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Amylase Production Optimization and Textile Application of a Novel Bacillus Halotolerans SH1 Isolate from Textile Wastewater

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ABSTRACT

Textile wastewater usually contains toxic compound and a few bacteria survive in this condition. Microorganisms in textile wastewater can produce a variety of enzymes and biotechnology products. This study aimed to isolate bacteria that produce amylase and polyhydroxyalkanoates (PHAs) from textile waste. Both of these compounds are used in the textile industry. Amylase enzyme is used to enhance thread strength and PHAs for the packaging and production of suture thread, surgical meshes, cardiovascular fabrics, and conventional fibers. The highest enzyme activity was optimized using the response surface method (RSM). The isolate was identified as Bacillus halotolerans SH1 by determining the 16S rRNA gene sequence. Optimum conditions for maximum enzyme production (0.26 U/mg) were starch 5.5 g/L, temperature 37 °C, and incubation period 48 hours. The results showed that a PHA concentration of 0.27 g/L is obtained from this bacterium. The reduction in BOD, COD by bacteria was found to be 54.93 and 51.92 %, respectively. This study reports a new strain of Bacillus halotolerans SH1, isolated from toxic textile wastewater, which is capable of producing both PHA and α-amylase enzyme, while also significantly reducing BOD and COD levels, showing its multifunctional potential in environmental and industrial applications, especially in the textile industry. Prog. Color Colorants Coat. 19 (2026), 83-95© Institute for Color Science and Technology.

1. Introduction

Amylases (α, β, and γ) are one of the most popular families of enzymes that have many biotechnological applications [1]. α-amylase breaks down the α-1,4 glycosidic bonds in starch [2]. Amylases comprise around 25% of the world enzyme market [3, 4]. Enzymes are widely used in the paper, detergent, and desizing industries [5, 6]. They play a crucial role in enhancing thread strength and are commonly used as sizing agents in the textile industry due to their easy availability, cost-effectiveness, and biodegradability [7, 8]. Starch accounts for over 75% of the sizing agents [9]. The use of enzymes, such as amylase, leads to starch decomposition and separation [10]. Enzymatic biotreatment of cotton fabrics enhances the physico-chemical properties of the surface and introduces functional groups on the surface. Thus, it improves the affinity of fabric dyeing [11]. α-amylases are produced from plants, animals, and microorganisms [12]. Microorganisms are the primary amylase producers due to their rapid growth and high enzyme production rate. Also, they can be changed by genetic engineering [13-15]. The most widely used microorganisms for α-amylase production are bacteria (Bacillus licheniformis, Bacillus amyloliquefaciens),

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actino-mycetes (Nocardiopsis aegyptia, Streptomyces fragilis), and fungi (Aspergillus niger, Aspergillus flavus) [12]. Textile industries are the largest source of water pollution, and the production of textile waste has increased. Some microorganisms present in textile wastewater may exhibit the ability to synthesize enzymes [16, 17]. Also, microorganisms can decrease biological oxygen demand (BOD) and chemical oxygen demand (COD) levels, making these effluents safe for disposal in the environment [18]. Polyhydroxy-alkanoates (PHAs) are polyesters produced by various microorganisms, and they have attracted much interest for their bio-degradability and biocompatibility [19]. In low concentrations of nutrients like nitrogen, phosphorus, and oxygen with an excess of carbon, certain microorganisms produce PHAs [20]. PHAs are highly persistent, hydrophobic, and bioaccumulative compounds [21]. PHAs are energy-storage bioplastics whose physical and chemical properties are similar to synthetic plastics [22]. RSM combines statistical and mathematical techniques for experimental design, assessing the impacts of independent factors, and identifying optimal response values [23-26]. Research by Sharif et al. screened and characterized thermostable amylase-producing bacteria from Kashmir hot spring water. The optimal enzyme production was observed by Anoxybacillus mongoliensis (MBT001) and authenticated by RSM at pH 7, 70 °C, 1.5 % soluble starch after 48 h of incubation [27]. In a research by Faten et al., microbial amylase production was done through fermentation of agro-industrial residues by a local bacterial isolate, Bacillus spp. NRC1. The highest amylase yield (6.99 U/mL) by CCD design was reached using potato peels. The produced amylase proved its potential in desize cotton fabric [11]. In another study by Osama et al., T. islandicus AUMC 11391 was used to produce the amylase enzyme. The obtained enzyme exhibited its optimum activity at pH 5.0 and 55 °C. Cu, Co, Fe, Ni, Ca, and Zn had an activating effect on the activity of the amylase enzyme at optimal conditions [28]. Commercial enzymes are typically extracted from the biomass of bacteria or different types of fungi. Industrial enzyme production uses carefully formulated culture media to provide all the essential nutrients necessary for microbial growth. Thus, more cost-effective methods for producing microbial biomass, avoiding the need for synthetic growth media, offer substantial commercial benefits for industrial enzyme production. The manufacturing of enzymes should be concentrated on using low-value wastes as substrates since this would lower the cost of production and assist in addressing the disposal and pollution issues associated with these materials [29]. In this study, a bacterial strain was isolated from toxic textile wastewater that, in addition to producing α-amylase, is capable of PHA production. Since the strain can survive in toxic conditions, it has higher capabilities than other common bacteria. The dual functionality of this bacterial strain enables efficient degradation of organic pollutants, highlighting its potential in environmental remediation and industrial biotechnology. In this research, the best α-amylase-producing bacteria were isolated from textile wastewater, and optimization of three parameters (incubation time, starch concentration, and incubation temperature) was investigated by the RSM method. Also, the ability of isolated bacteria to produce PHAs was determined.

2. Experimental

2.1. Sample collection, bacterial isolation

Sludge waste from Textile and Weaving Co. in Yazd, Iran, was utilized for bacterial isolation and stored at -20 °C. Serial dilutions (1:10) were made in sterile phosphate-buffered saline. Subsequently, 100 µL of the enriched broth was spread on nutrient agar plates (Merck) and incubated at 35 °C for 48 hours. The samples were stored at 4 °C on the NA plates [30].

2.2. Macroscopic and microscopic characterization of bacteria

Macroscopic identification includes the size, shape, and color of bacteria colonies grown on NA media was observed by a optical microscope (Olympus). The isolated bacteria were placed on the object glass and stained with Gentian Violet. They were then left for one minute, washed with water, and dried. Subsequently, they were treated with iodine and alcohol for one minute. The isolated bacteria on the object glass were examined by 1000x magnification objective. Gram-positive bacteria stain purple, while Gram-negative bacteria stain red. The endospore test is done in order to determine the evolutionary response of the bacteria in the environment. The isolated bacteria were stained with malachite green for 10 minutes, followed by safranin solution for 1 minute. They were then examined under a microscope at high magnification. Endospores appeared green, while areas without endospores appeared bright red [31].

2.3. DNA extraction, 16S rRNA gene amplification, identification of bacterial strain

DNA was extracted from bacteria by the boiling method for 10 minutes in a water bath, and then were centrifuged at 7000 rpm for 5 minutes. The extracted DNA was then amplified through polymerase chain reaction (PCR) with bacterial universal primers 27F forward and 1492R reverse. The PCR process involved 30 cycles, including denaturation at 95 °C for 1 minute, primer annealing at 56 °C for 1 minute, extension at 72 °C for 1 minute, and a final extension at 72°C for 7 minutes. The resulting PCR products were analyzed using 1% (w/v) agarose gel electrophoresis [32, 33]. Ethidium bromide staining, Gel doc visualization, and photography were used for detection. The sequences were compared to known bacterial strains in the NCBI database using the BLAST function. Finally, a phylogenetic tree was created with MEGA version 7 using a neighbor-joining algorithm [34].

2.4. Screening of amylase producing bacteria

Amylase-producing bacteria were screened by streaking isolates from nutrient agar slants onto starch agar plates (1 % soluble starch, 0.5 % peptone, 1.5 % yeast extract, 2% agar) and incubated at 37 ºC for 24 hours. After incubation, the iodine solution (1 % iodine in 2 % potassium iodide (w/v)) was flooded for 30 seconds. The clear zone of hydrolysis around the colony indicates a positive result.

2.5. Enzyme assay

The amylase assay was carried out in 250 mL flasks containing 50 mL production medium with the following composition (g/L) (Sigma-Aldrich, USA): starch 10, peptone 5.0, yeast extract 2.0, NaCl 1.5, KH_2PO_4 0.5, $MgSO_4$ 0.5, $CaCl_2$ 0.1, and 15 % glycerol (v/v). The flasks were kept in a shaker at 120 rpm at 37 ºC for 48 h. The screened bacterial isolates were inoculated into a 50 mL pre-sterilized production medium. Samples were centrifuged at 10,000 rpm for 20 min, and the resulting supernatants were used to estimate enzyme activity.

2.6. Dinitrosalicylic Acid Assay (DNS) to enzyme analysis

The Dinitrosalicylic Acid Assay (DNS) test was done using the method of Padhiar and Kommu [35]. DNS is an alkaline reagent that attaches to the reducing sugars and causes the color of the solution to change from yellow to red-brown [36]. A solution containing 0.5 mL of enzyme and 0.5 mL of starch (1 %) was prepared. The reaction was incubated at 37 ºC for 30 min, stopped by adding 1 mL of DNS (Sigma-Aldrich, USA), and boiled for 5 min. The amount of glucose was measured using a spectrometer (at 540 nm). A unit of enzyme activity is defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute [37, 38].

2.7. Statistical analysis

The central composite design (CCD) provides high-quality predictions of interaction effects of factors influencing a process [39-41]. The biggest advantage of this type of optimization model is that it is more accurate, and there is no need for a three-level factorial experiment for building a second-order quadratic model. [42]. In this article, optimization for maximal enzyme production was obtained using Design-Expert 7.0 statistical software, followed by CCD.

2.8. PHA production

PHA-producing bacteria were successfully isolated from waste material through the application of an enrichment technique aimed at promoting the growth of specific microorganisms. The samples collected were cultured in a nutrient-rich medium that contained 5 g/L of yeast extract, 8 g/L of tryptone, and 2.5 g/L of sodium chloride (NaCl). Following this initial cultivation phase, the bacteria underwent a process of inoculation into a specialized E2 medium designed for optimal PHA production. The inoculated cultures were then placed in a rotary shaker apparatus set to 120 revolutions per minute (rpm) and maintained at a constant temperature of 35 °C for 24 to 48 hours. The cultures were housed in 250 mL Erlenmeyer flasks containing 50 ml of the prepared medium to facilitate adequate growth conditions. The E2 medium, which is specifically formulated to support the production of polyhydroxy-alkanoates (PHA), was characterized by being nitrogen-limited. This nutrient medium contained the following components in grams per liter (g/L): 3.5 g/L of ammonium phosphate dodecahydrate ($NaNH_4HPO_4.4H_2O$), 7.5 g/L of potassium phosphate trihydrate ($K_2HPO_4.3H_2O$), and 3.7 g/L of potassium dihydrogen phosphate (KH_2PO_4). Additionally, it comprised 100 mM of magnesium sulfate heptahydrate

($MgSO_4.7H_2O$) as well as 10 mL of a trace elements solution. To serve as a source of carbon, the E2 medium also included 10 g/L of glucose. The trace element solution utilized in this medium was formulated with several essential microelements, which were present at specific concentrations (in g/L): 2.78 g/L of iron(II) sulfate heptahydrate ($FeSO_4.7H_2O$), 1.98 g/L of manganese (II) chloride tetrahydrate ($MnCl_4.4H_2O$), 2.81 g/L of cobalt(II) sulfate hepta-hydrate ($CoSO_4.7H_2O$), 1.47 g/L of calcium chloride dihydrate (CaCl2.2H2O), 0.17 g/L of copper(II) chloride dihydrate ($CuCl_2.2H_2O$), and 0.29 g/L of zinc sulfate heptahydrate ($ZnSO_4.7H_2O$), all sourced from Sigma in the USA. This carefully calibrated composition of the medium and trace elements provides an optimal environment for the growth and development of the PHA-producing bacterial strains [43].

2.9. Extraction of PHA

A 10 mL sample was extracted from the production media and centrifuged at 5000 rpm for 15 min. The supernatant was discarded and preserved for drying. The cell dry weight (CDW) of the pellet was considered as the biomass weight. The pellet was then resuspended in 5 ml of 2 % sodium hypochlorite solution and incubated at 37 °C for 1 hour to ensure complete digestion of cellular components. Following centrifugation and rinsing with an acetone: alcohol mixture (1:1 v/v), the PHAs were dissolved in boiling chloroform. Subsequently, the solution was evaporated through air drying to obtain the extracted PHAs in dried form [44]. Standard PHAs (PHB) from Sigma were employed for comparative purposes. PHA production was determined using Eq. 1.

$$\text{PHA production } (\%) = \frac{\text{Weight of purified PHA}}{\text{Dried weight of biomass}} * 100 \qquad (1)$$

2.10. BOD/COD concentration

Sample flasks were incubated in a shaking incubator at 120 rpm and 37 °C for 48 hours. Following incubation, COD and BOD were estimated using standard methods [45].

2.11. FTIR analysis

The chemical structure of the extracted PHA was analyzed using the FTIR spectrometer (Bruker-Equinox 55). The spectra of each sample were obtained within the 4000-500 cm^{-1} range.

3. Results and Discussion

3.1. Molecular detection and phylogenetic analysis

Microscopic characteristics of bacterial colonies included colonial pigmentation: White, cell shape: long, rod, cell arrangement: chain, Gram staining: negative, spore staining: positive. Figure 1a indicates the results of the 16SrRNA gene that was successfully amplified (1500bp), sequencing and blasting results revealed that the isolated bacteria had 100 % similarities with the *Bacillus halotolerans*. The isolate *Bacillus halotolerans SH1* was submitted, and the accession number is OR398703. Phylogenetic tree created using neighbor-joining analysis of 16S rRNA grouped (Figure1b).

3.2. Screening of α-amylase production

Based on colony morphology, using the iodine solution, the isolate showed a clear zone of hydrolysis that it produced around its colonies on starch agar plates. The presence of this clear zone indicates that the isolate is capable of producing enzymes that degrade starch, highlighting its metabolic capabilities in this particular environment.

3.3. Experimental design

In this design, we examined the quantitative impact of the most effective variables, including starch concentration, temperature, and incubation period (Table 1). As presented in Table 2, the total of experiments was 20 along with 8 factorial points, 6 axial points, and 6 replicate points.

Table 1: The number coded factor values.

Variables	Units	Levels		
		-1	0	+1
Starchconcentration	g/L	1	5.5	10
Temperature	ºC	37	43.5	50
Incubation period	h	24	48	72

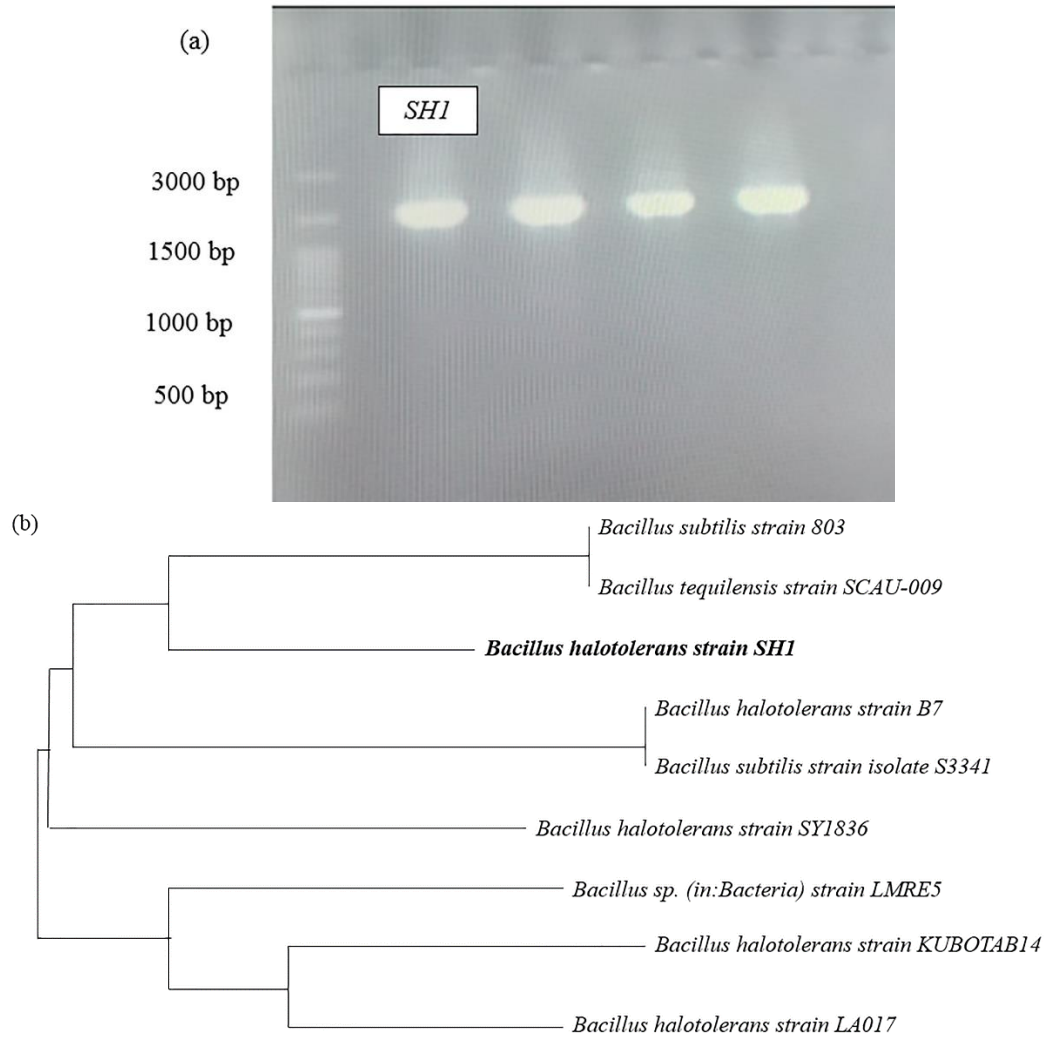

Figure 1: (a) DNA bands, (b) Phylogenetic analysis of *Bacillus halotolerans* with different 16S rRNA sequences bacteria, collected from NCBI-GenBank.

Table 2: Experimental design and corresponding responses.

Run	Temperature (ºC)	Incubation period (h)	Starch (g/L)	Amylaseactivity (U/mL/min)
1	-1	0	0	0.26
2	+1	0	0	0.14
3	0	0	0	0.20
4	-1	-1	+1	0.20
5	0	0	-1	0.19
6	+1	-1	-1	0.01
7	-1	+1	-1	0.16
8	0	-1	0	0.15
9	0	+1	0	0.21
10	-1	+1	+1	0.25

Table 2: Continue.

Run	Temperature (°C)	Incubation period (h)	Starch (g/L)	Amylaseactivity (U/mL/min)
11	0	0	+1	0.23
12	0	0	0	0.22
13	-1	-1	-1	0.14
14	0	0	0	0.22
15	+1	+1	-1	0.11
16	0	0	0	0.21
17	0	0	0	0.22
18	+1	+1	+1	0.12
19	0	0	0	0.20
20	+1	-1	+1	0.03

The best-fit model can be presumed after the quadratic model analysis by analysis of variance and *F*-value determination, as shown in Eq. 2, where *Y* is the α-amylase activity as a response; *n* the number of variables; x_i and x_j are the coded values of the factors and b_0, b_i, b_{ii}, and b_{ij} are the constant coefficient, the linear coefficient, the quadratic coefficient, and the interaction coefficient, respectively [46].

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j \quad (2)$$

In this research, based on a multiple regression analysis of experimental data, the final model was presented as follows (Eq. 3):

$$\text{α-amylase activity} = +0.22 + 0.022A - 0.060B + +0.032C - 0.015AB + 2.500E-003AC + 0.015BC - 0.016A^2 - 0.026B^2 - 0.046C^2 \quad (3)$$

Where A, B, C are temperature, incubation period, and starch, respectively. The analysis of variance (ANOVA) is described in Table 3, and 4. The ANOVA and the F test confirm the regression model is significant for enzyme production optimization. The F-value is the ratio of the mean squares to the mean squares error. The larger the F-value, the greater the variation between sample means relative to the variation within the samples. The p-value is the probability of obtaining an F-ratio as large or larger than the one observed, assuming that there is no difference between the group averages [47]. The values of Prob > F are lower than 0.05 (95% confidence) that this model is significant. To test the fit of the model, the regression equation and determination coefficient R^2 were evaluated. The model showed a high determination coefficient (R^2 = 0.9694), indicating a strong correlation between the experimental and predicted values.

Table 3: ANOVA for the quadratic model.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.084	9	0.010	81.02	< 0.0001	significant
Residual	1.423E-003	11	1.293E-004			
Lack of Fit	9.394E-004	6	1.566E-004	1.62	0.3066	
Pure Error	4.833E-004	5	9.667E-005			
Cor Total	0.085	19				

Table 4: ANOVA results.

Source model	Std. Dev.	R-Squared	Adj. R-Squared	
Linear	0.046	0.5991	0.5240	
2FI	0.048	0.6420	0.4767	
Quadratic	0.012	0.9839	0.9694	Suggested
Cubic	0.014	0.9865	0.9572	Aliased

The normal probability plot against the internally studentized residuals in Figure 2a shows that the data follows a straight line and not an S-shape, suggesting a normal distribution of the residuals [48]. Residuals represent the difference between observed and predicted values. A well-fitting model will typically show smaller residual values, demonstrating that the predictions align closely with the actual measurements. The predicted responses, along with the observed responses, are illustrated in Figure 2b, and the data points are located close to the diagonal line, suggesting a satisfactory correlation. The empirical models closely fit the observed data, demonstrating their effectiveness in capturing the trends and patterns present in the actual measurements.

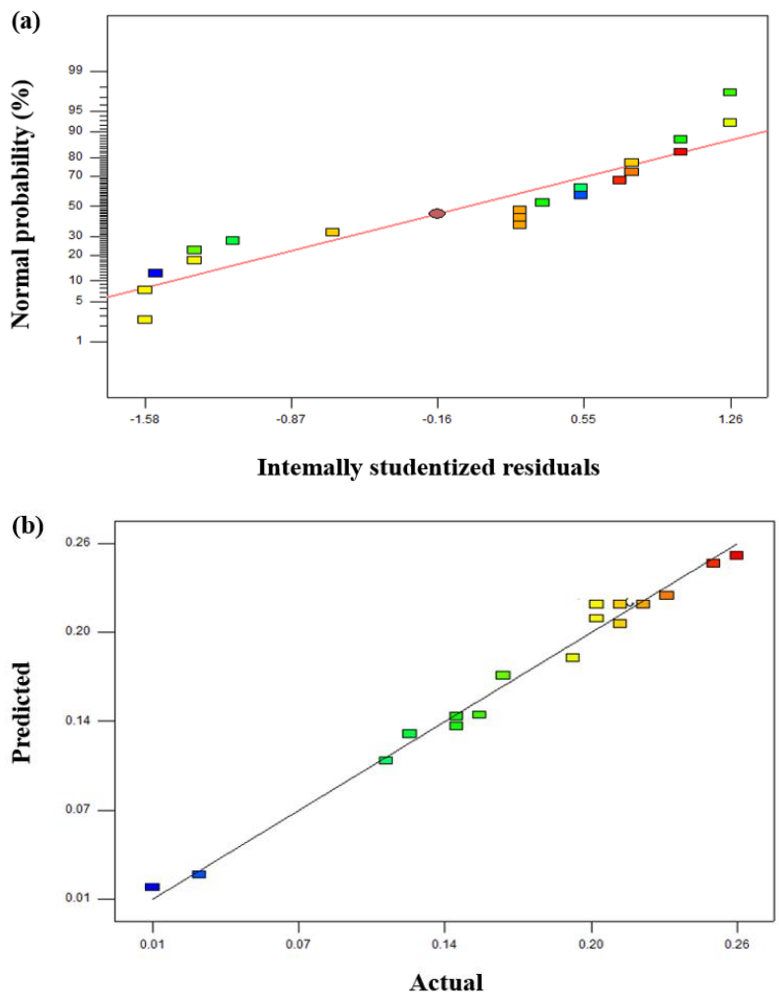

Figure 2: (a) Normal plot of residual and (b) Actual values versus predicted plot.

A contour plot visually represents a three-dimensional response surface based on two independent variables, helping to illustrate their main and interaction effects on the response. The blue color signified the lowest, the green color signified moderate, while the red color signified the highest yield, as shown in Figure 3. Also, the 3D plots can help understand how variables affect the response [49]. Figure 3 shows the amylase response and correlation between variables in three-dimensional plots. The incubation temperature plays a crucial role in microbial growth. The study found that 37 °C was the optimal temperature for production. As the temperature rises, enzyme structure denaturation occurs, leading to a decrease in α-amylase production. Additionally, longer incubation times decrease enzyme activity due to the decrease in nutrients in the culture. Also, the choice of carbon or energy source (e.g., starch, glucose, malt extract) is another key factor influencing microbial growth [50-52]. A numerical optimization to maximize α-amylase activity was performed, and the highest α-amylase activity (0.26 U/mg) was obtained using temperature (37 ºC), incubation period (48 hr), and starch (5.5 g/L). The experimental α-amylase activity is in agreement with the model prediction.

3.4. Isolate ability in reducing wastewater parameters

BOD is a crucial parameter that researchers examine when assessing water quality. A high BOD level signifies the presence of a substantial amount of organic matter in the water, which can lead to significant oxygen depletion. This depletion creates anaerobic conditions that can adversely affect aquatic ecosystems, resulting in a reduction of biodiversity and altering the distribution of various aquatic fauna [16]. In the current study, we observed a remarkable reduction in the levels of both BOD and COD in wastewater treated with *Bacillus halotolerans SH1*, with reductions of 54.93 and 51.92 % respectively (Table 5). This noteworthy decrease in BOD and COD can be attributed to the effective consumption of organic material in the wastewater by microbes. Applying such microbes can be a beneficial strategy in wastewater treatment efforts, highlighting their importance in environmental management and conservation.

Table 5: Percentage of BOD/COD reduction by using *Bacillus halotolerans SH1.*

Wastewater parameters	Initial	Final	Reduction (%)
BOD Reading (mg/L)	4160	1875	54.93
COD Reading (mg/L)	13000	6250	51.92

3.5. Fermentative production of PHA by Bacillus halotolerans *SH1*

The isolates were thoroughly assessed for their ability to accumulate polyhydroxyalkanoates (PHAs) using E2 medium, which was specifically designed for this purpose. The results indicated that the isolated strain identified as *Bacillus halotolerans SH1* demonstrated a remarkable capacity for growth as well as the production of PHAs. During the experimentation, it was observed that a substantial amount of cell dry weight (CDW) reaching 0.98 g/L was achieved. Furthermore, this strain exhibited a PHA production of 0.27 g/L, leading to an impressive PHA accumulation percentage of 27.55 % after 72 hours, as illustrated in Figure 5. These findings underscore the potential of *Bacillus halotolerans SH1* in the bioproduction of PHAs, highlighting its efficacy in utilizing E2 medium for growth and biopolymer accumulation (Figure 4).

3.6. Analysis of PHA

In 1300 to 1000 cm^{-1} region, C–O–C linkages indicative of ether stretching were observed. A peak between 1736 and 1650 cm^{-1} indicates C=O stretching from alkyl esters. The absorption band at 3400 cm^{-1} indicates the presence of a PHA hydroxyl group, while methyl (CH_3) and methylene (CH_2) peaks appear in the 3000–2600 cm^{-1} region. The presence of asymmetric CH_3 may indicate the formation of hydrogen bonds between C–H–O. Multiple absorption peaks from 1054 to 677 cm^{-1} correspond to C–O and C–C in the amorphous phase. Variations in peak intensity or the appearance of additional peaks compared to standard PHB may reflect compositional or structural differences due to the bacterial source. These distinct absorption bands confirm the PHA structure (Figure 5) [53, 54].

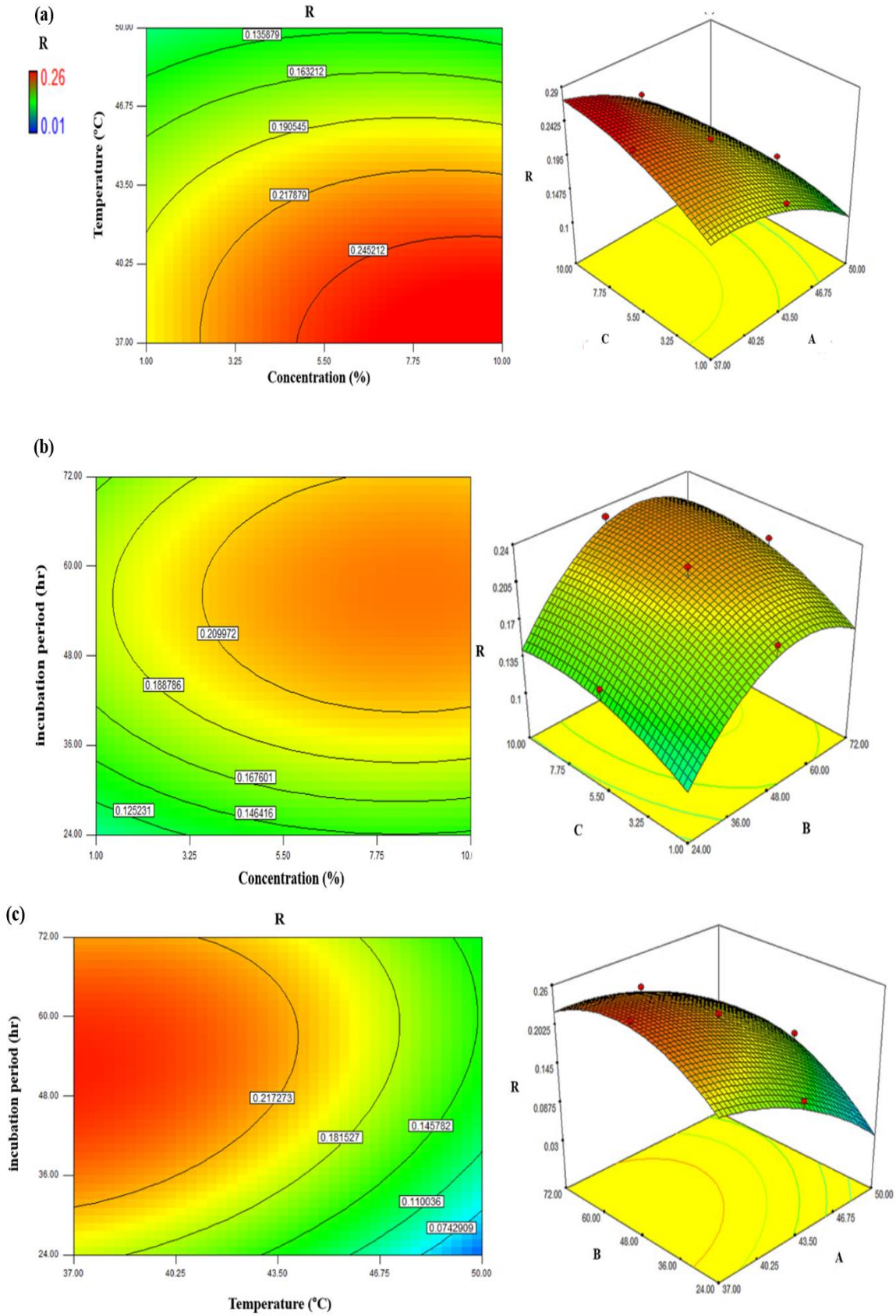

Figure 3: Contour and 3D plots for the effect of (a) concentration of starch and temperature (b) incubation period and concentration of starch and (c) temperature and incubation period.

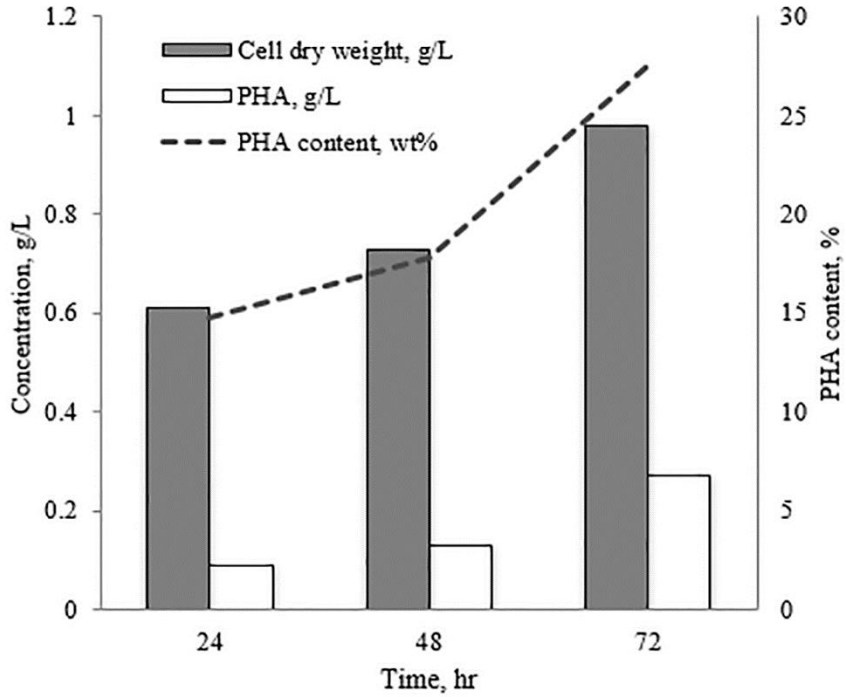

Figure 4: PHA content (%) and CDW (g/L) production by strain *Bacillus halotolerans SH1.*

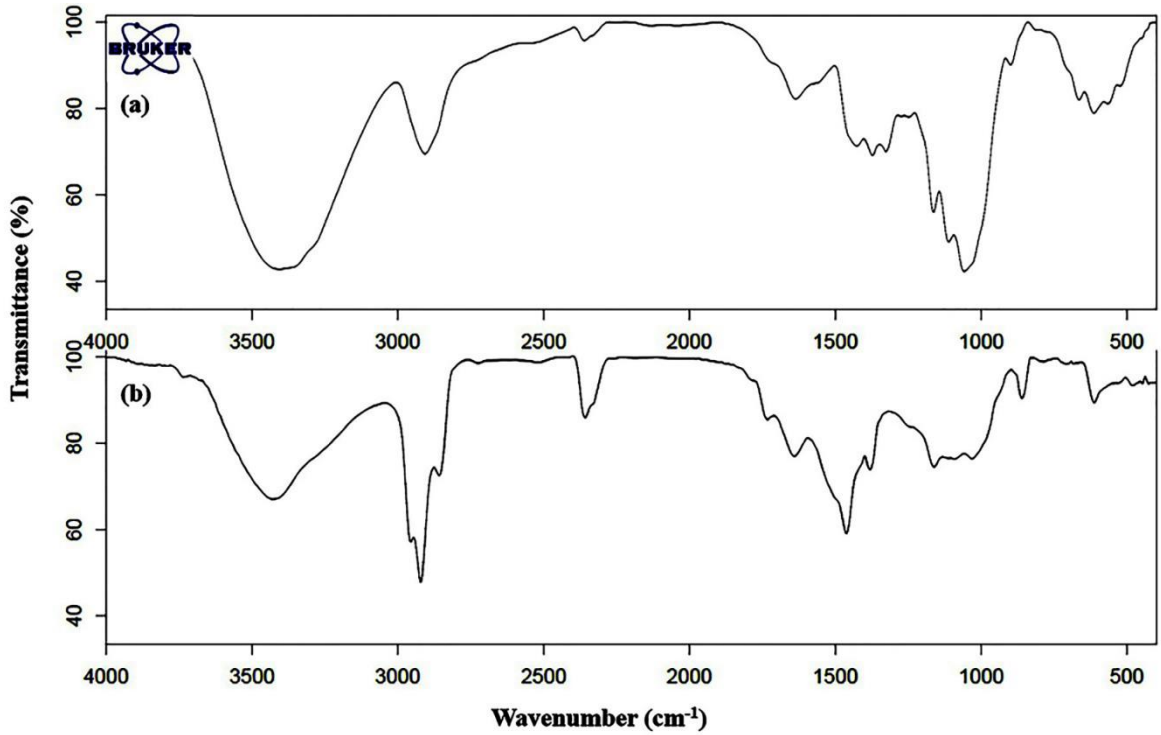

Figure 5: FTIR absorption spectra of PHA, a) standard (PHB), b) of bacterial isolate *Bacillus halotolerans SH1.*

4. Conclusion

Enzymes are extensively utilized in the textile sector. Waste from various industries, particularly textiles, serves as a cost-effective and viable option for α-amylase production. *Bacillus halotolerans SH1* is a bacterium that produces α-amylase and was isolated from textile waste. Optimization through RSM proved to be an effective, relatively straightforward method that saves time and resources. The optimal conditions for maximum enzyme activity (0.26 U/mg) were obtained at

a concentration of starch 5.5 g/L, a temperature of 37 °C, and a time of 48 hours. Additionally, numerous bacteria generate PHAs under stressful conditions, offering a promising alternative to petroleum-based plastics. The isolated bacterium exhibits significant potential for PHA production (0.27 g/L). The isolated bacterium was able to reduce BOD and COD levels by 54.93 and 51.92 %, respectively. Generally, in this study, a bacterial strain was isolated from toxic textile wastewater that, in addition to producing α-amylase, is capable of biopolymer production. Since the strain can survive in toxic conditions, it has higher capabilities compared to other common bacteria.

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