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Yeast-Produced S1 Recombinant Protein of SARS-CoV-2

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A R T I C L E I N F O

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A B S T R A C T

Introduction: COVID-19 pandemic caused by the emerging SARS-CoV-2, being the cause of COVID-19, leads to acute respiratory syndrome and is a vital threat to global health and the economy since it was identified in late December 2019 in China. Due to the limitations and long-time of vaccine production, most countries were forced to design and produce antigens, kits, and anti-viral drugs so that they might be able to prevent deaths caused by COVID-19. This study was conducted to design and express S1 protein of SARS-CoV-2 to be used as a vaccine candidate. **Methods:** Recombinant pPICZαA plasmid was replicated in *Escherichia coli* and the linearized plasmid was transfected into *Pichia pastoris* yeast as an endosomal fragment. After screening the colonies with Zeocin and confirming the presence of the gene and vector promoter inside the genome extracted from yeast, the expression of S1 protein was induced in BMMY medium with methanol. **Results:** The S1 protein was successfully expressed in *P. pastoris* and the results obtained on the SDS-PAGE indicated the presence of a protein with 130 kDa molecular weight, confirmed by Western blotting. **Conclusion:** The results of the present study showed that the yeast expression system of *P. pastoris* can be a suitable method to produce glycoprotein S1 as a vaccine candidate or a diagnostic antigen against COVID-19.

INTRODUCTION

The recent human coronavirus was first identified in Wuhan, China, at the end of 2019, and was named SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2). It quickly was spread around the world and caused a major pandemic which was called COVID-19 (coronavirus disease 19)[1, 2]. According to the World Health Organization data, there are 626,337,158 cumulative confirmed cases and 6,566,610 cumulative deaths associated with the disease until 28 October 2022. On February 19, 2020, two positive cases of COVID-19 in Qom province - Iran were confirmed by the World Health Organization representative for the first time in Iran. Due to the lack of sufficient information about the outbreak and the high risks of SARS-CoV-2 contagability [3], COVID-19 was spread rapidly in Tehran and the northern provinces [4].

Similar to previous human coronaviruses, SARS-CoV-2 often causes symptoms such as fever, weakness, and cough (known as flu-like symptoms) [5]. Severe lung infections have been reported at all ages; however, in the elderly or people with underlying medical conditions, SARS-CoV-2 infection leads to severe respiratory distress, caused by acute respiratory inflammation. This would ultimately increase the mortality rate, following an acute respiratory failure. In most of the affected individuals, variable amounts of dyspnea and radiographic symptoms have been reported [6, 7]. Due to the important role of SARS-Cov-2 spike glycoprotein (S) in binding and entering the virus into the cells, and subsequent stimulation of the production of neutralizing antibodies, the S protein is mainly used as a target for vaccine production [8]. The S protein has two important fragments, namely S1 and S2, which are involved in binding to the ACE2 receptor and the membrane fusion, respectively [9]. The structure of S1 subunit leads to the binding of viral particles to the cell receptors on the surface of the host cell and causes the onset of viral infection. Therefore, receptor identification is an important factor in viral entry and is considered a suitable target for designing antigens [9].

Nowadays, various expression systems are favored for the expression of recombinant proteins, including those which use mammals, molds, bacteria, plants, insects, and yeasts as hosts. Among yeast expression systems, those using *Pichia pastoris* and *Saccharomyces cerevisiae* are more popular. *P. pastoris* is one of the most common expression systems in molecular biology which is used to produce recombinant proteins. In general, the benefits of protein production by this system include the secretion of recombinant proteins into the external cellular environment and post-translational modifications such as proper folding [10]. COVID-19 caused by the new coronavirus is probably the most agile, aggressive, and ambitious disease so far

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[11]. To combat this threat, this study was conducted to design a pPICZαA-S1 construct for production of S1 protein using a simple, efficient, and cost-effective expression system to be used in subsequent studies for designing diagnostic kits and vaccine candidates after purification.

MATERIALS AND METHODS

Construction of pPICZαA-S1 Recombinant Plasmid

To design pPICZαA-S1construct with the desired characteristics, the S1 protein sequence of the Wuhan strain of SARS-CoV-2 was used (NCBI Accession No. O45512. 2). Then, 10 amino acids from the signal peptide of *S. cerevisiae* ('VSLEKREAEA' sequence) were selected as the primer sequence of the structure to improve the expression of the secretory proteins. The FC sequence of human immunoglobulin was used at the end of the construct for better compatibility of the expressed protein with human immunity. A GS linker was used to connect the S protein sequence and the FC sequence. After the initial design of the construct, the amino acid sequence prepared by <www.novoprolab.com> was codon-optimized for expression in *P. pastoris*. Finally, the expression of the obtained nucleotide sequence was examined *in silico* and then was sent to ProteoGenix (France) to be incorporated into pPICZαA vector with Zeocin antibiotic resistance gene and *Sac*I restriction site. To obtain a high copy amplification of the resulted pPICZA-S1 plasmid, it was transformed into competent *Escherichia coli* DH5α (Thermo Fisher Scientific, USA) via heat-shock method [12].

Extraction and Linearization of Recombinant pPICZαA-S1 Plasmid

The plasmids were extracted from *E. coli* using a miniprep DNA extraction kit (Favorgen, Taiwan). The presence of the inserted gene was approved by PCR. The chimeric pPICZαA-S1 plasmid was linearized with *Sac*I restriction enzyme (Fermentas, Lithuania), according to the manufacturer's protocol.

Transduction of Linearized pPICZαA-S1 Plasmid into *P. pastoris*

Ten µg of cold linearized DNA (in 10 µl sterile water) were combined with 80 µl of competent *P. pastoris* cells from KM71H strain (Invitrogen, USA). The hash was pulsed at 2000 V, 25 µF, and 200 Ω using a Gene Pulser Xcell electroporation device (Bio-Rad, USA). Another sample of the competent *P. pastoris* cells was pulsed with a non-linearized S1 gene as a negative control. The cells were cultured in plates, containing YPDS medium (1 g yeast extract, 2 g peptone, 2% dextrose, 1 M sorbitol, l.2 g agarose in 100 ml D.D.W., and 100 µg/ml Zeocin) and incubated at 29°C for 5-7 days.

PCR Analysis of Transduced *P. pastoris* Cells and Selection of Multi-Copy Colonies

The multicopy transformed colonies were screened in YPDS medium, containing 2000 µg/ml Zeocin. The genomic DNA was extracted from a colony of *P. pastoris* using lithium acetate (LiOAc)–SDS solution method to weaken the cell walls [13]. PCR amplification was performed using the proposed primers in Easy Select *P. pastoris* Kit, (Invitrogen, USA) and the PCR product was sent to Takapozist® (Tehran, Iran) for sequencing.

Expression of Recombinant S1 Protein

The *P. pastoris* cells were cultured in 6 baffled flasks, containing 150 ml of BMGY medium (2% peptone, 1% yeast extract, 1.34% yeast nitrogen base, 1% glycerol, 0.002% biotin). The flasks were capped with sterile cheesecloth and cotton balls. The flasks were then incubated at 29 °C in a shaking incubator (150 rpm) until the culture reaches an OD^{600} of 2–6. After centrifugation at 3000 x *g* for 10 min, the cells were transferred to the flasks containing 100 ml of BMMY medium (2% peptone, 1% yeast extract, 1.34% yeast nitrogen base, 0.5% methanol, and 0.002% biotin). The flasks were incubated at 29 °C under 150 rpm agitation, and every 24 h interval, 1 ml of the cells were collected from each flask, centrifuged, and stored at -80 °C. Subsequently, 0.5% methanol 100% was added to the flask. The flasks were incubated for six days and centrifuged at 3000 x *g* for 10 min.

Western-Blotting

After SDS-PAGE and transferring the proteins to a nitrocellulose membrane, the membrane was blocked by 5% skimmed-milk-TTBS solution for 2 h at RT. It was washed with 0.07% TTBS and kept with the COVID-19-positive serum at room temperature for 2 h. The protein reacted with goat anti-human secondary antibodies conjugated by HRP (Thermo Fisher Scientific, USA) at RT for 2 h. Finally, the reaction between the antibodies and the relevant protein was visualized using 5% diaminobenzidine, and 1% H_2O_2 in 50 mM Tris-HCl buffer (pH 7.5).

RESULTS

Production and Linearization of pPICZA-S1 Plasmid

After the synthesis of pPICZαA-S1recombinant plasmid, it was successfully transformed into *E. coli* DH5α competent cells. The growth of colonies on LSLB agar containing Zeocin indicated that the cells had received the recombinant plasmid (Fig. 1).

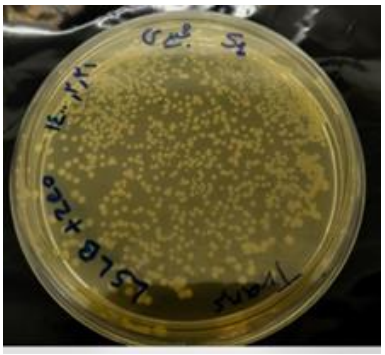

Fig. 1. Colonies grown with pPICZαA-S1plasmid on LSLB agar medium containing 25 µg/ml Zeocin.

Following extraction of the plasmid, analysis by agarose gel electrophoresis indicated the linearization of the plasmid by *Sac*I restriction enzyme (Fig. 2A). PCR analysis using AOX1 primers confirmed that the gene of interest was inserted into the correct position (Fig. 2B).

Transformation and Selection of Multi-Copy Colonies

After seven days of incubation, *P. pastoris* colonies harboring pPICZαA-S1 recombinant plasmid were observed on YPDS agar plates, supplemented with Zeocin (Fig. 3A). The

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result of colony-touch PCR demonstrated the transformation of the recombinant plasmid into *P. pastoris* cells (Fig. 3B). The sequencing results confirmed the presence of the construct (data not shown).

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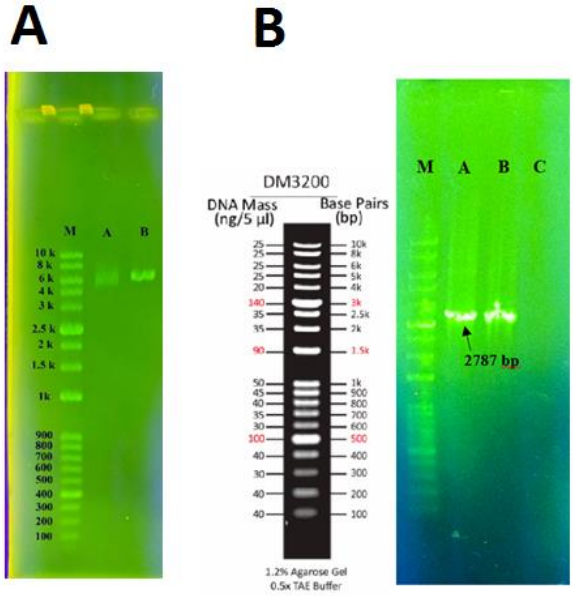

Fig. 2. (A) Linearized plasmid by *Sac*I restriction enzyme. M: 1 kb DNA ladder, A: Extracted pPICZαA-S1recombinant plasmid before linearization B: linearizated extracted pPICZαA-S1recombinant plasmid. **(B)** PCR results of the inserted gene. M: 1 kb DNA ladder; A: PCR product; B: positive control; C: negative control.

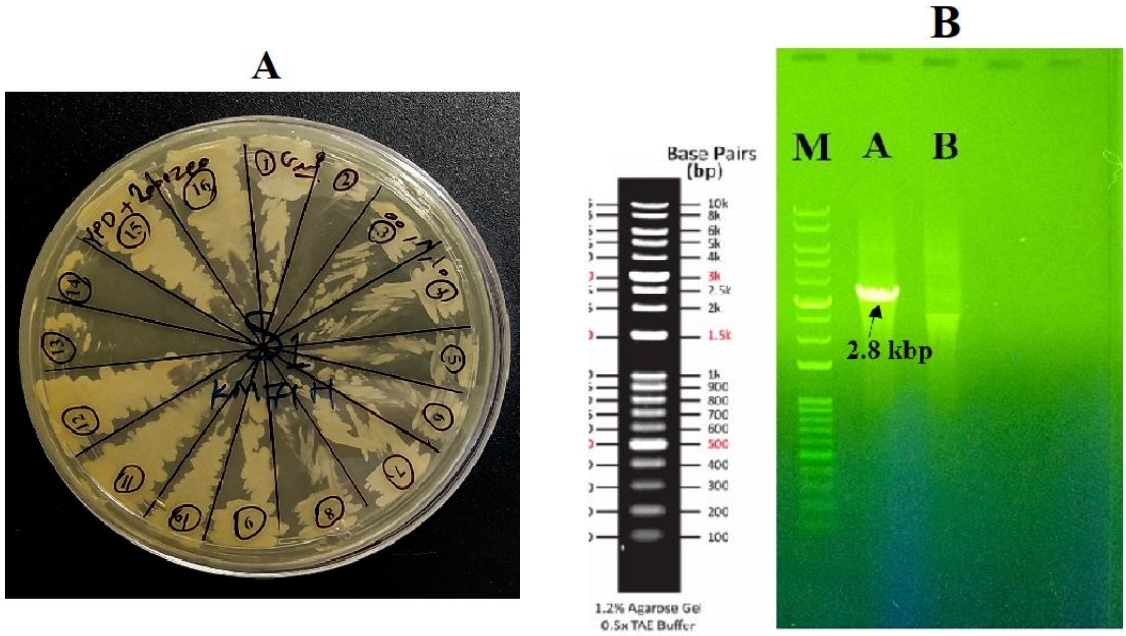

Fig. 3. (A) Grown colonies harboring pPICZαA-S1gene on YPD agar medium containing 2000 µg/ml Zeocin; The YPD agar plate was divided into 16 parts and 16 single colonies were cultured on it. Colonies number 2, 13 and 14 were considered as no growth and other colonies were considered as grown.. **(B)** Confirming the presence of pPICZαA-S1fragment with a 2.8 kbp amplicon in yeast by PCR. M: kb DNA ladder, A: Sample, B: Negative Control.

Protein Analysis of Yeast-Produced Recombinant S1

SDS-PAGE analysis exhibited an approximately 130 kDa band and confirmed the expression of S1 protein by the transformed *P. pastoris* cells (Fig. 4A). The molecular weight of the construct without glycosylation was calculated by <https://www.aatbio.com> website to be 103 kDa. Considering that the construct has 13 glycosylation sites (each estimated to be 2.5 kDa [14]), the molecular weight of the expressed protein was

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estimated to be 135.5 kDa. Therefore, a visible band within the range of 103 to 135 kDa was expected. The size and density of the band indicated increased rate of protein expression on consecutive days (0, 1, 2, 3, 4, and 6 days.

Moreover, 120 h was the best time to induce S1 protein expression. The detection of S1 protein by the anti-human antibodies was confirmed by Western blotting as shown in Fig. 4B.

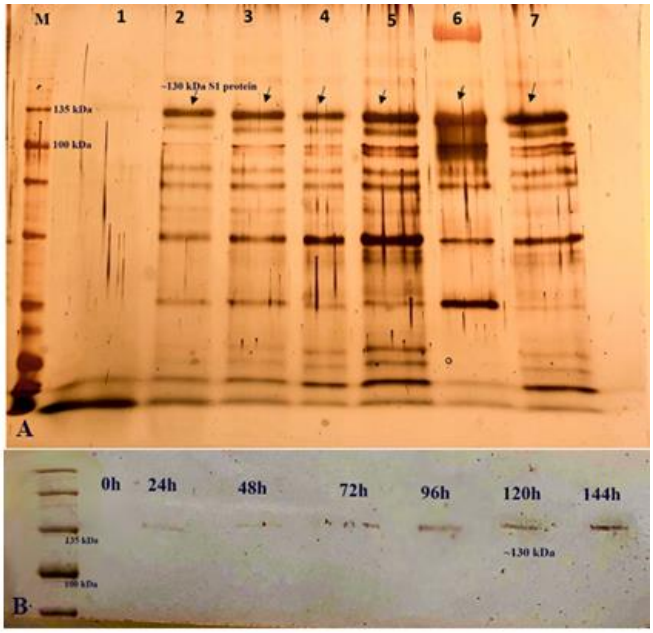

Fig. 4. Protein analysis of the recombinant S1 protein. **(A)** SDS-PAGE of the recombinant S1 protein, expressed by *P. pastoris*. 1- 7, expressed recombinant proteins in *P. pastoris* after different hours of expression (i.e. 0, 24, 48, 72, 96, 120, 144 h, respectively; M: Mw marker. **(B)** Western blot analysis using anti-human antibodies.

DISCUSSION

Recombinant protein expression by *P. pastoris* yeast is one of the most suitable and efficient systems for the production of various proteins of plants, bacteria, invertebrates, vertebrates, fungi, and viruses origins [9]. So far, different studies have indicated the ability to express different viral proteins by this system which include neuraminidase head domain of influenza virus [8], D glycoprotein of BHV-1[15, 16], E protein of Dengue virus [17], HBsAg of Hepatitis B virus [18, 19], gp41 epitope of HIV [20], ORF3 of Hepatitis E virus [21], gp120 HIV-1[22], large T antigen of Polyomavirus [23], 1 core protein of Reovirus λ [24], 1 protein of Reovirus σ [25], complement control protein of Vaccinia [26], chimeric L1&L2 protein HPV-16 VLPs [27], Recombinant botulinum neurotoxin binding domain of serotype A [28], E protein of dengue fever virus type 4[29], ASP-Na-2 Recombinant protein of human hookworm [30], chimeric LC3 protein of *Entamoeba histolytica* [31], envelope glycoprotein of Canine distemper virus [32], and HPV-16 L1 chimeric protein [33]. In the present study, the S1 protein of SARS-CoV-2 which is the binding site of the spike protein to the host's ACE2 receptor was expressed in a *P. pastoris* expression system. The results of the DNA sequence extracted from the yeast indicated the correct entry and replication of the endosomal fragment. The results of the SDS-PAGE gel showed the presence of a protein with a molecular weight of approximately 130 kDa, and the immunoreactivity of this protein was confirmed with anti-human antibodies by Western blotting. According to the obtained preliminary results, it appears that the expressed S1 protein can be produced with a good yield by *P. pastoris* expression system. It is envisaged that upon purification by protein G chromatography, this recombinant protein can be used in the future experiments as a vaccine candidate against COVID-19 and possibly as an antigen in COVID-19 diagnostic devices.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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