japan) and washed with Phosphate buffer saline (PBS).

The cell pellet was resuspended in PBS and the suspension (20×10^7 cells/mL) was poured into sterilized petri dishes and exposed to UV radiation (the distance between the UV source and irradiated suspension was 20 cm) from a bulb (Philips, TUV 15 W/G15) under sterile conditions, with gently agitation by magnetic stirrer; 0.5 mL of cell were taken every 10 s over 100 s. Irradiated cell suspensions were stored in foil-wrapped tubes at 4°C to avoid photoreactivation; then, plated on YPD agar and incubated at 37°C for 48 h to determine the viable count.

Survival yeasts were cultured, for one week, on yeast nitrogen base (YNB) containing 5-fluoroorotic acid (FOA) (YNB-FOA), prepared as follow: 0.67% YNB (Biobasic, Canada) supplemented with 2% glucose (Duchefa biochemie, Netherlands), 10 mM uridine, uracil (Sigma, USA) and 0.1% 5-FOA (Sigma, USA). Resistant colonies to 5-FOA were cultured on yeast nitrogen base with uracil and uridine (YNB-UU) and YNB.⁽¹⁵⁾

Cloning and transformation of *tcpA* gene by using the plasmid vector (pYES2.1/V5-His-TOPO®)

***Escherichia coli* transformation**

The cloning reaction was prepared by adding 2 µL of the fresh PCR product (*tcpA* gene), to 1 µL of salt solution, 2 µL of sterile water, and 1 µL of Topo vector (Invitrogen, USA); it was mixed gently and incubated for 5 min at room temperature. The reaction was placed on ice; then, 2 µL of the cloning reaction was added into a vial of TOP10 One Shot® Chemically Competent *E. coli* (Invitrogen, USA), mixed gently, and incubated on

ice for 5 to 30 min. The cells were heated for 30 s at 42°C without shaking. Immediately, the tubes were transferred to ice and 250 µL of room temperature Super Optimal broth with Catabolite repression medium (Invitrogen, USA) were added, the tubes were horizontally shaken (Lab companion, Ghana) at 200 rpm at 37°C for 1 h. Then, 10-50 µL were spread on pre-warmed Luria broth (LB) (Lab, UK) plates containing 50-100 µg/mL ampicillin (Sigma, USA) and incubated overnight at 37°C. Ten colonies were picked and cultured overnight in LB medium containing 50-100 µg/mL ampicillin. This culture was used to extract the transformed plasmid according to NucleoSpin® plasmid kit.

Yeast transformation

The yeast was transformed by electroporation.⁽¹⁶⁾ Cells were grown overnight, diluted to optical density reading 1.6 at 600 nm and were washed with icy water, buffer 1 M sorbitol and 1 mM CaCl₂ (Sigma, USA). Then, 100 mM of LiOAc (Sigma/USA) and 10 mM DTT (Invitrogen, USA) were used to suspend the cells and to agitate for 30 min. The pelleted cells were washed and resuspended in 1 M sorbitol and 1mM CaCl₂. A volume of 400 µL of cells was transformed with recombinant plasmid. Electroporation was performed using Gene Pulser Xcell TOTAL syst (Bio-Rad Laboratories, United States), voltage 1500, capacitance 25, and resistance 200. The transformation mixture was spread on the selective medium, and positive transformants were selected after 1-3 days of incubation at 30°C.

Expression of recombinant protein

The modified yeast cells containing the recombinant plasmid were cultured in 15 mL of Synthetic complete medium containing 2% raffinose and agitated overnight at 30°C. The overnight culture was centrifuged at 1500 g for 5 min at 4°C and the pellet cells were resuspended in 1-2 mL of induction medium (0.67% YNB, 2% carbon source, 0.01% adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan, uracil, and 0.005% aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine and valine). The volume was completed to 50 mL of induction medium and agitated under 30°C. The cells were harvested at 0, 4, 8, 12, 16, and 24 h by centrifugation, resuspended in 500 µL of sterile water and centrifuged for 30 s at maximum speed in the microcentrifuge (these methods depend on pYES2.1 TOPO TA Expression kit steps, Invitrogen, USA).

Detection of Recombinant Protein

The frozen cell pellet was resuspended in 500 μ L of breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol, and 1 mM PMSF) and centrifuged at 1500 g for 5 min at 4°C. The supernatant was removed and the cells were resuspended in a volume of breaking buffer to obtain an optical density reading at 600 nm. An equal volume of acid-washed glass beads was added and vortexed for 30 s, followed by 30 s on ice, and repeated four times for a total of 4 min to lyse the cells. Cells were lysed by shear force to release the recombinant proteins.

Purification of recombinant protein

The protein was purified using Ni-NTA spin Kit (Qiagen, Germany). Briefly, protein was resuspended in 630 μ L of lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 8.0) with 70 μ L of lysozyme stock solution (10 mg/mL) and 15 Units Benzonase[®] Nuclease that were added to each 5 mL cell pellet cultures lysate. This lysate was centrifuged at 12,000 g for 15–30 min at 4°C and 600 μ L of the cleared lysate supernatant containing the 6xHis-tagged protein was loaded up onto a pre-equilibrated Ni-NTA spin column and centrifuged for 5 min at 270 g; after that, the column was washed twice with 600 μ L of 50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole, pH 8.0 and centrifuged for 2 min at 890 g. The protein was eluted twice with 300 μ L of 50 mM NaH_2PO_4 , 300 mM NaCl, 500 mM imidazole, pH 8.0 and centrifuged for 2 min at 890 g.

Protein analysis by SDS-PAGE and Western blot

The recombinant TcpA produced by genetically engineered *S.bouhardii* was applied in polyacrylamide gel to determine the molecular weight. Protein ladder 250 KD (PageRuler[™] Plus Prestained Protein Ladder, thermo fisher Scientific, USA) was used to estimate the molecular weight of separated proteins. The gel plate was rinsed with water, 70% ethanol, and scrubbed with tissue paper. The separating gel (lower gel: 1.5 M Tris-Cl, pH 8.8 and 30% acrylamide, distilled water, 10 mg of ammonium persulfate and 5.0 μ L TEMED) was pipetted carefully into the glass up to 3/4th portion of the gel plates. Distilled water was pipetted into the free space and left in the glass purposely as an indicator for gel polymerization and to remove unwanted air bubbles. The gel was allowed to polymerise for 45 min. When the lower gel polymerized, the distilled water was rinsed out and excess water was removed using a paper towel. Then, the stacking gel (upper gel: 0.5 M Tris-

Cl, pH 6.8, 30% acrylamide, distilled water, 5 mg of ammonium persulfate and 3.5 μ L TEMED) was poured and a comb was inserted into the stacking gel. After gel solidification, the comb was removed. The solidified gel with plate was fixed in the gel running apparatus.

The gel running tank was filled with 1x running buffer prepared as followed: 0.5 g of SDS, 100 mL of running buffer (5x) (15.1 g Tris-base, 72 g glycine) and 400 mL of distilled water. Later, the ladder and 10 μ L of prepared samples were loaded into wells and 200 voltage electric field was applied for 1 h. The gel was transferred to a clean container and stained overnight with Coomassie stain (0.5 g of Coomassie brilliant blue R250, 800 mL methanol and 140 mL glacial acetic acid) at room temperature with constant shaking. After staining, the gel was washed twice with distilled water and destained using destaining solution (200 mL methanol and 70 mL glacial acetic acid) until a clear background was obtained. The gel then was soaked with running buffer to keep it hydrated and used it later in Western blot.⁽¹⁷⁾

Before applying the gel in Western blot, the filter paper and the cellulose membrane were wetted by transfer buffer (15.1 g Tris-base, 72 g glycine and final volume was made up to 1 L with distilled water and 20% methanol). The “transfer sandwich” was placed in semi-dry transfer chamber and the Trans-Blot-Turbo (Bio-Rad, USA) was run at 25 V for 5 min. The blotted membrane was blocked with skim milk (2 g in 40 mL PBS) and shaking for 1 h; later, the milk was removed and another skim milk (1 g in 10 mL PBS) was added. Two μ L of AntiHis mouse primary antibody (Transgen biotech, China) was added and incubated for 1 h with shaking; then, 5 mL PBS with Tween 20 (PBST) was added and shaking for 3 min, this step was repeated 3 times. After that, secondary antibody (Abs) was added to PBST and incubated for 1 h with shaking. Finally, 1mL of Abs in PBS was added and shaking until the bands appeared clearly.⁽¹⁸⁾

Results and Discussions

The *V. cholerae* strains used in this study as a source of chromosomal DNA gene (*tcpA*), were 99% identical when they were diagnosed by Vitek system, while the serological tests proved that all strains belonged to *V. cholerae* O1 Eltor Inaba. These results were confirmed at molecular level diagnosis. All strains belonged to *V. cholerae* O1 and the alignment rate reached to 99.38% for EUB338 primer and 97.68% for 16sRNA primer when compared with data in NCBI gene bank.

The *tcpA* gene was detected in all *V. cholerae* strains. A visible clumping of bacteria appeared as a pellet at the bottom of the tube, leaving a clear supernatant (phenomenon known as autoagglutination) and the hydrophobicity increased in broth culture, due to the expression of pili. These results were confirmed by PCR technique; *tcpA* gene was successfully amplified using the primer manufactured by IDT (Fig.1).

Molecular diagnosis of *S. boulardii* strain was confirmed by comparing the sequence results of the strains under study with the sequence in NCBI gene bank, where the alignment rate reached to 99.50%.

The *ura3*–auxotrophic yeasts are unable to grow on media lacking uracil and show resistance to 5-FOA. It was revealed that 5 colonies of the mutant transformed

yeasts showed the ability to grow on YNB-UU plates and on a plate containing 5-FOA, but not on YNB plates. Only two of the *S. boulardii* mutant’s revealed no reversion.

The transformation into TOP10F’ *E. coli* competent cells was successfully performed by chemical method; the insertion and orientation of *tcpA* gene was confirmed by digestion of the recombinant plasmid pYES2.1, using restriction enzymes EcoRV and XbaI (Promega, USA) as shown in Figure 2.

The sequence analysis results showed complete similarity between the *tcpA* sequences in Gene Bank (NCBI) under Accession Number: U09807 and the *tcpA* sequence (clone) isolated from the recombinant plasmid pYES2.1, where GAL1 (forward) and *tcpA* (reverse) primers were used (Fig. 3).

Mutants *S. boulardii* 1 and 2 were transformed by electroporation with a recombinant plasmid pYES2.1 encoding *tcpA* and the results showed the ability of transformed yeasts to grow on YNB medium and this confirms transformation was successfully done. The expression pattern analyzed showed that the time needed for *tcpA* expression in *S. boulardii* was 5 h (Fig. 4), while the Western blot results revealed a strong band of purified recombinant TcpA protein (Fig. 5).

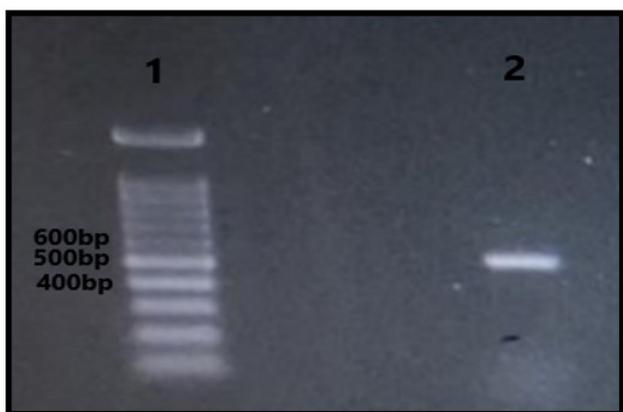


Fig. 1. Gel electrophoresis of the PCR products of *V. cholerae tcpA* gene on agarose gel (1%) for 1 h and 100 V using *tcpA* primer. Lane 1: DNA ladder 100 bp (Promega/USA). Lane 2: amplified *tcpA*.

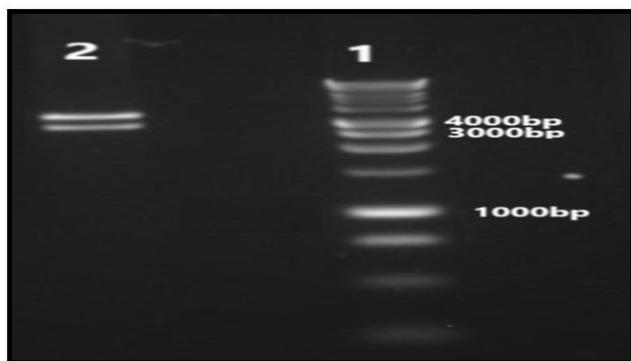


Fig. 2. Digestion of recombinant plasmid with restriction enzymes EcoRV and XbaI. From right to left: Lane1: DNA ladder RTU 1kb (GeneDirex, Taiwan). Lane2: shows two bands, first band (3576 bp), second band (2538 bp), the sum of these two bands (6414 bp) represents, *tcpA* product 528 bp + vector 5886 bp.



Fig. 3. DNA Sequencing between the subject *tcpA* sequences in Gene Bank (NCBI), Accession number: U09807 and query recombinant *tcpA* sequence isolated from the plasmid, where GAL1 is the forward primer and *tcpA*, the reverse primer.

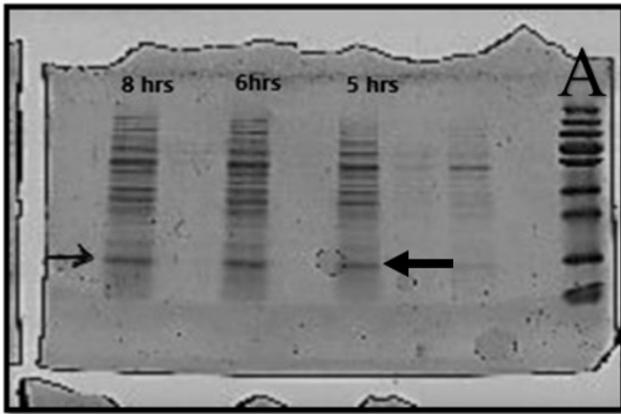


Fig. 4. Digestion SDS-PAGE (10%) of expressed recombinant TcpA from *S. boulardii* under galactose (2%) at different times of induction, at 200 V for 40 min. A: 250kb ladder.

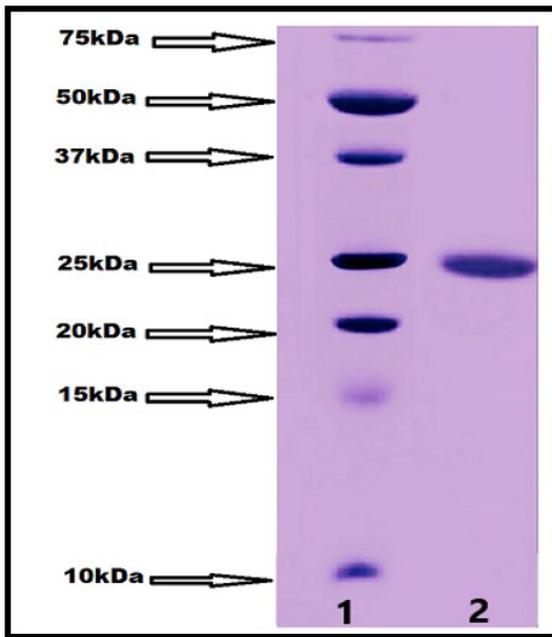


Fig. 5. Western blot analysis of purified recombinant TcpA ~25kDa. Lane 1 shows molecular masses (kDa) of specific markers and lane 2 indicates the TcpA protein.

Saccharomyces cerevisiae has been used as a host for expression of different kinds of heterogeneous proteins and provides an alternative approach for many problematic proteins that are not correctly expressed in *E. coli*. Also, the yeast expression system provides an excellent route for isolating protein complexes and for performing functional studies. In spite of its advantages as a host, in some studies, secretion of recombinant proteins into the culture medium of *S. cerevisiae* has not been observed. This state has to be followed by purification of recombinant proteins from cell debris

which is not very cost-effective. In Iran and Korea, the scientists were successful in produced recombinant cholera toxin subunit B (CTB) in *S. cerevisiae* as a step to produce a yeast-derived edible vaccine.⁽¹⁹⁾

The main step to use *S. boulardii* as a host for the transformation and expression of TcpA protein was the generation of an *ura3* auxotrophic strain. Several procedures (classical UV mutagenesis or molecular tools such as Cre-loxP system and CRISPR/Cas9) have been used to obtain an *ura3* auxotroph *S. boulardii*.⁽²⁰⁾ Here, we employed the UV mutagenesis and 5-FOA screening to produce two mutant strains that can be used without dependence on antibiotic resistance markers.

The 5-FOA is toxic to *S. boulardii* that has the *ura3* gene, therefore it makes them unable to grow on 5-FOA-containing media; this characteristic can act as a positive and negative selection marker at the same time, where the presence of URA3 generate sensitivity to FOA.⁽¹⁵⁾

In this study, the results showed *S. boulardii* as a promising host to express *V. cholerae* toxin TcpA as the first step in attempt to develop an oral *V. cholerae* vaccine, where YES2.1 has been used as a shuttle vector to express the *tcpA* gene with excellent results and the recombinant protein was induced after only 5 hours.

Conflicto de intereses

The authors declare no conflicts of interest.

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Expresión recombinante en *Saccharomyces boulardii* de la subunidad A del pili corregulado con la Toxina (TcpA) de *Vibrio cholerae* O1

Abstract

El cólera es endémico en más de 50 países. Se estima una mortalidad entre 100.000 – 120.000 debido a esta enfermedad. La vacunación se considera una medida complementaria para prevenir y controlar el cólera, por lo tanto, se necesitan preparaciones vacunales alternativas a las existentes. El Pili corregulado con la toxina, es parte del regulón de virulencia *toxR*, y es necesario para la colonización en la mucosa intestinal. Para expresar la proteína *tcpA* de *Vibrio cholerae* en *Saccharomyces boulardii*, se construyó el plásmido de expresión pYES2 insertando el gen *tcpA* obtenido a partir de aislamientos locales de *Vibrio cholerae* El Tor Inaba. La nueva construcción se transfirió a las células de *Saccharomyces boulardii* y se indujo la expresión del gen *tcpA* a partir del promotor GAL1 mediante la adición de galactosa al medio. El análisis mediante SDS-PAGE y Western blot demostró la presencia de TcpA en levaduras. Los resultados demostraron que *Saccharomyces boulardii* es un hospedero prometedor para expresar el gen *tcpA* de *Vibrio cholerae* como el primer paso en el intento de producir una vacuna oral contra *Vibrio cholerae*.

Keywords: *Saccharomyces boulardii*; *Vibrio cholera* O1; cólera; clonaje; ADN; *Escherichia coli*.

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