ISOLATION, SCREENING AND CHARACTERIZATION OF SILICASE PRODUCING BACTERIA FROM PADDY SOIL

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Abstract. Rice crop has great affinity to silica and can accumulate approximately 10% of the element to its dry mass. The absorption of silica by paddy roots was possible just due to the conversion of silica into soluble forms with the services of soil micro-fauna. The conversion of silicates into silicic acid by producing enzyme (silicase) is the most appropriate route carried by microbes to dissolve silicates. The paddy soils are potentially rich in silica solubilising bacteria which can be isolated, screened and utilized for silicase production. Silicase enzyme vividly crucial for many industrial application and exploration of natural paddy sites for its production is an eco-friendly and sustainable approach. This present work aimed at isolating, screening and identifying the silicase producing bacteria from paddy soil that solubilize silicate into uptake form. To enhance the microbial population, the soil was artificially enriched with different materials and found that soil enriched with rice straw produced enormous microbial colonies. The screening of isolated colonies was performed on different media which depicted that the Glucose silicate assay plate media was perfect for the screening of silicase producing bacteria (SPB). The production of silicase was carried out and resulted in highest activity (1.45U/mL/min) of SPB with production media comprising bagasse as siliceous material. The physiological and biochemical testing of SPB was performed which indicated that the bacteria might be *Klebsiella.* The molecular identification report confirmed that the isolated SPB culture highly resembled with *Klebsiella quasipneumoniae*.

Key words: enzyme; microbiota; paddy soil; silicase producing bacteria; siliceous material.

INTRODUCTION

Microbes play crucial role in solubilising various minerals present in soil, such as silicates and phosphates [9]. Plethora of advantageous microbial communities has been well documented for harnessing the uptake of different nutrient by plants even during harsh condition [18]. The insoluble silicon which is the major constituent of soil and rocks is available to plants after silicate dissolution into solubilised form by microbes [1, 2]. Silica in rice straw, acts as a physical barrier; it has been shown that insects and fungi preferentially attack regions of plant that are low in silica [27]. The naturally occurring microorganisms that colonize in paddy can be potentially explored for hydrolysing the desirable polysaccharides and reducing silica within rice straw without using the harsh chemicals and affecting environment. The identification of microbial communities that can degrade silica may lead to establishment of low-cost method to pre-treat rice straw by removing silica prior to downstream processes. The pre-treatment of the rice straw by xylanase [4, 11, 12, 14] had been reported widely due to their significant role in extracting the benefits from rice straw. Despite of bountiful quantity of silicon in soil, its bioavailable form (mono-silicic acid) is very low because of its high reactivity with metals like aluminium and potassium. A number of bacterial and fungal isolates have been reported for the dissolution of silicates in aqueous solutions in laboratory and field experiments. The bacteria belonging to genus *Bacillus, Pseudomonas, Rhizobia, Enterobacter* and *Burkholderia* have been reported for dissolution of silicates into soluble form and to make them accessible for plant use [3]. The fungal species viz *Aspergillus niger, Trichoderma, Beauveria caledonica* and *Serpulahima ntioides* had also been investigated for their potential of solubilizing silicates [13, 26].

Silicate solubilising microbes can transform the non-bioavailable form of silicon in soil into plant absorbable form [5]. The inter-conversion of silica (SiO_2) into silicic acid is performed by an enzyme known as "silicase," which comprises a long chain of peptides in structure. The hydrolysis of both crystalline and amorphous (SiO_2) is executed by silicases generating free silicic acid as the end product of the reaction. Silicase exhibit similar activity and mode of reaction to the carbonic anhydrases. A study on differential mRNA transcripts of sea sponge, *Suberites domuncula*, presented that silicase and carbonic anhydrases (CAs) carry the similarities, in their active sites, activities and reaction rates that's why silicases has been classified as a class of the CAs [19]. Silicase is a distinct sub-class of CAs and all sub classes of CAs have not been reported to solubilise silica by producing silicic acid. In a study a prokaryotic extremophile, *Methanosarcina thermophila,* was successfully explored to convert silica into its solubilised form at room pressure and temperature [22]. Schröder et al. [20] reported the activity of silicase in two of the oldest Porifera classes named, Demospongiae and Hexactinellida. Kang et al. [7] found that the rhizobacterium *Burkholderia eburnean*

CS4-2 was capable of dissolving silica into silicic acid. Ikeda et al. [6] discovered that some bacterial species have not only involved in the conversion of silica into a soluble form but also incorporate silicic acid for some crucial metabolic activities such as encapsulation of spores by *Bacillus* sps. Ribeiro et al. [17] reported the different bacterial communities that convert silica into dissolving minerals by weathering the silicate rocks naturally releasing silicic acid.

Kang et al. [7] screened the silica solubilising bacteria *Burkholderia eburn*ea CS4-2 on silicate glucose media and observed a clear zone formation. For screening silicate solubilizing bacteria (SSB), the literature supported the use of various screening media, such as agar media containing sugars, siliceous sources such as magnesium tri-silicate, and naturally occurring silicate minerals [8, 25]. Screening of SSB in several media, including nutrient agar with beef extract, soil extract agar, glucose agar medium and Bunt and Rovira medium, etc. was mentioned by [24]. An efficient approach to screen silicase producing bacteria (SPB) and to evaluate its activity via silicate kit was presented by [8]. The quantification of silicate and phosphorus solubilisation through molybdenum blue method involves a common mechanism [15] which poses hurdles in finding a concrete silicate solubilising bacteria screening media.

As rice straw is rich in silica, the probability of finding silicase producing organisms is higher in the paddy fields soils; however, only a few reports are available so far for isolation and screening of the SPB from paddy fields. To enhance the microbial growth and to supply sufficient nutrients to the microbial communities, the soil was enriched with biofertilizer and urea. The biofertilizer used in the study was procured from local farmer. To address the issue, the paddy soil from four different zones of Haryana State were enriched using (i) Paddy straw (ii) Paddy straw + bio+fertilizer (iii) Paddy straw + urea (iv) Paddy straw + bio-fertilizer + urea to isolate bacteria. The effect of different enrichment on SSB was studied and a comparative study on bacterial activity and their potential for silicase production was conducted. The isolated bacterium giving the best hydrolysis zone on plate assay was used for silicase production and identified and characterized for reporting the results.

MATERIALS AND METHODS

Raw material, chemicals, glassware and plastic ware

Soil samples from four different sites (paddy fields) Hisar, Narwana, Bata and Kurukshetra, Haryana, India were collected. The first step of study was the enrichment of soil samples with rice straw, biofertilizer, urea and their combinations. The enriching materials were collected from a farmer in Kurukshetra. The chemicals like beef extract, nacl, peptone, agar, magnesium tri-silicate, silicon dioxide, tris base, DL-Dithiothreitol (DL_DTT), zinc sulphate and magnesium sulphate etc. for media preparation were of high purity analytical grade and were procured from Hi Media Laboratories Ltd., India., Sigma Chemicals Ltd., USA and Merck and Co. Inc., USA. For analytical studies and to determine the silicase activity, spectrophotometer (Analytik jena) was utilized. For biochemical testing different reagents (hydrogen peroxide, Christenen's medium, gelatin, methyl red, Barrets's reagents, tryptone, Kovac'sindole reagent and Voges-Proskauer solution, etc.) were used and also procured from Hi Media Laboratories Ltd., India.

Isolation of silicase producing bacteria

Sample preparation and isolation

The soil samples were given four types of enrichment with i) paddy soil (250g) ii) paddy straw (50g) + biofertilizer (6.5g) iii) paddy straw (50g) + urea (1.8g) iv) paddy straw (250g) + biofertilizer (6.5g) + urea (1.8g). Small pits were dug at nursery of Kurukshetra University, Kurukshetra and were filled with soil and enriching material. The soil was left there for 20 days and then one gram of soil sample from those pits was suspended in 100mL of tap water and kept on shaker for 48 hours at 37ºC,100µL aliquot was spread on nutrient-agar medium plates and incubated at 37ºC for bacterial growth to occur. The bacteria were re-streaked on fresh nutrient agar plates for isolation of pure culture and these pure isolates were further screened for silicase production.

Screening of silicase producing bacteria

Three different media-glucose silicate agar and glucose silicate agar supplemented with beef extract and nutrient broth (Table 1) were utilized to screen the silicolytic potential of isolated bacteria. The cultures were kept for 1 to 4 days to examine the capability of isolated bacteria to dissolve silicates into silicic acid.

Table 1. Composition of different screening media for silica solublization

Media	Components	Quantity (g)
Glucose silicate (g/mL)	Glucose	1.0
	Agar	1.5
	Magnesiumtri-silicate	0.25
Beef extract glucose silicate (g/mL)	Beef extract agar	3.3
	Glucose	1.0
	Magnesiumtri-silicate	0.25
Nutrient agar supplemented with magnesium tri-silicate (g/mL) and glucose	Glucose	1.0
	Yeast extract	0.3
	Peptone	0.5
	NaCl	0.5
	Agar	1.5
	Magnesium-tri-silicate	0.25

The bacterial colonies showing the clear zone were stored on nutrient agar slants, and the bacterial culture showing zone of the maximum diameter was further taken for silicase production.

Physiological and biochemical testing of the screened bacterial culture

The morphological and biochemical testing of the culture were carried out using standard procedures [21]. Different parameters like amylase, catalase,

gelatinase, motility, Gram staining, endospore staining and methyl red etc. were evaluated to analyse the characteristics of the bacterial culture.The citrate utilization test was conducted to assess the sodium citrate consumption as sole carbon source and $NH_4H_2PO_4$ as nitrogen source by bacterium. Likewise, the Indole production experiment showing the ability of bacterium to break down the tryptophan, an amino acid, was carried out. The isolated culture was inoculated on Tryptone Broth and incubated for 48 hours at 37°C. To the culture, few drops of Kovac's indole reagent were poured and colour appearance was observed. To confirm the ability of SPB for converting the sulfur-containing substances to sulphides as part of their metabolic activity, H_2S production test was carried out. The oxidase test was used to assess the ability of SPB to produce the cytochrome c oxidase enzyme. To check the catalase activity the screened bacterial isolate was kept in a test tube containing hydrogen peroxide (3%). The urease experiment was carried out with 24 hour old culture. The culture was streaked to slant surface comprising Christensen's medium and incubated at 37°C. A test tube containing nutrient gelatin was used and inoculated stab of 24 hour bacterial culture was placed into it. The hydrolization of gelatin was observed for at-least 1 week. An organism's ability to produce acetylmethyl carbinol from the fermentation of glucose was assessed using the Voges-Proskauer (VP) test. Acetylmethyl carbinol formed can be converted into di-acetyl by adding naphthol, strong alkali (40% KOH), in the presence of oxygen from air. The bacterial culture was inoculated in Methyl Red-Voges Proskauer solution and was incubated 35°C for 2 days. Then 2 mL of the incubated culture was poured in a new test tube to which few drops of methyl red indicator were added. The yellow colour of the culture solution showed negative response to the test. To the same above incubated culture Barritt's reagents were added and kept on shaking for 1 min. Then the solution was kept undisturbed for 1 h to find Voges-Proskauer positivity.

Silicase production using different agro-waste

The modified Horikoshi medium, containing both synthetic and natural siliceous substrates, was used to produce silicase. The production medium was prepared by using 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 di hydrogen phosphate and 0.01 g of magnesium sulphate in 100 mL of distilled water. Different siliceous sources like silicon dioxide, magnesium trisilicate, cereal straw (wheat and paddy), bagasse and rice husk were investigated for silicase production. The agro-residues (wheat straw, paddy straw and bagasse) were ground into fine powder in a Wiley mill (Thomas Scientific, model ¼ HP) at 1440 rpm after being chopped into 1-2 cm pieces. Before being used as a substrate for silicase synthesis, the fine powdered materials were sieved through mesh (size 18mm, make- Perfit). Rice husk was powdered in mixer grinder sieved through 0.297 mm mesh. The silicase production media with different substrates were autoclaved at 121ºC at 15l psi for 20 minutes. The production media were inoculated with 2% of 16–18 hours aged inoculum and incubated at 37°C for four days at 150 rpm. Fermented broth was centrifuged at 10,000 rpm for 20 min at 4°C to get the crude enzyme. The silicase assay was performed using Merck silicate assay kit (1.14794). Reaction was carried out using silicon dioxide as substrate which was dissolved in Tris buffer (pH 7.2) to give final concentration of 100 µg/mL. The detailed method to perform silicase assay is mentioned in previous studies [8]. Different studies on silicase have presented the functional correlation of organic acids and acid phosphatase during silica dissolution. These studies have shown the bacterial silica dissolution using plate assay but silicase activity and silicic acid concentration have not been reported so far which originated a wide research gap. Present analysis was made according to a standard Molybedosilicate method (Standard Methods: 4500-SiO2 C, by National Environmental Methods Index) in which ammonium molybdate reacts with silica to produce heteropoly acids. The oxalic acid added during the reaction destroy all other organic acids except molybedosilicic acid. The appearance and intensity of the yellow colour is proportion to the concentration of solubilized silica in the form of silicic acid. Silicic acid standard curve was plotted for silicic acid concentration ranging from 0 to 10µg in 5mL of testing volume. One silicase unit is defined as released silicic acid in µg/mL/min.

Identification of isolate using 16S rDNA based molecular method

A culture plate and slant named AJS was sent to the Eurofins Genomics India Pvt. Ltd., Karnataka, India for molecular identification of isolated bacteria. The experimental procedure, as per the company, included:

During experimental procedure, DNA was extracted from the microbial culture and its qualitative analysis was performed on Agarose Gel (0.1%) and the single band of high molecular weight DNAwas observed. The amplifications of section of 16S rDNA gene were performed using 27F and 1492R primers. A sole distinct PCR amplicon range of 1500 bp was examined which resolute on Agarose gel.The contamination of the PCR amplicon was eliminated prior to evaluation. Using frontward primer and inverse primers, the DNA sequence reaction of amplicon was conducted using kit (BDT v3.1 Cycle sequencing). The instrument, ABI 3730xl Genetic Analyzer was used during the process. The software aligner was used to generate the accord sequence of 16S rDNA gene The 16S rDNA gene sequencing was further utilized to carry out BLAST with NCBI gene bank database. On the basis of highest identity score, top ten sequences were carefully chosen and aligned using software Clustal W. Distance ground was spawned, which helped in constructing the phylogenetic tree using MEGA 7.

RESULTS

Isolation of SPB from paddy soils

The paddy soils were supplemented with rice straw/biofertilizers/urea and combination of these all to enrich the soil microbes for silica dissolution. The soil was collected after 20 days of enrichment and 43 microbial isolates were isolated and purified on nutrient agar medium. The isolated cultures were different in their morphological appearance and zone formation. The details related to the total number of isolated SSB with different enrichments is given in Table 2. The study found that enrichment with paddy straw was sufficient for isolating the SSB. No significant difference was observed on additionally adding the biofertilizer, urea and their combinations during soil enrichment. So, the experminets were further conducted only by enriching the soil with paddy straw.

These bacterial cultures maintained on nutrient agar were screened for silicase production. A clear zone of silica dissolution was formed by isolated bacterium on glucose silicate agar, as shown in figure 1 which showed the bacteria has utilized the silica source (magnesium trisilicate) from the substrate exhibited by a clear zone in the silicate medium. It depicted that the magnesium tri silicate was broken down by microbial attack and resulted into the release of water-soluble silicic acid. A clear zone of silica dissolution was formed by isolated bacterium on glucose silicate agar as shown in figure 1.

Screening of silicase producing micro-organisms

The isolated 43 cultures were further screened to evaluate their silica dissolution potential using different media, as mentioned in Table 3. Though, all the 43 isolates were carrying potential for silica dissolution yet 2 cultures showed high response which were further tested for getting the best silicase activity. After final screening bacterial culture named AJS was selected. The bacterial growth appeared on all the plate assay media after 24 h incubation. However, zone of silica dissolution by SPB was visualised on second day on all media. The clearest and the maximum zone of silica dissolution of 3 mm diameter was observed in glucose silicate agar while the minimum zone was observed on Beef extract glucose silicate agar as shown in table 2. Moreover, the zone of silica dissolution on nutrient glucose silicate agar was of 1.5 mm diameter. It was also observed that over the time, repeated sub culturing of bacterial isolate and repeated screening on glucose silicate agar resulted in induced feature in bacterium to exhibit dissolution zone as early as 16 h. This may correspond to the early dissolution of silica by SPB due to some genetic variation or drift induced during repeated sub culturing and screening on glucose silicate medium. The zone formation pattern was found to be different for different bacterial isolates due to various pathways followed by microbes for silica dissolution in an intricate manner.

Table 2. Numeric representation of isolated SSB from different soil sites with enrichment

	Paddy Straw		Paddy straw + Biofertilizer		Paddy straw + Urea		Paddy straw + Biofertilizer + Urea	
	Isolates	SSB	Isolates	SSB	Isolates	SSB	Isolates	SSB
Hisar	8	5	7	3	7	2	9	3
Kurukshetra	7	4	8	3	5	1	6	2
Bata	9	4	6	4	7	1	7	2
Narwana	8	3	4	2	6	2	5	2

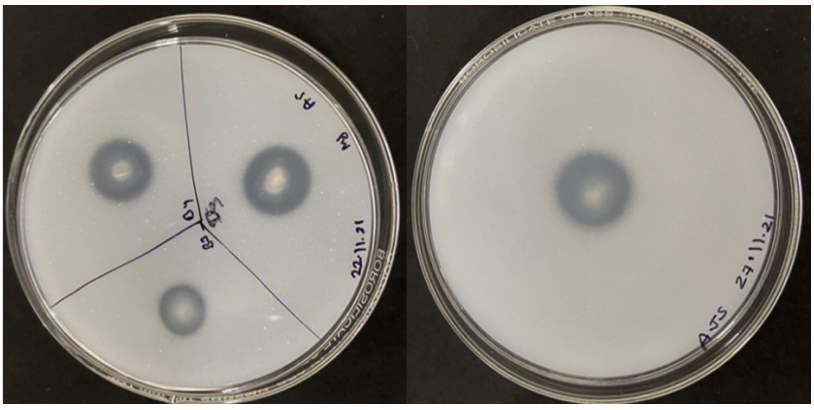

Figure 1. Clear zone formation by the isolated bacteria

Table 3. Screening media results of bacterial culture on different media

Media	Inoculum age (hour)	Growth	Zone	Diameter of zone observed on 2nd day
Silicate glucose agar media	18-22	+	++++	3mm
Beef extract glucose silicate media			+	1.0 mm
Nutrient agar glucose silicate media			++	1.5 mm

Silicase activity of isolated bacteria in production media

Crude silicase enzyme was obtained from the supernatant of centrifuged production media. The silica in its soluble form as silicic acid was evaluated before and after incubation of inoculated production media and is given in Table 4. The silica in its soluble form as silicic acid was evaluated before and after incubation of inoculated production media. The production media was supplemented with different siliceous substances to enhance the activity and to find the suitable substrate. The silicic acid content was calculated to be good in all substrates as shown in Table 3. It was observed that isolated bacterium was highly efficient in producing silicase with production media containing rice straw and rice husk. With both the substrates 0.88U/mL/min and 0.52U/mL/min of silicase activity was detected (table 3). The silicase production was found to be less with production media containing wheat straw and bagasse as a source of silica. The rice straw which is rich in silica showed high potential for silicase production i.e. 0.88 U/mL/min.

Furthermore, the production media containing magnesium trisilicate and silicon dioxide also resulted in significant silicase activity.

Preliminary morphological and biochemical characterisation of SPB

The screened bacterial isolate was non-motile and produced very large, round, mucoid, opaque and confluent colonies. The colonies were whitish cream in colour and showed convex mucoid appearance. The polysaccharide capsules were formed by the bacterium. The gram staining of bacterium showed that it was gram negative. The bacterium was found to be efficient at fermenting sugars and produced acid and gas. Further, it was found to be catalase and amylase positive but showed negative results for oxidase and gelatinase test. The production of bubbles during peroxide addition confirmed it as catalase +ve. The detailed observations related to biochemical characterization are given in Table 5.

The hydrolization of gelatin was observed for at-least 1 week but no change was observed. The bacterium was observed to be methyl red negative, Voges-Proskauer and citrate positive. The microbe used glucose and citrate agar, conversely, it did not perform for hydrogen sulphide and indole test. To the culture, few drops of Kovac's indole reagent were poured and appearance of yellow colour on reagent layer showed negative activity for indole test.

Citrate agar medium was prepared in a test tube slant and to this bacterial culture was inoculated. On incubating the test tube at 37°C for 1day, blue colour was observed. The detailed results of the biochemical testing are given in Table 4. The tests results on relating the literature indicated that the screened SSB may belong to the genera *Klebsiella* and for confirmation, the culture was sent to Eurofins Genomics Pvt. Ltd., Karnataka, for 16S rDNA cataloguing.

Molecular identification of SPB

On the basis of results obtained during isolation and screening related to highest zone of hydrolysis and highest activity for silicase production, the selected microbial culture was analysed microscopically. The identification and characterization were performed on the basis of similar nucleotide appearance and genetic analysis of the bacterium. The report on 16S rDNA gene amplification was prepared by Eurofins Genomics Pvt. Ltd., Karnataka, which showed high resemblance of the culture with *Klebsiella quasipneumoniae* and confirmed it.

The nucleotide chain similarity can be measured by creating a diagram (phylogenetic tree) that represents the relatedness and estimates the evolutionary spaces among different sequences [16]. The complete report is attached as supplementary data with the manuscript.

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. Biochemical characteristics of the screened SPB

Test Name	Results
Gram Staining	-ve
Endospore Staining	-ve
Catalase Test	+ve
Amylase Test	+ve
Gelatinase Test	-ve
Motility Test	-ve
Methyl red Test	-ve
Voges – Proskauer Test	+ve
Citrate Agar Utilization Test	+ve
H_2S Test	-ve
Indole Test	-ve
Oxidase Test	-ve

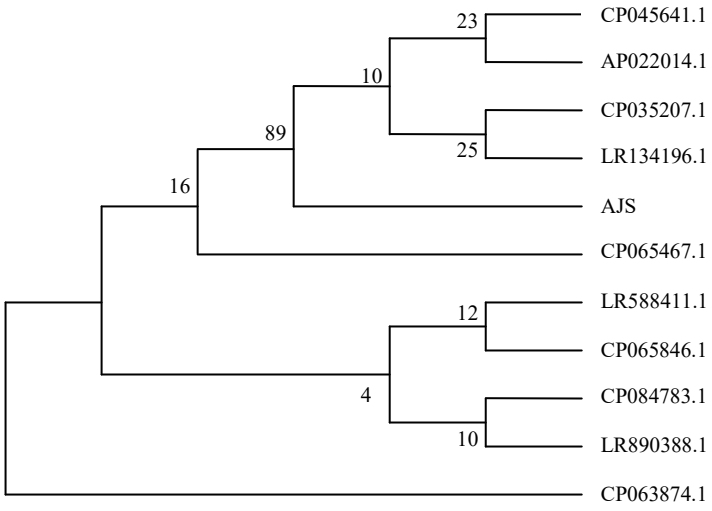

Figure 2. Molecular Phylogenetic tree based on Maximum Likelihood method (AJS)

DISCUSSION

In our study, 43 SPB microbial isolates with the maximum and the clearest zone were isolated from paddy soil. A similar study [8] also reported the isolation and screening of silicolytic bacteria from different soil samples. The isolates were subjected to screening which resulted in hallow zone formation on a medium containing siliceous substrate and glucose. The SPB gave positive results on all types of screening media but the clearest response was observed in silicate glucose agar media. Different studies revealed that SPB solubilise the amorphous silica into monosilicic acid which is indicated by a hallow zone formulation on a siliceous medium [7-9, 25] which appeared in present study also. The study concluded that the isolation of SPB from paddy soil and silicase production from these SPB is a novel, cost effective and an environmental friendly route. Ikeda et al [6] found that approximately ten per cent of soil bacteria required silicic acid for their growth these bacteria utilize the silicic acid for their structural formulation in early static phase and spore production. The enzyme produced during present study followed the similar mechanism usually shown by carbonic anhydrase which was in strong agreement with the studies [20, 22]. This depicted that silicase belongs to the gamma subclass of carbonic anhydrase and shows morphological and behavioural homology to carbonic anhydrase. This further ensures that a particular subclass of carbonic anhydrase is involved in dissolution of silica in a reaction similar to CO_2 conversion to carbonic acid by carbonic anhydrases. The dissolution of silica into silicic acid is also associated with release of organic acids that contributes to the silicase production mechanism [22]. The release of organic acids in present study was similar to the mechanism of carbonic anhydrase which supports the results. Moreover, out of all isolates, only specific were showing the potential to solubilize silica which evident that only a few carbonic anhydrase show the silicase activity. Apart from it, silica dissolution is a tough task and is usually done at high temperature using strong acid. In soil, the dissolution of silica into solubilised form can be possible just due to enzymatic action. The dissolution of silica into silicic acid is also associated with release of organic acids that contributes to the silicase production mechanism [22]. However, the mechanism is yet to be established and it may be hypothesized that evolution may have directed ubiquitous carbonic anhydrases for silica dissolution and forming a distinct sub-class with silicase activity. Further, the silicase activity was analysed and found to be high on using agrowastes such as rice straw and husk in production media. This is in consent with the earlier study that presented the potential of ligno-cellulosic agro-wastes as substrate for silicase production [8]. On the basis of morphological and biochemical characterization, it was concluded that isolated bacteria belongs to *Klebsiella* which was further confirmed by molecular characterization of the culture. The isolated SPB was *Klebsiella quasipneumoniae*. The study presented a novel way to produce silicase enzyme which have high potential to convert silicates into their solubilised forms. The study will be fruitful to disintegrate or manage the wastes containing silicate components such as agro-wastes (rice straw), electronic wastes (microchips), glass and ceramics using a biological and environmental friendly fashion. The application of the microbial silicases to manage the silica containing wastes will be the part of future studies.

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