OPTIMIZATION OF PROCESS FOR ENHANCED SILICOLYTIC ENZYME PRODUCTION FROM *Klebsiella quasipneumoniae* (AJS) *via* SUBMERGED FERMENTATION

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Abstract. Silicases, enzymes that facilitate silica dissolution, have garnered considerable interest due to their potential utility in agricultural industries. This study investigates the production, optimization, and characterization of silicase from *Klebsiella quasipneumoniae* (AJS), a bacterium exhibiting silicolytic activity isolated from a paddy field ecosystem. Employing submerged fermentation (SmF), we optimized the culture conditions for enhanced silicase production, assessing factors such as inoculum age, pH, temperature, incubation duration, and the influence of nitrogen and carbon sources. Highest silicase activity of 0.88 U/mL/min was observed with rice straw, a rich silica source, as the substrate. Maximum silicase activity of 0.88 U/mL/min was achieved using rice straw, a substrate rich in silica as the primary substrate. Optimization trials indicated that silicase production peaked with a 24-hour inoculum age, 1.0% inoculum volume, pH 7.0, and an incubation temperature of 40°C. Among evaluated nitrogen sources, urea and yeast extract proved most effective, resulting in silicase activities of 0.83 U/mL/min and 0.86 U/mL/min, respectively. Additionally, incorporating Triton X-100 as a supplement notably enhanced enzyme activity. Our results highlight the feasibility of leveraging agro-industrial by-products, particularly rice straw, for cost-effective silicase production, supporting sustainable biotechnological applications. This investigation delivers key insights into optimizing process parameters for scalable silicase sbiofuels.

Keywords: agricultural waste; enzyme optimization; *Klebsiella quasipneumoniae*; rice straw; silicase.

INTRODUCTION

The management of agricultural waste has emerged as a major global challenge, with the problem becoming particularly acute in countries such as India, where residue generation and disposal pressures have escalated in recent years. Rice straw, a major by-product of paddy cultivation, has become a notable concern. India produces roughly 62 million tons of agricultural waste each year, with a substantial portion comprising crop residues like rice straw. Although these residues contain considerable amounts of organic carbon and plant nutrients, the high silica content of rice straw (typically 12–18%) renders it unsuitable for livestock feed, thereby complicating waste disposal [12]. Due to the adverse effects on digestibility, farmers are unable to utilize this waste as fodder and frequently opt to burn the residues to expedite field clearance for subsequent crop cycles. This burning practice leads to severe air pollution, harms soil health by disturbing the microbial balance, and consequently impacts long-term agricultural productivity. Additionally, it contributes to increased healthcare costs stemming from pollution-related health problems.

A core aspect of this challenge is the elevated silica content in rice straw. Silica enhances paddy growth by improving resistance to biotic and abiotic stresses; however, its high accumulation in crop residues simultaneously inhibits the microbial decomposition of lignocellulosic biomass, creating significant challenges for effective agricultural waste management [3, 23]. Consequently, identifying efficient strategies to either reduce silica levels or improve biodegradation of these residues is vital for environmental sustainability and optimizing agricultural practices. A potential solution involves the production of silicases, enzymes that can break down silicate compounds by microorganisms, which could aid in decomposing silica-rich biomass.

Silicases have emerged as key enzymes in addressing the challenges related to agricultural waste management. Various studies have reported the identification of bacteria capable of solubilizing silicate. Most of these bacteria belong to genera such as *Bacillus, Pseudomonas, Acidobacillus, Burkholderia*, and *Enterobacter* [3, 26]. Recent research has pinpointed microorganisms like *Klebsiella quasipneumoniae* (AJS) [23] that produce silicase and are adept at thriving in environments rich in silicate. Table 1 lists some of the identified bacterial isolates. These organisms hold considerable promise for large-scale silicase production via submerged fermentation. Nevertheless, despite the potential of these bacteria, there is still a need for enhanced process optimization to boost silicase yield and activity. Conventional fermentation techniques often fall short of achieving high enzyme production levels. Moreover, current assay methods for measuring silicase activity lack the sensitivity and precision needed for effective monitoring and process control.

To address these limitations, this research presents a novel, highly sensitive and cost effective assay method specifically designed for the accurate measurement of silicase activity. By combining this cutting-edge assay with methodical fermentation optimization, the study seeks to determine the ideal conditions for maximizing silicase production from *K. quasipneumoniae*. Critical process variables including pH, temperature, aeration, and nutrient makeup are investigated to refine fermentation conditions for improved enzyme yield and activity.

To further enhance the sustainability of this process, the research examines the utilization of rice straw as a cost-effective alternative carbon source for fermen-

DOI: 10.61215/AUOFB.2026.1.05

Table 1. Different identified bacterial isolates and their sources of isolation

Bacteria	Source of Isolation	Reference
Bacillus sp.	Magnesite ore	[18]
Bacillus mucilaginous	Soil sample	[15]
Bacillus globisporus	Weathered feldspar minerals	[24]
Bacillus sp.	Clay from mountain territory	[17]
Burkholderia cenocepacia	Zeolite mineral	[20]
Burkholderia aeburneaus CS4-2	Rice rhizosphere soil	[10]
Burkholderia vietnamiensi	Soil samples	[20]
Pseudomonas fluorescens	Paddy rhizospheric soil	[26]
Bacillus flexus	Paddy rhizospheric soil	[26]
Enterobacter ludwigii	Forest and paddy soil	[14]
Bacillus mucilaginosus	Soil samples	[27]
Pseudomonas psychrotolerans	Lab isolates	[13]
Klebsiella quasipneumoniae	Paddy rhizospheric soil	[22]
Pseudomonas aeruginosa	Soil Samples	[25]

tation. Incorporating rice straw into the fermentation medium yields dual advantages: it supplies a sustainable substrate for microbial growth and simultaneously addresses the environmental issue of disposing of agricultural waste. The elevated silica content in rice straw presents a distinct challenge. However, by harnessing the capabilities of silicase-producing bacteria, this study investigates a method for decomposing the intricate lignocellulosic structure of rice straw and promoting its effective biodegradation.

By optimizing silicase production by fine-tuning process parameters and utilizing agricultural waste as a carbon source, this research delivers a sustainable approach to addressing challenges in agricultural waste management and enzyme production. The outcomes hold considerable implications for the biotechnological sector, as they present a scalable, economical, and eco-friendly method for producing silicase. The findings advance enzyme technology and contribute to the overarching objective of enhancing waste management strategies in agriculture. This fosters a circular economy that minimizes waste, boosts soil health, and generates economic advantages for farmers.

MATERIALS AND METHODS

This investigation focused on identifying silicase-producing organisms and optimizing culture conditions for silicase enzyme production *via* submerged fermentation. The research was carried out in the Microbial Biotechnology Laboratory, Department of Biotechnology, Kurukshetra University, India.

Raw materials, chemicals, plastic wares and glass wares. Raw materials used for production included two synthetic siliceous compounds i.e. silicon dioxide, magnesium tri-silicate and agro-residues namely rice straw, rice husk, wheat straw, sugarcane bagasse and wild grass *Tripidium bengalense* (sakanda) were procured locally. Another substrate fly ash used was collected from Rajiv Gandhi Thermal Power Plant, Hisar (India). The Chemicals beef extract, NaCl, peptone, magnesium trisilicate, silicon dioxide, Tris base, DL-dithiothreitol (DL-DTT), zinc sulfate, magnesium sulfate, potassium nitrate, D-xylose, hydrochloric acid, ammonium molybdate, oxalic acid, and 1-amino-2-naphthol-4-sulfonic acid (ANSA) were procured for media preparation and various pretreatment analyses. A silicase assay kit was purchased from Merck (1.14794). All reagents and chemicals were of high-purity analytical grade and were supplied by HiMedia Laboratories Ltd., India; Sigma Chemicals Ltd., USA; Rankem, Gurugram; and Merck and Co. Inc., USA. Glassware from Perfit and plasticware from Tarson were employed for silicase production and enzyme assays. A spectrophotometer (Analytik Jena) was used for analytical studies and determining silicase activity.

Silicase production using different agro-waste and its activity using kit method. Silicase was produced using a modified Horikoshi medium, originally formulated for the isolation and cultivation of alkaliphilic microorganisms. In this study, the medium was adapted to include both synthetic and natural siliceous substrates to facilitate enzyme production. The microorganism exhibiting the largest zone of dissolution on screening media, as noted in previous studies by Sharma *et al.* [22], was selected for further quantitative evaluation. The production medium consisted of 1 g of siliceous substrate, 0.5 g of peptone, 0.3 g of yeast extract, 0.5 g of potassium nitrate, 0.1 g of potassium dihydrogen phosphate, and 0.01 g of magnesium sulfate in 100 mL of distilled water (Table 2).

Table 2. Composition of medium used for production of silicase under submerged fermentation

Media components	Volume (g/100mL)
Peptone	0.5
Yeast extract	0.3
Potassium nitrate	0.5
Potassium dihydrogen phosphate	0.1
Magnesium sulphate	0.1
Siliceous substrate	1 %

Various siliceous sources including silicon dioxide, magnesium trisilicate, cereal straws (wheat and paddy), bagasse, and rice husk were examined for silicase production. Agro-residues like wheat straw, paddy straw, sugarcane trash, and bagasse were chopped into 1-2 cm pieces and then ground into a fine powder using a Wiley mill (Thomas Scientific, model ¼ HP) at 1440 rpm. Prior to use as a substrate for silicase synthesis, the powdered materials were sieved through an 18 mm mesh (Perfit). Rice husk was

DOI: 10.61215/AUOFB.2026.1.05

powdered in a mixer grinder and sieved through a 0.297 mm mesh. Silicase production media containing different substrates were sterilized by autoclaving at 121°C and 15 psi for 20 minutes. The production media were inoculated with a 16-18 hour-old inoculum and incubated at 37°C for four days at 150 rpm. Following fermentation, the broth was centrifuged at 10,000 rpm for 20 minutes at 4°C to obtain the crude enzyme. Silicase activity was determined using a Merck silicate assay kit (1.14794). The reaction utilized silicon dioxide as the substrate, dissolved in Tris buffer (pH 7.2) to achieve a final concentration of 100 µg/mL.

The current analysis followed the standard Molybdenum silicate method (Standard Methods: 4500-SiO_2 C, as per the National Environmental Methods Index), where ammonium molybdate reacts with silica to form heteropoly acids. The reaction mixture for the silicase assay consisted of 100 µL of substrate containing 100 µg/mL silicon dioxide dissolved in Tris buffer (pH 7.2). To this substrate, 900 µL of Tris buffer was added, followed by 50 µL of crude enzyme supernatant. The mixture was incubated at 30°C for 30 minutes, and the reaction was terminated by adding a reagent from the silicate kit, which lowered the pH to acidic levels. Three drops of Reagent Si-I were added to the mixture and left to stand for 3 minutes at room temperature. Next, Reagent Si-II was added and allowed to stand for 2 minutes. Finally, 0.5 mL of Reagent Si-III was added and left for 10 minutes. The resulting soluble silicic acid, a heteropoly acid formed from the reaction between silicate and molybdate ions, was reduced to silicomolybdenum blue. This was quantified by measuring absorbance at 810 nm. A standard curve for silicic acid was plotted using concentrations ranging from 0 to 10 µg in a 5 mL test volume. One silicase unit is defined as the amount of enzyme releasing 1 µg/mL/min of silicic acid.

Silicase activity was quantified using a Merck silicate assay kit (1.14794), which served as the standardized kit-based method prior to the implementation of the lab-established assay described later.

Lab established assay method for silicase activity. Due to the high cost of estimating silicase activity using a silicase kit, a cost-efficient and rapid method was developed in the lab for this purpose. This method was based on the same principle as the kit- the heteropoly blue method. In this approach, silica was solubilized as silicic acid and then measured using colorimetry. The enzyme assay reaction mixture contained 100 µL of 1 mg/mL silicon substrate in 900 µL of Tris-HCl buffer (pH 7.2). To this, 50 µL of crude enzyme extract was added and incubated at 37°C with shaking at 120 rpm for 30 minutes [12]. Upon reaction completion, 4 mL of distilled water was added. The reaction mixture's pH was adjusted to acidic conditions using 12 M HCl, followed by the addition of 200 µL of 10% ammonium molybdate. The mixture was left to stand for 5-10 minutes. Next, 200 µL of 7.5% oxalic acid was added and left for 1 minute. Finally, 200 µL of 1-amino-2-naphthol-4-sulfonic acid (ANSA) was added, and color development was observed after 10 minutes. Experiments were conducted in triplicate, and mean values were reported. The absorbance of the resulting color was measured at 810 nm against a control using a UV-Vis spectrophotometer. The absolute amount of silicic acid was determined using a calibration curve constructed with standards.

Optimization of process conditions for obtaining tiers of silicase production. Various process variables including incubation time, incubation temperature, pH, carbon source, nitrogen source (both organic and inorganic), metal ions, and additives were considered and optimized to achieve maximum silicase production (Table 3).

Effect of inoculum age and size. To examine and optimize inoculum age, 1% of culture aged 12-48 hours was used to inoculate 250 mL Erlenmeyer flasks containing 100 mL of production medium. For optimizing inoculum size, a 24-hour-old culture was used at levels of 1-4% for inoculation. The flasks were incubated at 37°C and 120 rpm for 24 hours, after which the contents were harvested and analyzed for silicase activity.

Impact of pH on silicase production. Different enzymes function optimally at varying pH levels. Therefore, to maximize silicase yield, the pH of the production medium was optimized. Production media with pH values of 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 were inoculated and incubated at the optimal temperature of 37°C with shaking at 120 rpm.

Effect of incubation temperature on silicase production. Incubation temperature significantly impacts enzyme production and activity. Therefore, the effect of temperature on silicase production was examined. Incubation temperatures of 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C were tested for optimization. Erlenmeyer flasks (250 mL) containing 100 mL of modified medium were inoculated with 1% (v/v) of a 24-hour-old culture using Tarsons plasticware. The flasks were incubated at 120 rpm shaking. After 4 days, the crude enzyme was harvested and analyzed for silicase activity.

Effect of incubation time on silicase production. To identify the optimal incubation period for enzyme production, Erlenmeyer flasks (250 mL) containing 100 mL of production medium were inoculated with 1 mL of a 24-hour-old culture. The flasks were incubated for varying time intervals (0-8 days) at 37°C with shaking at 120 rpm using plasticware. The crude enzyme was then analyzed for silicase activity.

Effect of agro-residues as carbon source. Erlenmeyer flasks (250 mL) made of Tarsons plasticware containing 100 mL of production medium (pH 7.0) without a carbon source were supplemented with various carbon sources including agro-residues like rice straw, rice husk, bagasse, sugarcane trash, wheat

DOI: 10.61215/AUOFB.2026.1.05

Table 3. Optimization of various parameters at different ranges

Parameter	Range
Incubation time (d)	0, 1, 2, 3 and 4
Inoculum size (v/v)	1 %, 2 %, 3 % and 4 %
Inoculum age (h)	12, 18, 20 and 24
Temperature (°C)	20, 25, 30, 35, 40, 45 and 50
pH	5.5, 6, 6.5, 7, 7.5, 8.0 and 8.5
Substrates	● **Agro-residue:** Rice straw, Rice husk, Baggase, Sugarcane trash, Wheat straw ● **Wild Grasses:** Sarkanda ● **Industrial waste**: Fly ash ● **Chemicals:** Silicon dioxide and Magnesium trisilicate
Sugars	● Glucose ● Xylose ● Starch ● Maltose ● Sucrose ● Galactose
Nitrogen In-organic source	● Potassium nitrate ● Ammonium chloride ● Ammonium sulphate ● Sodium nitrate ● Urea ● Ammonium nitrate
Nitrogen Orgnic source	● Yeast extract, ● Beef extract ● Soyabean meal
Metal ions	● Calcium chloride ● Sodium chloride ● Potassium chloride, Magnesium sulphate ● Zinc dust ● Cadmium chloride
Additives	● EDTA ● Triton X- 100 ● Tween 80

straw; industrial waste (fly ash); wild grasses like Tripidium bengalense (sarkanda); and chemicals such as silicon dioxide and magnesium trisilicate (1% w/v). Following autoclaving, the flasks were inoculated with 1% of a 24-hour-old culture and incubated at 37°C with shaking at 120 rpm for 4 days. Silicase activity of the extracted crude enzyme was then measured. The optimal concentration of the selected carbon source was determined by testing concentrations of 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5% (w/v) in the medium.

Effect of sugars in the medium. Various sugars including glucose, xylose, starch, maltose, sucrose, and galactose (0.5% w/v) were added to Erlenmeyer flasks (250 mL) made of plasticware. Each flask contained 100 mL of production medium (pH 7.0) with 1% rice straw and was inoculated with 1% of a 24-hour-old culture. Incubation was carried out at 37°C for 4 days with shaking at 120 rpm. The optimal concentration of the chosen sugar was determined by testing levels of 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5% (w/v) in the medium.

Effect of the nitrogen source. The effect of different organic and inorganic nitrogen sources like potassium nitrate, ammonium chloride, ammonium sulfate, sodium nitrate, urea, ammonium nitrate, yeast extract, beef extract, and soybean meal on silicase production was examined. These nitrogen sources (0.5% w/v) were added to the medium containing 1% rice straw and sucrose as carbon sources, with no other nitrogen source present. Erlenmeyer flasks (250 mL) with 100 mL of production medium were inoculated with 1% of a 24-hour-old culture and incubated at 37°C for 4 days with shaking at 120 rpm. Additionally, the chosen nitrogen source was tested at concentrations of 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5% (w/v) in the production medium (pH 7.0) to determine the optimal concentration.

Effect of metal ions and additives on silicase production. To examine the impact of different metal ions and additives on silicase production, Erlenmeyer flasks (250 mL) made of Tarsons plasticware containing 100 mL of production medium were supplemented with various metal salts including calcium chloride, sodium chloride, potassium chloride, magnesium sulfate, zinc dust, and cadmium chloride, as well as additives like EDTA, Triton X-100, and Tween 80, each at a concentration of 1%. The flasks were incubated at 37°C with shaking at 120 rpm for 4 days, followed by analysis of silicase activity. Subsequently, the effect of varying concentrations (0.25, 0.5, 1.0, 1.5, 2.0, 2.5% w/v) of the selected metal salt and additive on enzyme production was investigated in the medium.

RESULTS

Silicase activity: Enzyme assay. Silicase from silicolytic microbes can be quantitatively detected using a colorimetric method based on heteropoly acid formation. This requires cultivating the microbe in

DOI: 10.61215/AUOFB.2026.1.05

Horikoshi's production medium under optimal conditions (37°C) for enzyme production, followed by extraction of crude enzyme from the supernatant of centrifuged production medium. Soluble silica in the form of silicic acid was assessed before and after incubation of inoculated production medium. Different siliceous substances were added to the production medium to enhance activity and identify a suitable substrate. Silicic acid content was found to be satisfactory across all substrates (Table 4). In prior research, silicase activity for a bacterial isolate (AJS) from paddy field samples was 0.71 U/mL/min, and from wild grasses was 0.73 U/mL/min [23]. The isolated bacterium was highly effective in producing silicase in production medium containing rice straw and rice husk. Silicase activities of 0.88 U/mL/min and 0.52 U/mL/min were detected with these substrates (Table 4). Silicase production was lower in production medium containing wheat straw and bagasse as silica sources. Rice straw, which is silica-rich, showed high potential for silicase production (0.88 U/mL/min).

Optimization of process conditions for silicase production. Various process parameters including pH, temperature, incubation time, carbon source, nitrogen source, and substrates were optimized to maximize silicase production, with results detailed in subsequent sections. Based on these optimized conditions, the components of the silicase production medium were chosen for further analysis.

Effect of inoculum age and size. Inoculums aged between 12 and 48 hours were tested at levels ranging from 0.5% to 2.0%. Comparable silicase activity results were obtained with inoculum ages of 12 and 36 hours. The highest silicase activity (0.62 U/mL/min) was achieved with a 24-hour-old inoculum. Silicase activity decreased with inoculums older than 48 hours (Table 5). Using a 1.0% culture yielded the maximum silicase activity (0.58 U/mL/min), which decreased with larger inoculum sizes, possibly due to overcrowding of bacteria and limited nutrient availability in the medium.

Table 4. Effect of Siliceous substrates on silicase activity

Siliceous substrate in production media	Silicase activity (U/mL/min)	Silicic acid before inoculation (µg/mL)	Silicic acid after inoculation (µg/mL)
Rice straw	0.88±0.04	400	328
Rice husk	0.52±0.06	260	180
Bagasse	0.30±0.05	120	90
Silicon dioxide	0.56±0.07	370	197
Wheat straw	0.45±0.06	160	152
Magnesium tri-silicate	0.50±0.04	390	172

Table 5. Effect of inoculum age and size on silicase production

Inoculum age (h)	Silicase activity (U/mL/min)	Inoculum size (%)	Silicase activity (U/mL/min)
12	0.32	0.5	0.38
24	0.62	1.0	0.58
36	0.36	1.5	0.50
48	0.24	2.0	0.42

Effect of pH. To examine the impact of pH on silicase production, the organism was grown at pH levels ranging from 5.5 to 8.5. Maximum silicase activity (0.82 U/mL/min) was observed at pH 7.0. At pH 5.5, silicase activity was 0.26 U/mL/min, increasing by 68.2% when pH was adjusted to 7.0. Above pH 7.0, silicase activity gradually declined (Fig. 1).

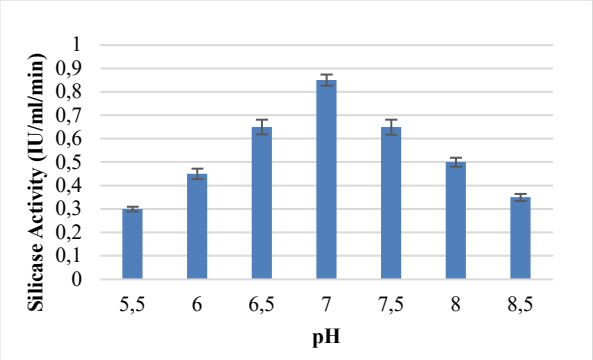

Figure 1. Effect of pH silicase production by *Klebsiella quasipneumoniae* (AJS)

Effect of incubation temperature. The effect of incubation temperature on silicase activity was examined using a temperature range of 20°C to 50°C at 5°C intervals. The isolated bacterial culture exhibited silicase activity at all tested temperatures, with activity increasing as temperature rose to 35°C. At 20°C, silicase activity was 0.37 U/mL/min, increasing by 53.75% when temperature was raised to 35°C. Further temperature increases did not significantly impact silicase activity. Above 40°C, silicase activity sharply declined, indicating an optimal temperature range of 35°C to 40°C for silicase production by bacterial culture (AJS) under submerged fermentation (Fig. 2).

Effect of incubation time of silicase production. To assess the effect of incubation duration on silicase production, a range of 0 to 8 days was tested. The isolated bacterial culture demonstrated silicase activity throughout the tested days, with activity increasing as incubation time progressed. Maximum silicase activity (0.84 U/mL/min) was achieved after 4 days of incubation. On the first day, silicase activity was 0.02 U/mL/min, increasing by 97.6% by day 4 (Fig. 3). On

DOI: 10.61215/AUOFB.2026.1.05

further increase in the time of incubation, silicase activity declined sharply.

Effect of various agro- industrial residues as carbon sources. Silicase activity in production medium with fly ash was 0.29 U/mL/min, lower than with magnesium trisilicate (0.50 U/mL/min). Among the agro-residues tested, sugarcane trash yielded the lowest silicase activity (0.22 U/mL/min). Wheat straw and rice husk proved effective as substrates, with silicase activity increasing by 52.1% and 69.86% respectively compared to sugarcane trash (Fig. 4). Rice straw was the most effective substrate, with silicase activity of 0.88 U/mL/min in production medium containing rice straw.

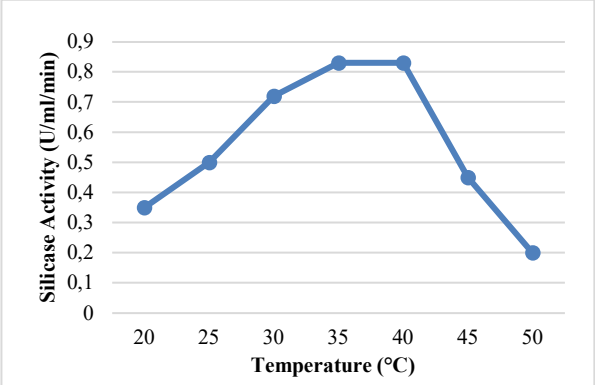

Figure 2. Effect of incubation temperature on silicase production by *Klebsiella quasipneumoniae* under SmF (AJS)

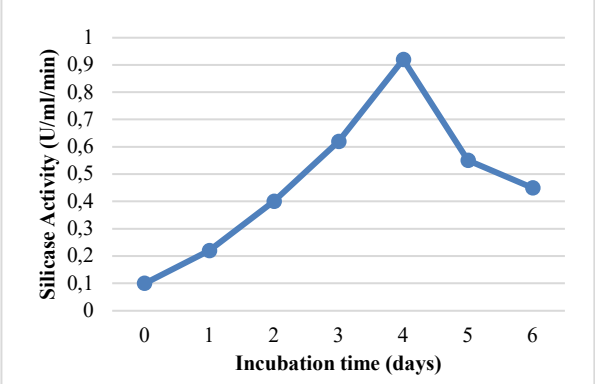

Figure 3. Effect of incubation time on silicase production by *Klebsiella quasipneumoniae* under SmF (AJS)

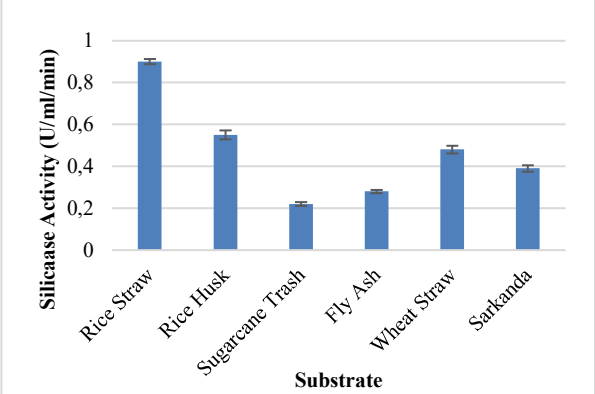

Figure 4. Silicase production using various agro-industrial residues as carbon source by *Klebsiella quasipneumoniae* (AJS) under smF

Effect of sugars of silicase production. The effects of various sugars including maltose, xylose, starch, glucose, sucrose, and galactose on silicase production in the production medium were examined. Medium containing sucrose yielded the highest silicase activity (0.76 U/mL/min), followed by maltose (0.72 U/mL/min). In contrast, the lowest silicase activity was observed with galactose (0.28 U/mL/min). Xylose and starch resulted in comparable silicase activities (Fig. 5).

Nitrogen sources are vital components of production media. Various inorganic nitrogen sources including urea, potassium nitrate, ammonium chloride, sodium nitrate, ammonium sulfate, and ammonium nitrate were evaluated. Production medium with ammonium sulfate yielded the lowest silicase activity. Ammonium chloride resulted in high silicase activity, followed by sodium nitrate. Media containing ammonium nitrate, potassium nitrate, and urea showed favorable results for silicase activity (Fig. 6a). The highest silicase activity (0.83 U/mL/min) was obtained with urea. Activity levels with ammonium nitrate and potassium nitrate were 22.8% and 6.0% lower than with urea, respectively. Thus, urea was chosen as the inorganic nitrogen source for production media. For organic nitrogen sources, yeast extract, beef extract, and soybean meal were tested. Results for the three organic sources were comparable, but yeast extract yielded

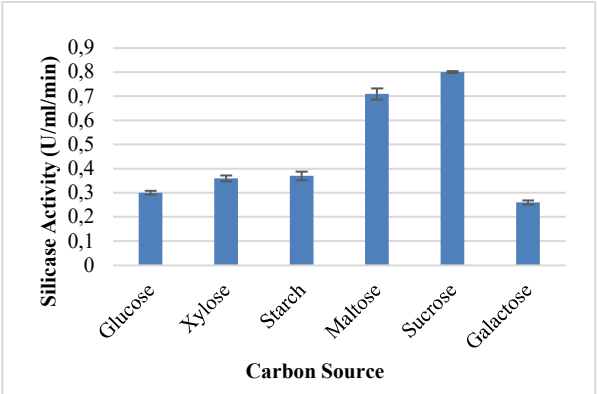

Figure 5. Effect of concentration of sugars source on silicase production by *Klebsiella quasipneumoniae* (AJS) under smF

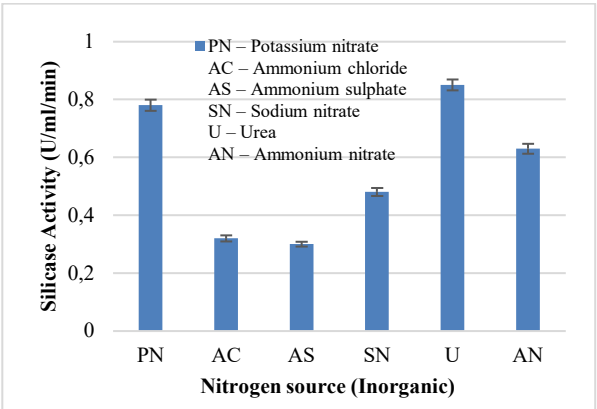

Figure 6a. Effect of inorganic nitrogen source (Inorganic) on silicase production by *Klebsiella quasipneumoniae* (AJS) under smF

DOI: 10.61215/AUOFB.2026.1.05

slightly higher silicase activity, leading to its selection as the organic component for production media (Fig. 6b).

Effect of metal ions on enzyme production. The effects of various metal ions including zinc dust, cadmium chloride, potassium chloride, sodium chloride, magnesium sulfate, and calcium chloride on silicase activity in production medium were assessed. The highest silicase activity (0.42 U/mL/min) was observed with potassium chloride, followed by zinc dust (0.39 U/mL/min) and cadmium chloride (0.38 U/mL/min). The lowest silicase activity was found with magnesium sulfate (0.24 U/mL/min) (Fig. 7).

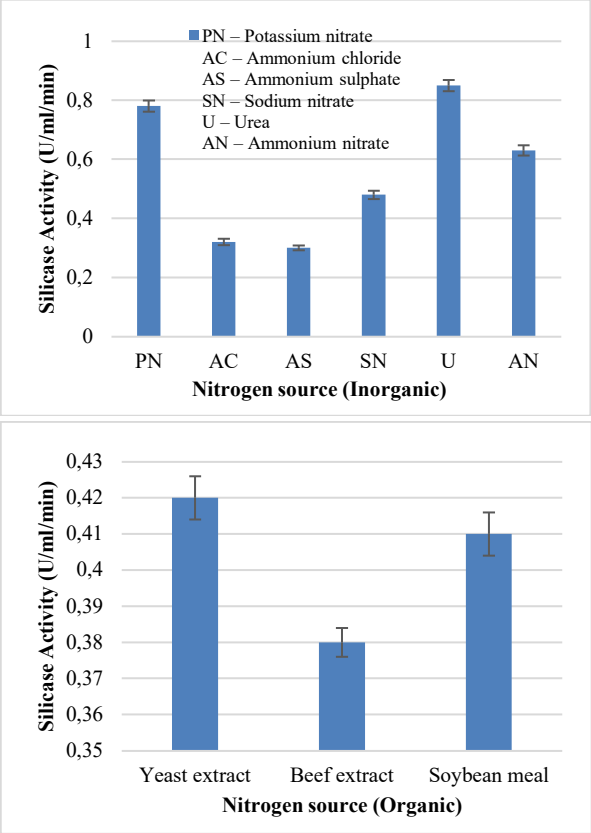

Figure 6b. Effect of organic nitrogen source (Organic) on silicase production by *Klebsiella quasipneumoniae* (AJS) under smF

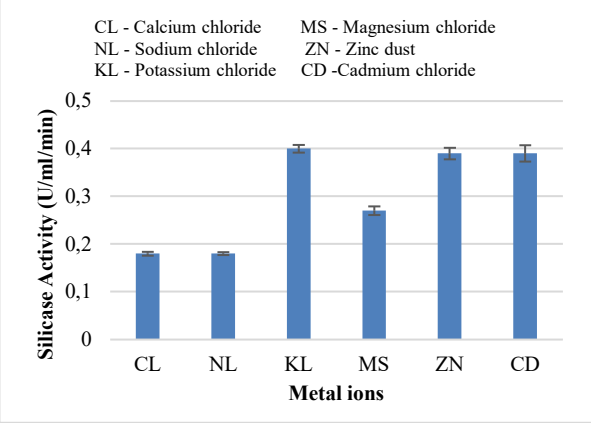

Figure 7. Effect of metal ions on silicase production by *Klebsiella quasipneumoniae* (AJS) under smF

Effect of additives on silicase production. The impact of additives like Triton X-100, EDTA, and Tween 80 on silicase production in the production medium was examined. Adding Triton X-100 to the production medium resulted in the highest silicase activity (0.84 U/mL/min), followed by Tween 80 (0.58 U/mL/min) and EDTA (0.56 U/mL/min). Although EDTA significantly enhanced silicase activity, it was 33.3% lower compared to Triton X-100 (Fig. 8).

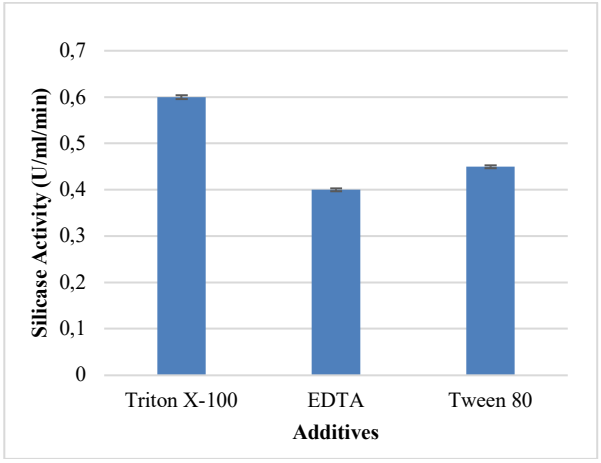

Figure 8. Effect of additives on silicase production by *Klebsiella quasipneumoniae* (AJS) under smF

Optimization of concentrations of different nutrients and additives components for silicase production. Following optimization, the selected parameters for silicase production were: inoculum age 24 h, inoculum size 1.0%, temperature 40°C, incubation time 4 days, and pH 7.0. These parameters were used to further optimize other factors. Urea and yeast extract were the most effective inorganic and organic nitrogen sources for enzyme production. Among tested substrates, rice straw yielded the highest silicase activity. Sucrose, KCl, and Triton X-100 were identified as the optimal sugar, metal ion, and additive. Concentrations of urea, yeast extract, substrate, sugar, metal ion, and additives were then optimized. Parameters like substrate, nitrogen sources, sugars, metal ions, and additives were optimized using concentrations of 0.25%, 0.5%, 1.0%, 1.5%, 2.0%, and 2.5%. Maximum silicase activity (0.86 U/mL/min) occurred with 1.0% rice straw in production medium. Optimal silicase activities of 0.87 U/mL/min and 0.44 U/mL/min were obtained with 0.5% urea and yeast extract. Activity levels of 0.42 U/mL/min and 0.85 U/mL/min were achieved with 1.0% KCl and 1.5% Triton X-100. Further increases or decreases in concentration of these factors led to reduced silicase activity (Table 6).

Summarized results for the final optimized parameters for silicase production are given in the Table 7.

Optimal conditions for silicase production by AJS were found to be a 24-hour-old inoculum at 1.0% level in production medium at 40°C with a 4-day incubation period. Urea and yeast extract were the most effective

DOI: 10.61215/AUOFB.2026.1.05

Table 6. Optimization of concentrations of different media components for silicase production

Parameter	Concentration (%)					
	0.25	0.5	1.0	1.5	2.0	2.5
Rice Straw	0.2113	0.4774	0.8612	0.4943	0.3145	0.2321
Sucrose	0.2143	0.23911	0.7541	0.5402	0.2691	0.2412
Urea	0.3128	0.8796	0.5483	0.4243	0.3142	0.2589
Yeast Extract	0.2031	0.4420	0.3398	0.2141	0.2021	0.1340
KCL	0.2346	0.4349	0.4291	0.2143	0.2143	0.2014
Triton X-100	0.3151	0.5880	0.8219	0.8541	0.6431	0.3051

Table 7. Optimized conditions of process parameters for silicase production

Parameter	Value (g/L)
Incubation time	4 d
Temperature	37 ºC
pH	7.0
Rice straw	10.0g
Sucrose	10.0g
Urea	5.0g
Yeast extract	5.0g
KCL	10.0g
Triton X-100	15.0mL

inorganic and organic nitrogen sources. Rice straw yielded the highest silicase activity among tested substrates. Sucrose, KCl, and Triton X-100 were determined to be the optimal sugar, metal ion, and additive for silicase production. Following media optimization, concentrations were further refined, leading to the development of Modified Horikoshi's medium. The optimized Modified Horikoshi's medium for silicase production consists of (per liter): rice straw 10.0 g, sucrose 10.0 g, urea 5.0 g, yeast extract 5.0 g, KCl 10.0 g, Triton X-100 15.0 mL, with an incubation time of 4 days, temperature of 37°C, and pH 7.0.

DISCUSSION

Optimized conditions for silicase production were determined, leading to the development of a Modified Horikoshi's medium. The findings highlight the potential of silicase for reducing silica content in rice straw, thereby enhancing its utility as a feedstock for various applications and as animal fodder.

The decrease in silicic acid concentration from initial higher levels may result from bacterial uptake of silicic acid for spore formation during the early stationary phase, as noted in a recent study [7]. Research indicates that approximately 10% of bacteria undergo biosilicification, with silica being utilized for spore coat formation in specific species like *Bacillus cereus* and related taxa. This contributes to the high resistance of spores in this genus. Additionally, a newly identified subclass of enzymes, iota CA, has been discovered in the genus *Burkholderia*, a common rhizobacteria [10]. Another enzyme subclass, gamma CA, has been termed silicase due to its role in silica dissolution when the recombinant gene is expressed in *E. coli*. Silica dissolution has also been associated with gluconic acid production, leading to the hypothesis that organic acids play a role in silica dissolution mechanisms. However, while a correlation between organic acid production and silica dissolution exists, transcriptomic analysis of *S. domuncula* suggests alternative interpretations.

Schröder *et al*. [21] discovered that silica dissolution in sponges is mediated by an enzyme with significant structural and functional homology to gamma carbonic anhydrase (CA), which they termed silicase. While silicases exhibit CA activity and CAs are widespread across all domains of life, not all CAs possess silicase activity. As shown in the study, despite the presence of three different CA subclasses in bacteria, not all isolates demonstrated silicase activity. This suggests that a specific CA has evolved to hydrolyze silica using a mechanism similar to that of CA.

Inoculum age and size were key factors affecting enzyme production. Similar findings indicate that higher inoculum ages can impact enzyme activities [16], potentially due to nutrient competition among the microbial population, as observed in *Bacillus coagulans* [1] and *Thermomucor indicae-seudaticae* [11].

Variations in enzyme production due to pH changes may result from optimal nutrient availability at specific pH levels [9]. Consequently, pH is a primary controlling factor among these variables [8, 28].

Substrate costs account for approximately 30-40% of industrial enzyme production expenses [4]. Selecting a low-cost substrate is therefore crucial for economical enzyme production. The type of growth substrate significantly impacted enzyme production. In this study, agro-wastes like rice straw, wheat straw, rice husk, sugarcane trash, bagasse, and sarkanda were tested. These agro-residues were cost-effective and readily available. In addition to agro-wastes, fly ash—a byproduct of thermal power plants—was also used as a substrate. For comparison, magnesium trisilicate, a silica source, was included in the evaluation. Results showed that all other waste materials had greater potential for silicase production compared to sugarcane trash. Consequently, rice straw was chosen as the optimal substrate for silicase production medium [12, 22].

For organic nitrogen sources, yeast extract yielded slightly higher silicase activity. It may be due to yeast extract's content of B-complex vitamins and the presence of essential amino acids in peptone [6]. Yeast extract has also been reported to enhance enzyme production in *Kluyveromyces cerevisiae* [2].

Minimal inhibition indicates the enzyme's potential for applications like flax retting and plant DNA extraction, where EDTA is used as a chelating agent [5, 19]. These additives likely increase cell membrane permeability, promoting enzyme secretion.

DOI: 10.61215/AUOFB.2026.1.05

In summary, this study establishes the assay of silicase-producing bacteria Klebsiella quasipneumoniae (AJS) under submerged fermentation. Production medium containing rice straw as a siliceous substrate proved optimal for silicase production. However, the high silica content in rice straw limits its use as a raw material at commercial scale for various applications. Thus, developing an effective method for silica reduction is a key challenge. This study demonstrates the potential of silicase for removing silica from rice straw, enabling its use as a valuable feedstock and suitable animal fodder.

Acknowledgments. The authors wish to express their sincere gratitude to HSCSIT, Haryana (HSCSIT/R&D/2022/152), for funding this research project. Our thanks also extend to Kurukshetra University for providing the necessary infrastructure that facilitated the successful completion of this study. The valuable contributions of the research team and collaborators, whose expertise and assistance were critical to this work's success, are greatly appreciated.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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Received: July 23, 2025
Accepted: January 20, 2026
Published Online: January 24, 2026
Analele Universităţii din Oradea, Fascicula Biologie
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433
Oradea University Press
https://www.bioresearch.ro/revistaen.html
https://doi.org/10.61215/auofb.2026.1.05

DOI: 10.61215/AUOFB.2026.1.05