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Original Article

Saccharomyces cerevisiae OS303 expression of an alkaline protease from a newly isolated *Bacillus subtilis* D9

Expressão de *Saccharomyces cerevisiae* OS303 de uma protease alcalina de um *Bacillus subtilis* D9 recém-isolado

A. I. Alqosaibi[a,b*], A. Mahmoud[a,b*], E. Kotb[a,b], Y. Huang[c], I. S. Al-Dhuayan[a,b], S. Alhazmi[d], A. A. Bahloul[a,b], S. T. Okasha[a,b], H. Otaibi[a,b], N. AlYami[a,b] and E. Louis[c]

[a]Imam Abdulrahman Bin Faisal University, College of Science, Department of Biology, Dammam, Saudi Arabia
[b]Imam Abdulrahman Bin Faisal University, Basic & Applied Scientific Research Center, Dammam, Saudi Arabia
[c]University of Leicester, Centre for Genetic Architecture of Complex Traits, Leicester, United Kingdom
[d]King Abdulaziz University, College of Science, Department of Biological Sciences, Jeddah, Saudi Arabia

Abstract

The aim of this study was to produce high yield of a local bacterial alkaline protease in the yeast system because the scientific involvement of microorganisms in enzyme production is still not given enough attention in Saudi Arabia. Soil samples were collected from the rhizosphere of some desert plants in Saudi Arabia. Ninety-three alkaline protease producing bacterial isolates were recovered on skimmed-milk agar at pH 9.4 and 45°C for 48 hr. Isolate D9 obtained from the rhizosphere of *Heliotropium digynum* at Dhahran City was the most potent isolate in respect to enzyme productivity (184.6 U/ml). The full gene of alkaline protease was amplified and showed the expected size (1300 bp). Restriction enzymes analysis also verified the integrity of the PCR product. The sequence of the protease gene revealed an open reading frame of 1329 nt correspond to the full length of the protease gene of isolate D9 encoding a 443 aa protein. After ligation of the amplified gene by the TA cloning method, digestion with appropriate restriction enzymes confirmed the integrity of the cloned gene. The insert was prepared by two PCRs that were conducted with a pair of primers specifically designed for this purpose. The digested and purified cloning vector pRS426/GAL1p-207-Glu-MS was ligated with the insert then transformed into various strains of *Saccharomyces cerevisiae* via the electroporation method. Maximum protease expression was done by recombinant OS303 in galactose containing media (145.5 U/ml) with an approximately 2-fold increase when compared with the wild OS303 strain., this may be due to ability to activate gal operon.

Keywords: microbial enzymes, alkaline protease, restriction enzymes, cloning, enzyme expression, *Bacillus subtilis*, *Saccharomyces cerevisiae*.

Resumo

O objetivo deste estudo foi produzir alto rendimento de uma protease alcalina bacteriana local no sistema de leveduras, já que o envolvimento científico de microrganismos na produção de enzimas ainda não recebe atenção suficiente na Arábia Saudita. Amostras de solo foram coletadas da rizosfera de algumas plantas do deserto na Arábia Saudita. Noventa e três isolados bacterianos produtores de protease alcalina foram recuperados em ágar de leite desnatado a pH 9,4 e 45°C por 48 horas. O isolado D9 obtido da rizosfera de *Heliotropium digynum* na cidade de Dhahran foi o mais potente em relação à produtividade da enzima (184,6 U/ml). O gene completo da protease alcalina foi amplificado e apresentou o tamanho esperado (1300 pb). A análise de enzimas de restrição também verificou a integridade do produto de PCR. A sequência do gene da protease revelou uma fase de leitura aberta de 1329 nt, correspondendo ao comprimento total do gene da protease do isolado D9 que codifica uma proteína 443 aa. Após a ligação do gene amplificado pelo método de clonagem TA, a digestão com enzimas de restrição apropriadas confirmou a integridade do gene clonado. A inserção foi preparada por dois PCRs que foram conduzidos com um par de primers projetados especificamente para esta finalidade. O vetor de clonagem digerido e purificado pRS426/GAL1p-207-Glu-MS foi ligado com a inserção e então transformado em várias cepas de *Saccharomyces cerevisiae* por meio do método de eletroporação. A expressão máxima da protease foi feita por OS303 recombinante em meio contendo galactose (145,5 U/ml) com um aumento de aproximadamente duas vezes quando comparado com a cepa OS303 selvagem, e isso pode ser por causa da capacidade de ativar o operon gal.

Palavras-chave: enzimas microbianas, protease alcalina, enzimas de restrição, clonagem, expressão enzimática, *Bacillus subtilis*, *Saccharomyces cerevisiae*.

*e-mail: amgosaibi@iau.edu.sa; amhsalem@iau.edu.sa
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1. Introduction

Proteases are the major industrial enzymes in worldwide markets. Microbial proteases contribute 60% of the total world market share and became inevitable in the production of detergents, food, medical formulations, and leather (Razzaq et al., 2019; Varia et al., 2019; Sharma et al., 2017).

Different reports have focused on the characterization and applications of alkaline proteases produced by *B. subtilis* group, recovered from Saudi Arabia, because of their industrial importance in feather degradation, wound healing, and detergents (Al-Dhuayan et al., 2021; Mahmoud et al., 2021; Almahasheer et al., 2022).

Alkaline proteases are widely used in the detergent industry because they can withstand a wide pH range (9.0–12.0) of laundry solution (Haddar et al., 2009). Furthermore, they are stable at elevated temperatures in the presence of non-ionic detergents, surfactants, and peroxide agents in order to be a successful component in modern bleach-based detergent formulations (Maurer, 2004).

It is recommended that, for the isolation of the microorganisms to be used in the industrial oriented applications, exploitation of the sources such as alkaline habitats are much preferred as the strains produce enzymes that are stable naturally in high alkaline conditions and could resist chemical denaturants present in the detergents (Jaouadi et al., 2008).

The global market for industrial proteases enzymes was estimated to be $4.4 billion in 2015 and is expected to be worth $6.30 Billion by 2022 (Gupta et al., 2002; Singh et al., 2016; Chapman et al., 2018). Alkaline proteases have been found in many fungi, and bacteria (Giongo et al., 2007; Haddar et al., 2009). Among the bacteria, many species of the genus *Bacillus* are widely used for proteases production on an industrial scale (Jørgensen et al., 2000).

Bacilli represents 35% of total microbial enzyme sale and it was widely used in the detergent formulation since the 1960s. Furthermore, protease from *Bacillus* sp. was also used as cleansing additives in detergents to facilitate the release of proteinaceous materials in stains such as grime, blood, milk, etc (Chapman et al., 2018; Razzaq et al., 2019). *Bacillus subtilis* is the most common genus producing enzymes including alkaline proteases. It is a gram-positive aerobic bacterium with capability to produce endospore and lives in soil, water, and plant residues (Kunst et al., 1997; Rivolta and Pagni, 1999). *B. subtilis* produces an extracellular protease at the end of the exponential phase of its growth (Stephenson et al., 1999; Kodama et al., 2007). Also, produces alkaline protease X which is an intracellular alkaline protease, significantly expressed in the stationary phase of bacterial growth (Valbuzzi et al., 1999).

Despite the fact that variety of microorganisms are known to produce alkaline proteases, there is still a need for novel enzymes with specific properties. Furthermore, protease production varies significantly depending on the strain and the environment from which it was isolated.

To the best of our knowledge, there is insufficient information regarding the capability of local Saudi Arabian microorganisms to produce alkaline proteases. In this work, we performed a potential screening program for proteolytic bacteria from environmental samples collected from the Kingdom of Saudi Arabia. It also included the cloning of the effective gene in yeast for further enzyme productivity.

2. Materials and Methods

2.1. Materials

Oligonucleotides were prepared to a final concentration of 100 µM in 1×TE and stored at -20°C. Molecular weight markers were used at 500 ng per lane. 1kb DNA hyper ladder I was obtained from Bioline Co. (UK) and used at a concentration of 100 ng/µl. All restriction enzymes used in this study were obtained from New England Biolabs (NEB) Co. (UK) according to manufacturer's recommendation. General laboratory chemicals were purchased from Sigma-Aldrich Co. (USA), unless otherwise stated.

2.2. Survey for alkaline protease producing bacteria

During the second half of the year 2017, many soil samples were collected from the rhizosphere region of some desert plants in certain localities of Saudi Arabia. The survey included Dammam, Khobar, Qatif, Hail, Sehat, Dhahran, Shiba, Shaqra, Dahna desert, and Nafud desert. Differential isolation of alkaline proteases producing bacteria was carried on skimmed-milk agar comprising of (g/L) agar (15), peptone (5), yeast extract (1), and skimmed milk (150 ml v/v) at pH 9.4. Then, dishes were incubated at 45°C for 48 hr. Cleared halos surrounding the growing colonies are indicative of alkaline protease productivity. Exactly ninety-three alkaline protease producing bacterial isolates were obtained.

2.3. Enzyme production and bacterial characterization

Inoculate of the most positive isolates were subcultured into Luria-Bertani broth consisting of 0.5% yeast extract, 0.5% NaCl, and 1.0% tryptone with pH 7.0. One percent (v/v) 12 h inoculum (~3×10^8 cfu/mL), was transported into a fermentation broth developed by Kotb (2015). It consists of 1% lactose, 0.5% soybean powder, 0.2% $CaCl_2$, 0.15% KH_2PO_4, 0.05% K_2HPO_4, and 0.05% $MgSO_4.7H_2O$. Incubation was done under shaking conditions of 120 revolutions per minute shaking speed at 45°C for 48 hr in 250 mL Erlenmeyer flasks holding 50 mL broth at pH 9.5. Short-term bacterial cultures were preserved on nutrient agar at 4°C while, long-term cultures were preserved in 25% glycerol at -80°C.

2.4. Protease assay and protein quantification

Proteolytic activity was assessed according to Kotb (2015) by modifications. Initially, 1 mL of culture supernatant was mixed with1 mL of 1% (w/v) azocasein solution of pH 9.4 by 0.2 M glycine-NaOH buffer. The enzyme-substrate reaction was done for 30 min at 45°C. The enzymatic reactivity was ended by adding 2 mL of 10% trichloroacetic acid solution and incubating for 60 min in crushed ice bath. Centrifugation at 5000 rpm for 20 minutes was done. Exactly 1 ml of supernatant was mixed well with 1 N NaOH before reading absorbance at A_{440}. The amount of soluble degradation proteins (C) was

measured (mg/mL) following this calculation; C (mg/mL) = 1.55 A_{280} - 0.76 A_{260}. The unit (U) of proteolytic enzyme activity was corresponding to 1µg of L-tyrosine released mL^{-1} min^{-1} at the standard conditions of experimentations (Kotb et al., 2013).

2.5. Yeast strains and plasmids

Yeast strains were kindly gifted from Edward Louis lab, Leicester University (Table 1). The vectors pRS426/GAL1p-207-Glu-MS and pRS426/GAL1p-MS were kindly gifted by Dr. Yan Huang, Leicester University.

2.6. Amplification of the Alkaline protease gene by PCR

The bacterial isolate D9 with the highest alkaline protease productivity was selected for cloning of its alkaline protease gene. Based on *16S rRNA*, the D9 isolate was classified as *B. subtilis* in previous work (Mahmoud et al., 2021) (accession number MK819972.1). An overnight *B. subtilis* culture was grown in 10ml LB broth in a shaking incubator at 37°C, the total DNA of the bacterium was extracted by DNeasy Mini Kit (Qiagen). PCR was performed with the he forward primer 5'-CATATGTTTGGGTACTCTATGG-3' and the reverse primer 5'- GGATCCTTATTGGCCGGGAACGGAA- 3' (Sadeghi et al., 2009). 1µl of ~100ng of *B. subtilis* extracted Genomic DNA template was amplified in a 100µl reaction mixture containing 71µl of PCR water, 5µl of 5µM of each Forward and Reverse primers, 4µl of 5 mM dNTPs, 10µl of 10× NH4 buffer (Taq buffer), 3µl of 50 mM MgCl2, 1µl of Taq Polymerase (5U/µl) in total reaction 100µl. PCR SuperMix was purchased from Invitrogen. Taq DNA polymerase was obtained from Bioline. The thermocycling profile was as fallow: initial denaturation at 95°C for 2 minutes; 30 repeated cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 68°C for one minute and a half; with a final extension of 68°C for 7 minutes. The PCR products were visualized using 0.8% agarose gel electrophoresis to check the size of the products. Sequencing of alkaline protease gene was performed through Macrogen (https://dna.macrogen.com/eng/).

2.7. Cloning vector construction containing alkaline protease gene

The PCR product (containing alkaline protease gene) was extracted from the 0.8% agarose gel using QIA quick Gel Extraction Kit (Qiagen). The amplified gene was inserted into the cloning vector by the TA cloning method using the TA Cloning® Kit with pCR®2.1 from Invitrogen. For this purpose, the insert and vector (molar ratio of 1/1, respectively), Following the manufacturer's instructions. The ligation mix was incubated overnight at 14°C then was transformed into competent cells using One Shot® chemical transformation protocol (heat shock). 2µl of the ligation reaction was added to One Shot® TOP10 Chemically Competen E. coli (Invitrogen™) and incubated on ice for 30 minutes. The samples were then heat shocked at 42ºC for 30 seconds then placed on ice immediately for 2 minutes. 250µl of preheated SOC medium was added to recovery the cells and incubation at 37ºC for 1 hour at 225 rpm in a shaking incubator. 200µl of cells were then plated on LB media plates containing 100µg/ml ampicillin and incubated overnight at 37°C. Colony PCR of the transformants was performed to confirm cloning and ligation of the PCR products and confirmed by sequencing using M13 forward and reverse primers. The Cloning vector that contains alkaline protease gene was digested with SpeI, EcoRI, XbaI, and NheI- NdeI- BamHI restriction enzymes according to New England Biolabs (NEB) manufactures recommendation and was run on 0.8% agarose gel to confirm the integrity of the PCR product and to design the appropriate primers to construct the expression vector.

2.8. Construction of the expression cloning vector

The cloning vector pRS426/GAL1p-207-Glu-MS was linearized with BamHI and SpeI according to New England Biolabs (NEB) manufactures recommendation. DNA fragments for cloning were separated by electrophoresis. The correct sized bands around 5000bp were excised from the agarose gels using a clean scalpel blade. DNA was purified from the gel fragment using the MinElute Gel Extraction kit (Qiagen) following the manufacturer's instructions.

To prepare the insert, two PCRs were conducted. 1st PCR was performed with a pair of primers specifically designed for this purpose. The forward primer (5'-**TCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCA***TTTGGGTACTCTATGG*-3') containing SUC2 leader (signal peptide) sequence without START codon (bold) and our gene forward primer sequence (italic) and the reverse primer (*5'GG*ACTAGT*TTGGCCGGGAACGGAA*-3')

Table 1. Yeast strains used in this study.

Strain	Genotype and Relevant Modification	Source
OS617	*(Matα, HO/hoΔ::HYG, ura3Δ::KanMX, leu2Δ1, lys2Δ202)*	Ed. Louis lab is derived from the Sake strain (Y12) used in Cubillos et al. (2009).
OS603	*(Mat a, HO/hoΔ::HYG, ura3Δ::KanMX, leu2Δ1)*	Ed. Louis lab is derived from the North American strain (YPS128) used in Cubillos et al. (2009).
OS595	*(Mat a, HO/hoΔ::HYG, ura3Δ::KanMX, leu2Δ1)*	Ed. Louis lab is derived from the West African strain (DBVPG6044) used in Cubillos et al. (2009).
OS591	*(Mat a, HO/hoΔ::HYG, ura3Δ::KanMX, leu2Δ1)*	Ed. Louis lab is derived from the Wine/European strain (DBVPG6765) used in Cubillos et al. (2009).
OS303	*(Mat a, ura3Δ::KanMX)*	Ed. Louis lab is a clinical isolate YJM978 (Wei et al., 2007).

containing SpeI restriction site (underlined) and our gene reverse primer sequence (italic) without Stop codon. 1µl of ~100ng of TA recombinant plasmid containing alkaline protease template was amplified in a 100µl PCR reaction as described above. The PCR product was then Cleaned up by using GenElute™ PCR Clean-Up Kit from Sigma-Aldrich. Then, the purified PCR product was used as a template for the 2nd PCR that was performed with a pair of primers specifically designed for this purpose. The forward primer **(5'**CGGGATCCAAAAA **TGCTTTTGCAAGCTTTCCTTTTCCTTTTGGCTGGTTTTGC'3)** containing BamHI restriction site (underlined), the SUC2 leader, and the reverse primer (5'GGACTAGTTTGGCCGGGAACGGAA-3') was amplified in a 100µl reaction as described above. The PCR product was extracted from the 0.8% agarose gel using QIA quick Gel Extraction Kit (Qiagen). The insert was digested with BamHI and SpeI in 50µl total reaction for 1 hour.

The digested and purified cloning vector pRS426/GAL1p-207-Glu-MS was ligated with the insert. 4µl of fresh PCR product was used. The ligation mix was incubated overnight at room temperature then was transformed into competent cells using One Shot® chemical transformation protocol (heat shock) as described above. 200µl of cells were then plated on LB media plates containing 100µg/ml ampicillin and incubated overnight at 37°C. Colony PCR of the transformants was performed to confirm cloning and ligation of the PCR products using M13 forward and reverse primers. Further analysis, Plasmid DNA extraction was carried out and used by several resection enzymes to confirm the successful cloning of the protease gene from *B. Subtilis*. Then, the expression construct was used for transformation.

2.9. Yeast transformation

The strains of *Saccharomyces cerevisiae* used in this study are listed in Table 1. Yeasts were transformed using the high efficiency lithium acetate (LiAc) transformation method (Gietz and Schiestl, 2007). Briefly, cells were grown overnight in 5 mL of yeast peptone dextrose (YPD) to exponential phase. The cells were harvested and resuspended in 1 mL of sterile water. Cells pellet was washed in water, then in 0.1 M LiAc and the pellet was recovered after centrifugation. A transformation mix was made for the number of transformations and negative control. Ingredients were added according to the following order: 240µl (50% w/v) of polyethylene glycol (PEG), 36µl of 1M lithium acetate (LiAc), 40µl of (2.5 mg/ml) denatured salmon sperm (Single-stranded carrier DNA, Invitrogen), 1µg of the Expression construct transforming DNA and dH2O up to total volume 360µl. Tubes were incubated at 30°C for 30 minutes following by heat shocked at 42°C for 20 minutes. Cells were centrifuged and finally washed in water and incubated in YPD at 4°C overnight. The cell suspension was plated on a uracil drop out media and transformants were isolated after 2–3 days.

2.10. Protease production by the yeast strains

Yeast strains were inoculated in 250 ml Erlenmeyer flask containing 50 ml of a fermentation medium containing (per 1000 ml distilled water) 20 g carbon source (glucose or galactose), 10 g yeast extract, and 20 g of peptone. Fermentation was allowed for 42 hours at 27°C and pH of 5.85 under shaking conditions of 130 rpm.

2.11. Statistical analysis

Unless otherwise stated, all treatments and experimentation were done in triplicates and the final data were expressed in the form of averages ± standard deviations. The software SPSS Statistics V24 was used for all analytics.

3. Results

3.1. Selection of a native bacterial producer of alkaline protease

Several reports were published concerning the potentiality of alkaline proteases in many industrial applications. For this reason, the present research was focused on the isolation of a native bacterial isolate from Saudi Arabia with potent alkaline protease productivity (Table 2) for further expressing its protease gene in the yeast system. For this, an extensive screening of local bacterial producers was done during the second half of the year 2017. Many soil samples were collected from the rhizosphere region of some desert plants in certain localities of Saudi Arabia including Dammam, Khobar, Qatif, Hail, Sehat, Dhahran, Shiba, Shaqra, Dahna desert, and Nafud desert (Table 2). Among ninety-three bacterial isolates, isolate D9 recovered from the rhizosphere of *Heliotropium digynum* plant found at Dhahran City was the most potent isolate in respect to enzyme productivity (184.6 U/ml).

3.2. Protease gene amplification

The electrophoresis of the PCR product observed a band of approximately 1300 bp was as shown in Figure 1.

3.3. Cloning vector construction containing alkaline protease gene

The prepared plasmid was digested by several restriction enzymes SpeI, EcoRI, XbaI, and NheI- NdeI- BamHI respectively. SpeI showed one band of nearly 5000-6000 bp. The EcoRI, XbaI, and NheI- NdeI- BamHI restriction enzymes showed two bands of approximately 3000bp and 1300bp and less, as shown in Figure 2. The protease gene does not contain either of SpeI or BamHI site. Because of that we include these sites in our designed primers. Restriction analysis confirmed the integrity of the PCR product of the protease gene. According to our previous work (Mahmoud et al., 2021), the cloned PCR product was sequenced, sequence analysis indicated the protease gene is classified as alkaline serine protease (accession number MK814958). In addition, sequence analysis confirmed the full-length sequence the alkaline protease gene; the deduced amino acid sequence showed an open reading frame encoding 442aa, an estimated molecular weight of about 47,789 Da.

Table 2. The most potent alkaline-protease producers recovered from the rhizospheric region of some desert plants of Saudi Arabia.

Isolate no.	Source rhizosphere	Alkaline protease productivity (U/ml)	Id	*16S rRNA* gene accession no.
D2	*Tamarix gallica, Dhahran*	120.5±7.5	*Bacillus cereus*	MK819971.1
D9	*Heliotropium digynum, Dhahran*	184.6±6.0	*Bacillus subtilis*	MK819972.1
D10	*Cistanche tubulosa, Dhahran*	171.3±2.1	*Bacillus subtilis*	MK819973.1
D14	*Suaeda vermiculata, Khobar*	142.3±6.4	*Bacillus cereus*	MK819974.1
D26	*Acacia mellifera, Dhahran*	145.2±8.0	*Bacillus cereus*	MK819975.1
D30	*Convolvulus buschiricus, Dhahran*	119.2±4.2	*Bacillus subtilis*	MK819976.1
D35	*Datura innoxia, Dhahran*	181.0±4.6	*Bacillus cereus*	MK819977.1
D40	*Zygophyllum amblyocarpum, Shaqra*	173.5±8.1	*Bacillus cereus*	MK819978.1
D42	*Teucrium oliverianum, Shaqra*	135.2±2.2	*Bacillus subtilis*	MK819979.1
D44	*Peganum harmala L., Shaqra*	115.2±4.6	*Bacillus cereus*	MK819980.1
D46	*Adenium obesum, Nafud desert*	102.6±7.8	*Bacillus cereus*	MK819981.1
D48	*Diplotaxis acris (Forssk) Boiss, Dahna desert*	103.6±6.0	*Bacillus cereus*	MK819982.1

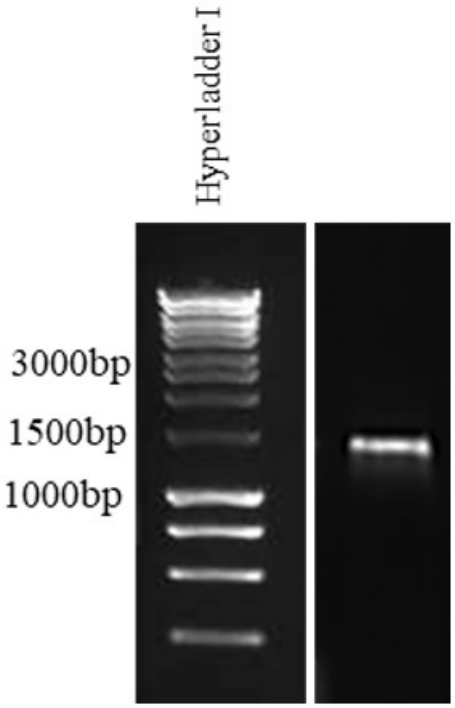

Figure 1. The electrophoresis of PCR product of alkaline protease gene from *Bacillus subtilis* D9. After PCR, 5µl of the product was run on 1% agarose gel electrophoresis. The expected band of approximately1300bp was observed. Lane 1: DNA size marker hyperladder I; Lane 2: PCR product sample.

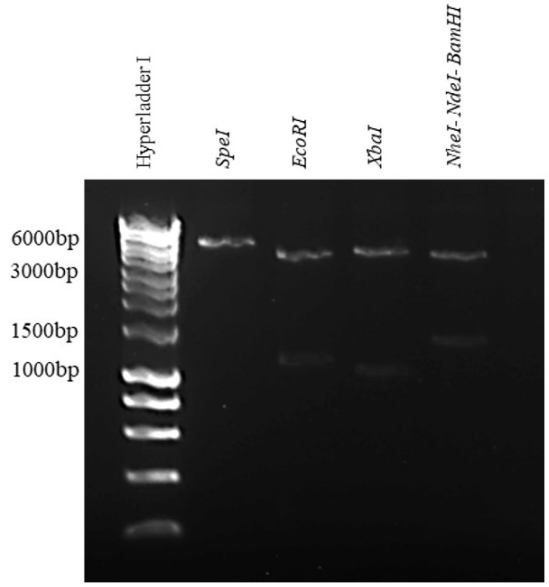

Figure 2. Analysis cloning vector construction containing alkaline protease gene with restriction enzymes to confirm the integrity of the PCR product of alkaline protease gene and to design the appropriate primers to construct the expression vector. By the enzyme combination of NheI- NdeI- BamHI restriction enzymes showed two bands of approximately 3000bp and ~1300bp must be shown as alkaline protease gene. Lane 1: DNA size marker hyperladder I; Lane 2, 3, 4, and 5: Digested sample of TA Cloning vector construction containing alkaline protease with enzyme SpeI, EcoRI, XbaI, and NheI- NdeI- BamHI respectively.

3.4. Construction of the expression cloning vector

The expression cloning vector (digested and purified cloning vector pRS426/GAL1p-207-Glu-MS ligated with the insert containing alkaline protease) was transformed into competent cells using heat shock transformation protocol. Fifty-seven colonies were seen on the plate. Plasmid DNA extraction was carried out for number of colonies and used by restriction enzymes BamHI and SpeI digestion, causing isolation of insert from the vector. The electrophoresis showed two bands of approximately

>6000bp and <1500bp observed as shown in Figure 3. The lower band around <1500bp includes protease gene sequences and SUC2 leader. Figure 4 illustrates the electrophoresis of expression cloning vector (digested and purified cloning vector pRS426/GAL1p-207-Glu-MS ligated with alkaline protease called in this study pRS426/GAL1p-207-Glu-MS/alkaline-protease plasmids around 8000bp, pRS426/GAL1p-207-Glu-MS vector around 1Kb and pRS426/GAL1p-MS vector that was used as a negative control in yeast transformation. Successful cloning of the protease gene from *B. Subtilis* could pave the way for the expression studies in yeast.

3.5. Alkaline protease assay for yeast strains

In media with glucose as a carbon source, it appears that alkaline protease productivity by yeast strains with expression plasmids were better than those without expression plasmids. Maximum protease production was achieved by recombinant strain OS591 (82.4 U/ml). In media with galactose as a carbon source, it appears that alkaline protease productivity by recombinants OS617, OS303, and OS603 were better than wild except for recombinants OS591 and OS595. In most cases, enzyme productivity was better in the presence of galactose (Table 3). Maximum protease production was achieved by recombinant OS303 in galactose containing media (145.5 U/ml). This may be due to the ability to activate the gal operon.

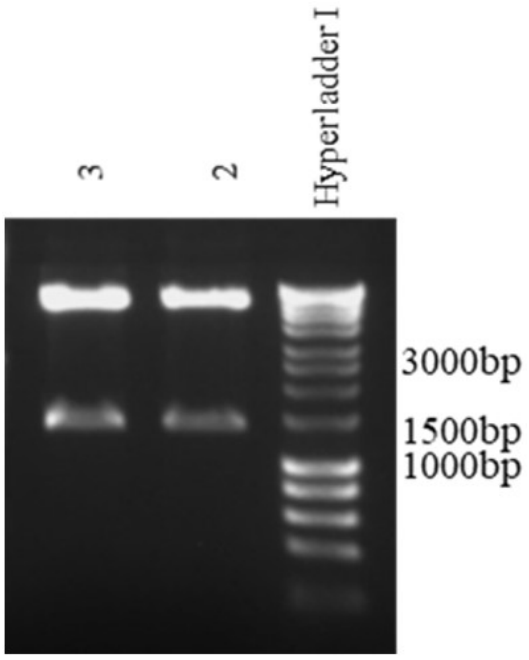

Figure 3. Analysis of expression cloning vector (digested and purified cloning vector pRS426/GAL1p-207-Glu-MS ligated with alkaline protease called in this study pRS426/GAL1p-207-Glu-MS/alkaline-protease plasmids. To screen the recombinant plasmids, the plasmids prepared from colonies were digested with double restriction enzymes and then were run on 0.8% agarose gel electrophoresis. Lane 1: DNA size marker hyperladder I; Lane 2, 3: pRS426/GAL1p-207-Glu-MS/alkaline-protease plasmid digested with BamHI and SpeI, after digestion, two bands of >6000bp and <1500bp observed.

4. Discussion

Microbial proteases are the most important industrial enzymes in worldwide markets. Among the world sales, they are found in nutrition supplements, beverages, and infant formulae. The use of them as detergent additives represent the largest application among the industrial enzymes. Another industrial process that has received attention is the enzyme aided dehairing of animal hides and skin in the leather industry (Al-Dhuayan et al., 2021).

Table 3. Protease productivity from the tested yeasts in the presence of glucose and galactose.

Yeast strain	*Protease activity (U/ml)*	
	Glucose	*Galactose*
OS617[c]	*32.1±2.1*	*40.5±3.5*
OS617[t]	*54.3±3.2*	*70.7±4.1*
OS303[c]	*78.0±4.2*	*73.0±4.0*
OS303[t]	*79.8±6.4*	*145.5±12.0*
OS591[c]	*40.5±2.6*	*24.0±1.8*
OS591[t]	*82.4±6.2*	*37.5±2.2*
OS603[c]	*34.5±1.5*	*38.2±2.7*
OS603[t]	*48.1±2.5*	*64.4±1.0*
OS595[c]	*39.5±3.4*	*45.0±4.0*
OS595[t]	*64.0±4.1*	*42.9±1.0*

[c]Native strains (no expression plasmid). [t]Cloned strains (has expression plasmid).

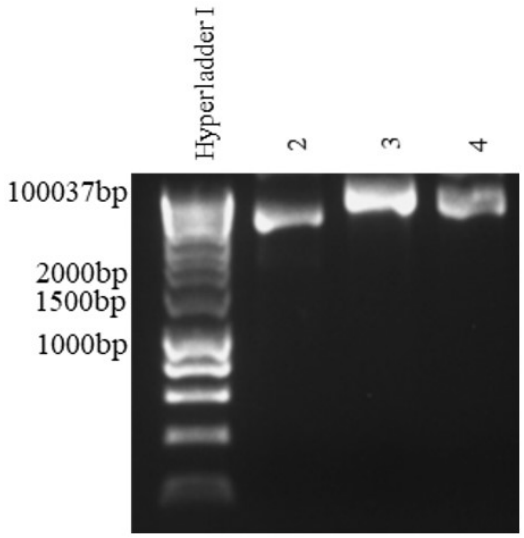

Figure 4. Analysis of expression cloning vector (digested and purified cloning vector pRS426/GAL1p-207-Glu-MS ligated with alkaline protease called in this study pRS426/GAL1p-207-Glu-MS/alkaline-protease plasmids. Lane 1: DNA size marker hyperladder I; Lane 2: pRS426/GAL1p-207-Glu-MS/alkaline-protease linear plasmid around 8000bp; Lane 3: pRS426/GAL1p-207-Glu-MS vector. Lane 4: pRS426/GAL1p-MS vector.

The goal of this study was to clone the local *Bacillus subtilis* D9 alkaline protease gene in the yeast system for high expression level of D9 alkaline protease. The PCR product of the protease gene was sequenced, submitted to Genbank MK814958, and characterized according to our previous work (Mahmoud et al., 2021). The sequence of the protease gene revealed an open reading frame of 1329 nt correspond to the full length of the protease gene of isolate D9 encoding a 443 aa protein. The results achieved from the PCR product digestion with the appropriate restriction enzymes confirmed the gene amplification fidelity. After gene cloning, restriction of plasmid product with appropriate enzymes with BamHI and SpeI, confirmed the integrity of the cloning process.

Many studies have been achieved on cloning of alkaline protease gene from *Bacillus* species. Sadeghi et al. study revealed that the *Bacillus subtilis* 168 alkaline protease gene cloned in pTZ57R cloning vector by of TA cloning method a 1326 bp ORF encoding a 442 aa protein (Sadeghi et al., 2009). A novel protease gene (nprB) of *Bacillus subtilis* encoding a neutral protease was cloned by using a shotgun cloning approach, a molecular mass of 60 kDa was determined (Tran et al., 1991). The *Bacillus subtilis* RD7 was isolated and cloned into pET15b vector and transformed into *Escherichia coli* DH5α (Suberu et al., 2019). The recombinant expression of serine alkaline protease gene had an estimated of 43 kDa and the insert from selected recombinant clone demonstrated it to be a 1203bp gene encoding a protein of 400 amino acids. Moreover, molecular cloning and sequence of the gene for alkaline protease from *Bacillus circulans* MTCC 7906 by Kaur et al. (2012) revealed that the molecular size of alkaline protease protein had an estimated of 46 kDa. Gene sequencing of the insert from selected recombinant clone demonstrated it to be a 1329 bp gene encoding a protein of 442 amino acids.

The recombinant expression of serine alkaline protease gene that was constructed in this study was transformed to *Saccharomyces cerevisiae*. In media enriched with glucose as a carbon source, it illustrates that alkaline protease productivity by yeast strains with expression plasmids was better than those without expression plasmids. In addition, in media with galactose as carbon source, it shows that alkaline protease productivity of strains with expression plasmids OS617, OS303, and OS603 were better than those without expression plasmids except for strains OS591 and OS595. In most cases, enzyme productivity was better in the presence of galactose. This may be due to ability to activate gal operon. The expressed serine protease enzyme showed maximum protease activity of 79.8±6.4 U/ml and 145.5±12.0 U/ml was achieved by strain OS303 in both glucose and galactose containing media, respectively as shown in Table 2. Our results confirm that the cloned gene was highly expressed in active form, therefore fulfilling the goal of cloning to produce large quantities of a specific protein (serine alkaline protease) with high enzyme activity.

On an industrial scale, optimization of parameters are essential in the successful production of exoproteases that shows cultivation conditions such as temperature, pH, and media composition such as glucose, galactose, and other costly substrates must be controlled in process development (Abidi et al., 2011).

Recently, many attempts have been conducted to produce different types of proteases from a number of fungal strains such as *Aspergillus, Rhizopus, Thermomyces, Penicillium, Humicola, Thermoascus, Mucor,* among others. Several substrates have been employed for cultivating enzyme production. The microbial proteases of Aspergillus species have been widely studied. Many of these secreted enzymes have been widely used in the food and beverage industry for decades (Wu et al., 2006).

Latiffi et al. have been attempted to produce the thermostable alkaline protease from *Bacillus stearothermophilus* F1 in yeast for system higher yield. They amplified the full genes of F1 protease and cloned into *Pichia pastoris* expression vectors (pPICZαB and pGAPZαB), then and transformed into *P. pastoris* strains. They found that the highest expression with the highest yield of 4.13 U/mL was achieved under regulation constitutive GAP promoter in contrast to inducible AOX promoter which proves that constitutive expression strategy was better than inducible system (Latiffi et al., 2013).

5. Conclusions

This project was undertaken to apply biotechnology perspectives in the overexpression of a protease enzyme by the yeast system. We successfully isolated 93 bacterial isolates from the environment of Saudi Arabia. The most potent isolate (D9) was characterized molecularly and the protease gene for D9 isolate was also characterized and deposited in the GenBank database. The alkaline protease gene from isolate D9 was successfully cloned in various strains of *Saccharomyces cerevisiae*. Strain OS303 gave the best expression results regarding alkaline protease production, especially in the presence of galactose. Production by strain reached OS303 145.5 U/ml with an approximately 2-fold increase when compared with the wild OS303 strain. Future research will be focused on the industrial production of this enzyme for many applications, especially as a detergent additive.

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