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RESEARCH ARTICLE

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Characterization and optimization of glutaminase enzyme from *Bacillus albus* isolated from agriculture waste

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ABSTRACT: Glutaminase is an enzyme that catalyzes the hydrolysis of glutamine to ammonia and glutamate. Although it can be isolated from bacteria, fungi, plants, and animals, microorganisms remain the preferred source due to their biochemical diversity and ease of cultivation. In this study, 34 soil samples were collected from agricultural waste areas in district Kohat to isolate glutaminase-producing bacteria. Primary screening using phenol red indicator and subsequent biochemical characterization identified 10 positive isolates (30% positivity rate). Among these, four representative strains (A1, G2, P3, and 1R) were selected for detailed analysis, with the majority belonging to the *Bacillus* genus. Optimal enzyme activity was observed at neutral pH (7.0) and 37°C. SDS-PAGE (12%) analysis was used to estimate the molecular weight of the purified glutaminase. 16S rRNA gene sequencing identified isolate A1 as *Bacillus albus*, isolates G2 and P3 as closely related to *Bacillus anthracis*, and isolate 1R as *Alkalibacillus bogoriensis*. Phylogenetic analysis revealed that *Alkalibacillus bogoriensis* (1R) clustered distinctly from other Bacillus species. Among all isolates, *Bacillus albus* demonstrated the highest glutaminase activity, showing good pH and temperature stability. These findings suggest that the isolated strains, particularly *Bacillus albus*, have potential for future pilot-scale studies and industrial applications in glutaminase production.

KEYWORDS: Glutaminase enzyme, Optimization, *Bacillus albus*, Agriculture waste, SDS-PAGE

INTRODUCTION

Glutaminase is an amino hydrolase enzyme that catalyses the conversion of glutamine to glutamate and ammonia [1]. This enzyme plays a crucial role in nitrogen metabolism in both prokaryotes and eukaryotes and has garnered attention for its potential applications in the pharmaceutical and food industries [2]. The demand for the glutaminase enzyme is very high in industries. The significance of glutaminase extends beyond its applications. Its role as a biosensor to measure glutamine levels showcases its versatility and importance across various fields [3]. Glutaminase enhances the flavour of fermented foods and exhibits anti-cancer properties, making it valuable in both sectors [4, 5]. Cancer cells utilize glutamine for their energy needs and growth [6], more avidly than normal cells, making glutaminase an effective anti-cancer agent by depleting glutamine from cancerous cells [7, 8, 9].

Microorganisms remain a good choice due to their diverse biochemistry and ease of handling although enzymes are sometimes isolated from plant and animal tissues [10, 11]. This enzyme is abundant in bacteria, actinomycetes, yeast, and fungi, which makes microorganisms a primary source for industrial production of glutaminase due to their efficient and economical production methods [12, 10, 13].

Thus, Soil microbes, known for their characteristics and ability to exhibit high enzyme activity and stability are being explored as a promising alternative, for the industrial synthesis of glutaminase [14]. The majority of the glutaminase-producing microorganisms have been isolated from soil, with the exception of a small number that have been found in aquatic and marine environments [15, 16].

It has been documented that soil Pseudomonas species, *Vibrio costicola species*, and *Micrococcus species* all produce extracellular glutaminase [17]. Extracellular glutaminase-producing bacteria were identified by Gomaa *et al.* [18] from seawater and marine sediments in Cochin settings, and they noted that their numbers were on the order of 105 cells/per ml or gm. additionally, glutaminase-producing *Pseudomonas, Vibrio, Bacillus, Moraxella, Aeromonas*, and *Acinetobacter* species were discovered.

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However, there is research on isolating bacteria that produce glutaminase from soil contaminated by agricultural residue, which could offer ingenious and efficient strains. Understanding the characteristics and identification of these bacteria is crucial in grasping how they synthesize glutaminase once isolated. The molecular characterization process involves analyzing the metabolic mechanisms in which enzyme is synthesised. These bacterial strains are typically characterized through methods such as genetic sequencing and biochemical tests. For the production of glutaminase especially from marine bacteria, submerged fermentation (SmF) is recommended [19, 20]. Achieving high volume production and enhanced enzyme yields rely on manufacturing processes and strain optimization strategies, including statistical optimization and adaptive mutation [21, 22].

Researchers are actively seeking out microbial strains capable of producing glutaminase due to the enzyme's diverse applications. Despite the potential of glutaminase in both industry and medicine there remains limited knowledge about identifying and characterizing glutaminase producing bacteria in soil contaminated by agricultural residue.

The current research aimed to isolate and characterize bacteria capable of producing the enzyme glutaminase from soil polluted by agricultural waste. The study objectives included isolating the enzyme from selected strains evaluating its activity under different pH and temperature conditions and optimizing the synthesis conditions, for glutaminase production.

This research study greatly enhances our knowledge of bacteria that produce glutaminase in soil contaminated by agricultural waste. It highlights *Bacillus albus* as a strong enzyme producer, performing best at neutral pH and 30 ºC. This discovery opens up possibilities for large-scale production and potential industrial and medical uses of glutaminase enzymes.

MATERIALS AND METHODS

Sample collection and isolation

In order to isolate potential glutaminase-generating bacteria, loamy soil samples were taken from agricultural locations in District Kohat, Khyber Pakhtunkhwa, Pakistan, and sent to the laboratory of microbiology at Kohat University of Science and Technology. All samples were stored in refrigerator in an aseptic environment at 4 °C for further research. A typical serial dilution plating approach was employed to isolate glutaminase producing bacteria as reported by Saleem & Ahmed [11].

Screening of bacteria

Standard microbiological procedures were utilized to isolate powerful glutaminase generating bacteria from soil samples, where phenol red was used as glutaminase activity indicator.

Characterization and Identification

The chosen bacterial isolates were identified using the conventional approach based on morphological (i.e., Gram staining and spore staining) and biochemical tests, such as catalase, oxidase and citrate test [23, 24, 25].

Partial purification and Protein size confirmation of enzymes through SDS-PAGE

Ammonium sulphate technique was used to purify the crude enzyme. While liquid medium culture was used to partially purify isolates that produce glutaminase as reported by Jozala *et al.* [26]. Then, SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) was used to visualize and estimate the protein size of the purified enzyme. On a 12% SDS-PAGE under a reducing environment, the Laemmli method was used to evaluate the purity and molecular weight of partially purified glutaminase as followed by Kumar *et al.* [27].

Optimization of Culture Conditions for Glutaminase Production

The effect of temperature and pH on glutaminase-producing bacteria was determined by incubating the bacteria at varying temperatures (20^oC–50^oC) and pH levels (between 4 and 11). The bacterial isolates were grown in broth and incubated for 24 hours at varying temperatures and pH levels. At 540 nm wavelength, the turbidity of a bacterial culture was evaluated with a spectrophotometer.

DNA extraction for molecular identification

DNA was extracted by following the protocol of Johar *et al* [28]. To isolate the nucleic acids from a bacterial culture, we inoculated 5 ml of liquid culture with the strain of interest and grew it until saturated. Then, we centrifuged 1.5 ml of the culture, discarded the supernatant, and resuspended the pellet in 567 µl TE buffer. We added 30 µl 10% SDS, 3 µl 20 mg/ml proteinase K, and incubated for 1 hour at 37°C. Next, we mixed 100 µl of 5 M NaCl, followed by 80 µl CTAB/NaCl solution, and incubated for 10 minutes at 65°C. After that, we extracted with chloroform/isoamyl alcohol, removed the supernatant, and added phenol/chloroform/isoamyl alcohol. Then, we precipitated nucleic acids with isopropanol, washed with ethanol, dried, and redissolved the pellet in 100 µl TE buffer for further processing. This method efficiently isolated nucleic acids while removing unwanted contaminants.

DNA confirmation and 16srRNA amplification

A 1500 base pair segment of the 16S rRNA gene using primers specifically the 27F forward primer (5 AGAGTTTGATCMTGGCTCAG 3) and the 1492 R reverse primer (5 TACGGYTACC TTGTTACGACTT 3) was identified. The PCR process took place in a volume of 25 µL containing various components, like PCR buffer, dNTPs, MgSO4, Taq DNA Polymerase, forward and reverse primers, bacterial template DNA and double distilled H_2O. Over a period of 1 hour and 40 minutes we conducted a total of 30 DNA amplifications using specific temperature cycles. After incubating at 72°C for a minute and then allowing it to rest for 10 minutes at the same temperature. Then we employed a commercially available technique to sequence the amplified product of the 16S rDNA. The order was discovered using nucleotide BLAST on the NCBI website. Sequences aligned in Fasta were used to create a phylogenetic tree through the neighbour joining method. The strains evolutionary history was investigated using Mega software giving insights [11].

Phylogenetic Analysis

Using the MEGA 7 software, a phylogenetic tree was built for the selected isolates based on the sequences of the 16S rRNA gene. Phylogenetic analysis indicated the evolutionary connection of the selected strains.

Statistical Analysis

The data was arranged and was analysed through Statistix version 9 by applying simple T test and ONE WAY ANOVA on different pH and Temperature which were changed during the experimental work. The p value <0.05 was considered statistically significant.

RESULTS

Primary screening of glutaminase producing bacteria

The rapid plate assay technique was used, in which the phenol red was used as a pH indicator, for the first screening of glutaminase producing bacteria. Soil samples were put into the media, and colonies that turned pink around their edges were picked, sub-cultured, and then tested again for enzyme production on each strain. After being cleansed, 10 bacterial colonies out of 34 samples produced positive findings, turning the yellow medium pink, shown in (Figure 1). Among these 10 positive samples, a total four types of bacteria namely A1, G2, P3, and 1R were identified, which were mostly *Bacillus* species. After the plate rapid assay, the same results were seen when the strains were tested in liquid media.

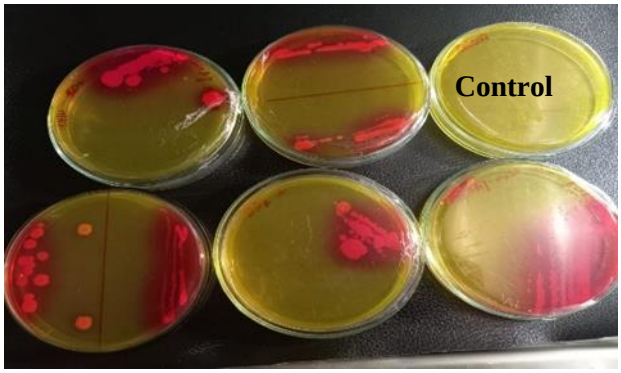

Figure 1. Pink colour showed the bacteria having potential to produce glutaminase enzyme.

Microscopic and morphological identification of glutaminase producing bacteria

The cultural characteristics provide valuable information for the identification and classification of bacterial isolates based on their growth patterns and appearance on culture media. These characteristics include size, shape, margins, elevation, consistency, opacity, and pigmentation. Isolate A1 and G2 have dome-like elevation, moist consistency, opacity, and lack of pigmentation. However, A1 has medium-sized, rhizoid, lobated, and irregular serrated margins, while G2 is smaller, circular with entire margins. While G2 and P3 isolates share circular shape, entire margins, dome-like elevation, moist consistency, opacity, and lack of pigmentation. They differ in size, with G2 being smaller and P3 larger. 1R isolate is unique in its large size, filamentous, filiform shape, and flat elevation but shares the moist consistency, opacity, and lack of pigmentation with the other isolates.

Biochemical identification

The biochemical results provide valuable information of each isolate, helping in their identification and classification based on their metabolic capabilities. The results of biochemical tests conducted on four bacterial isolates: A1, G2, P3, and 1R were as follow., all isolates tested negative for oxidase activity, indicating the absence of certain enzyme systems. However, they all displayed positive catalase activity, indicating the presence of catalase enzymes that break down hydrogen peroxide. In terms of citrate utilization, all isolates tested negative, suggesting their inability to use citrate as a sole carbon source. Indole production varied among the isolates, with only A1 demonstrating a positive result, indicating the production of indole from tryptophan. All isolates showed positive results for glucose fermentation, indicating their ability to utilize glucose as an energy source. Furthermore, all isolates tested positive for urease activity, indicating the presence of urease enzymes capable of hydrolyzing urea.

Partial purification of glutaminase enzyme and SDS Page

On 12 percent SDS-PAGE, the molecular weight and enzyme pattern of partly purified glutaminase were observed. Each sample band on the gel was examined on its own line, and the results were compared to a standard protein marker, shown in the (Figure 2) below. The sizes of AI, G2, P3 and 1R protein were 33 KDa, 37KDa, 34 KDa and 38 KDa respectively.

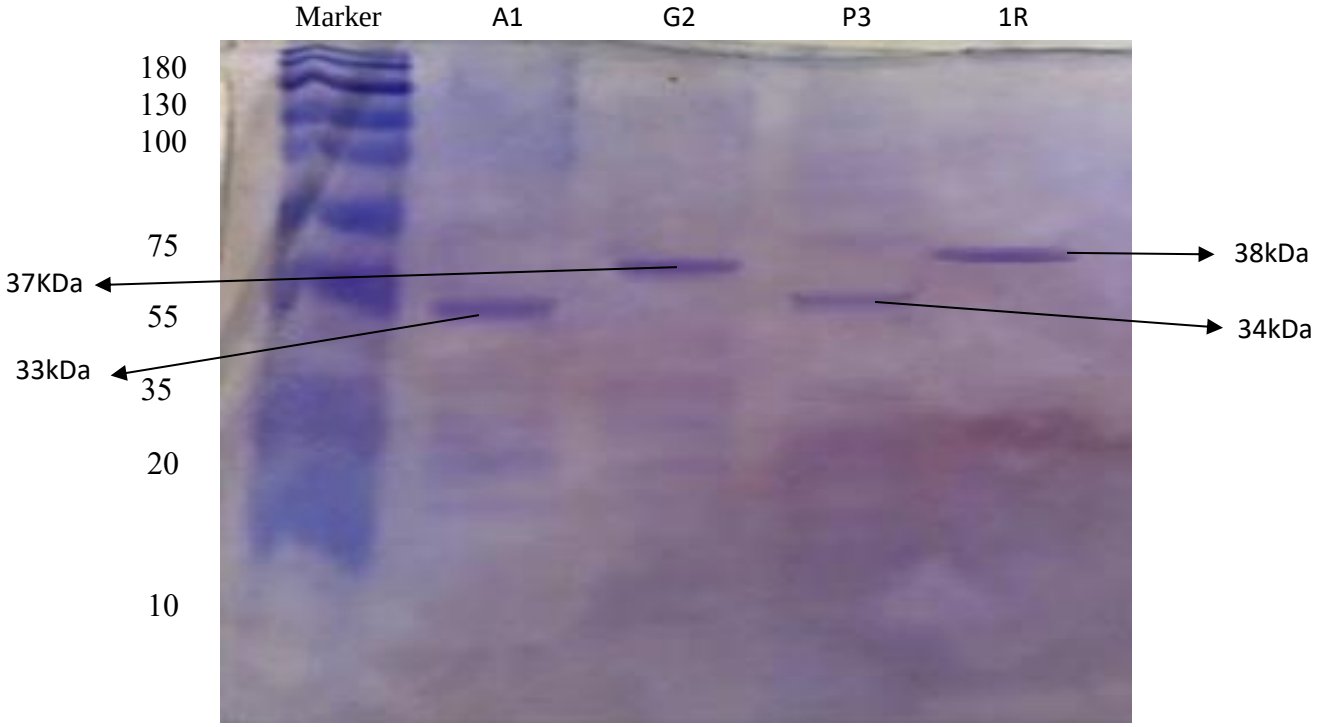

Figure 2: Protein confirmation on the basis of molecular weight using 12 % SDS-PAGE gel electrophoresis.

Optimization of pH for the production of glutaminase

The (Figure 3) presents enzyme activity data at various pH levels for four different bacterial isolates: AI, G2, P3, and IR. Our findings indicate that enzyme activity varies among the isolates tested and is influenced by pH conditions. The IR isolate appears to be particularly efficient at pH 9, while the other isolates demonstrate their highest activity at pH of 8.

Comparing the values of all these isolates, it's notable that the IR isolate exhibited the highest enzyme activity at pH 9, with a value of 0.65 OD. The P3 isolate also demonstrated significant enzyme activity at pH 8, although slightly lower at 0.61 OD. Meanwhile, the G2 isolate exhibited its highest activity at pH 8, with a value of 0.68 OD, and the AI isolate displayed its maximum activity at pH 8 with a value of 0.54 OD. From the above perspective, almost all the selected isolates show highest glutaminase activity at alkaline pH 8. Surprisingly, from highest to lowest activity comparison, it is clear from (Figure 3) that G2 isolate displayed highest activity at pH 8 and lowest activity at pH 4 amongst all selected 4 isolates.

Optimization of temperature for production of glutaminase

The (Figure 4) details the optimization of temperature for enzyme activity, measured by spectrophotometer, across four different samples: A1, G2, 1R, and P3. Comparing the values among these samples, it can be clearly seen that Sample A1 displayed its maximum activity at a slightly lower temperature, 30ºC, with a value of 0.79 OD. G2 exhibited the highest enzyme activity at 40ºC, with a value of 0.62 OD. The P3 sample demonstrated significant enzyme activity at 40ºC, albeit slightly lower at 0.78 OD. Meanwhile, sample 1R exhibited its highest activity at the highest temperature tested, 45ºC, with a value of 0.71 OD. Considering the temperature conditions, it's noteworthy that G2 and P3 displayed optimal enzyme activity at 40ºC, while A1 showed its maximum activity at a lower temperature of 30ºC. Sample 1R exhibited its highest activity at the highest temperature tested, 45ºC.

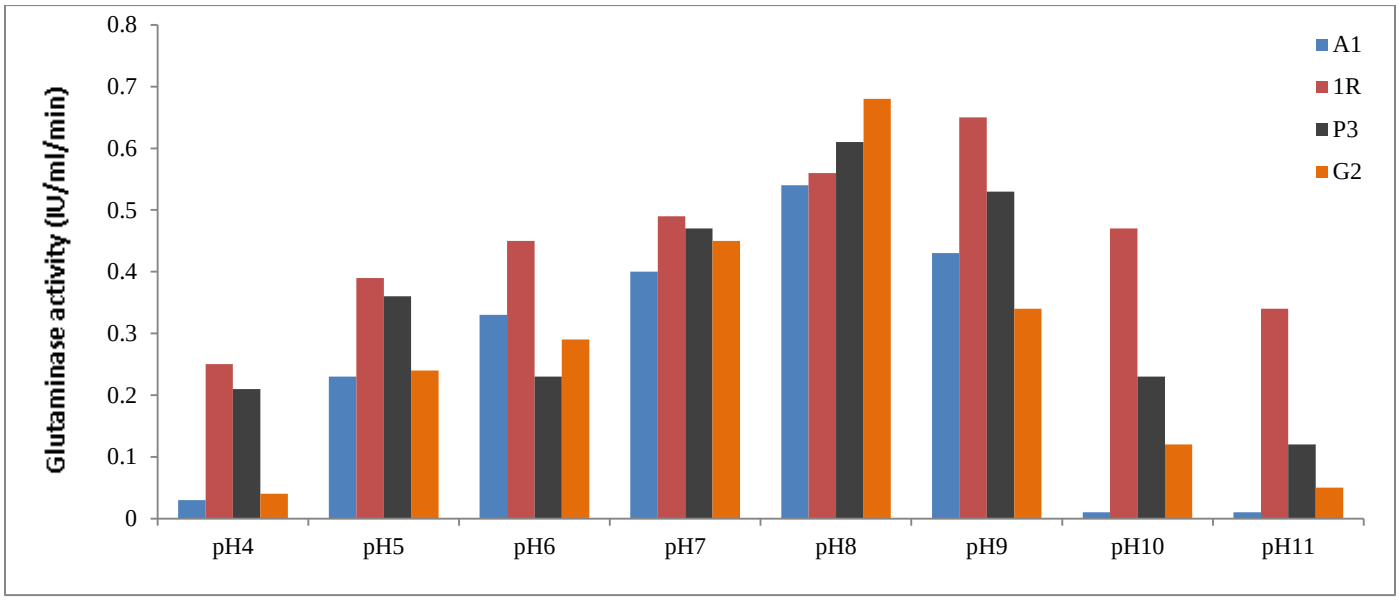

Figure 3. Effect of pH on production of glutaminase by selected isolates. (On X axis the pH ranges and Y axis the value of enzyme production)

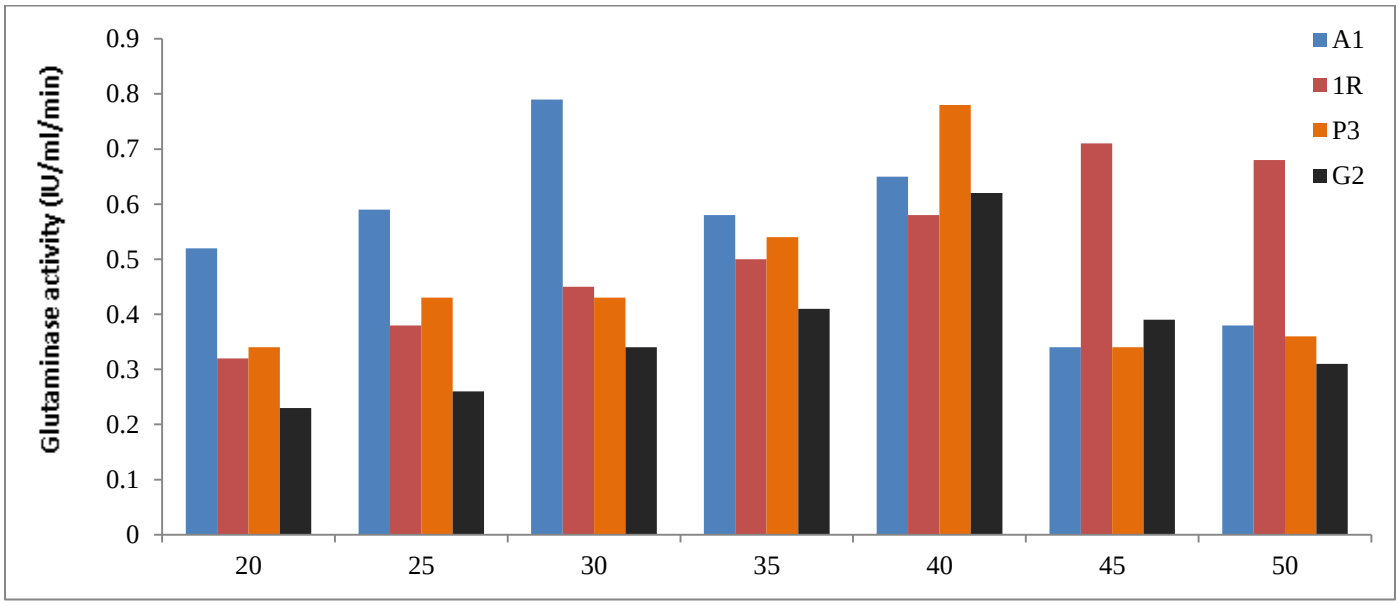

Figure 4. Effect of temperature on production of glutaminase by selected isolates. (On X axis the temperatures ranges and Y axis the value of enzyme production).

Molecular identification

The Figure 5 indicates the molecular identification process of the selected bacterial isolates, focusing on the confirmation of glutaminase production through the amplification of the 16S rRNA gene via PCR. The resulting PCR products were visualized on a 2% agarose gel, alongside a 1000bp ladder for size comparison. In Figure 4, the gel image displays distinct bands corresponding to PCR products of approximately 965bp, indicative of successful amplification of the targeted gene fragment. This confirms the presence of the desired genetic material in the isolated DNA of the bacterial isolates. The ladder serves as a reference for estimating the size of the PCR products, ensuring accuracy in identifying the amplified fragments.

Phylogenetic Analysis

The phylogenetic analysis designed to explain the evolutionary relationships among the isolates. According to

the phylogenetic analysis, strains A1 and G2 were identified as belonging to specific bacterial species. The identification was apparently based on the clustering of their 16S rRNA gene sequences with reference sequences of known bacterial species. G2 and P3 (Figures 7 and 9, respectively), both identified as *Bacillus anthracis*, show significant similarity in colony morphology (circular shape, entire margins, dome-like elevation). A1, identified as *Bacillus albus* (Figure.6), shares some morphological characteristics with G2 and P3 (dome-like elevation, moist consistency, opacity, and lack of pigmentation) but differs in size, shape, and margin details. 1R, identified as *Alkilobacillus bogonensis* (Figure 8), exhibits unique morphological characteristics (filamentous, filiform shape, flat elevation) distinct from the other isolates. The similarity search results for all the four isolate by 16S rRNA gene sequences are presented in Tables 1- 4.

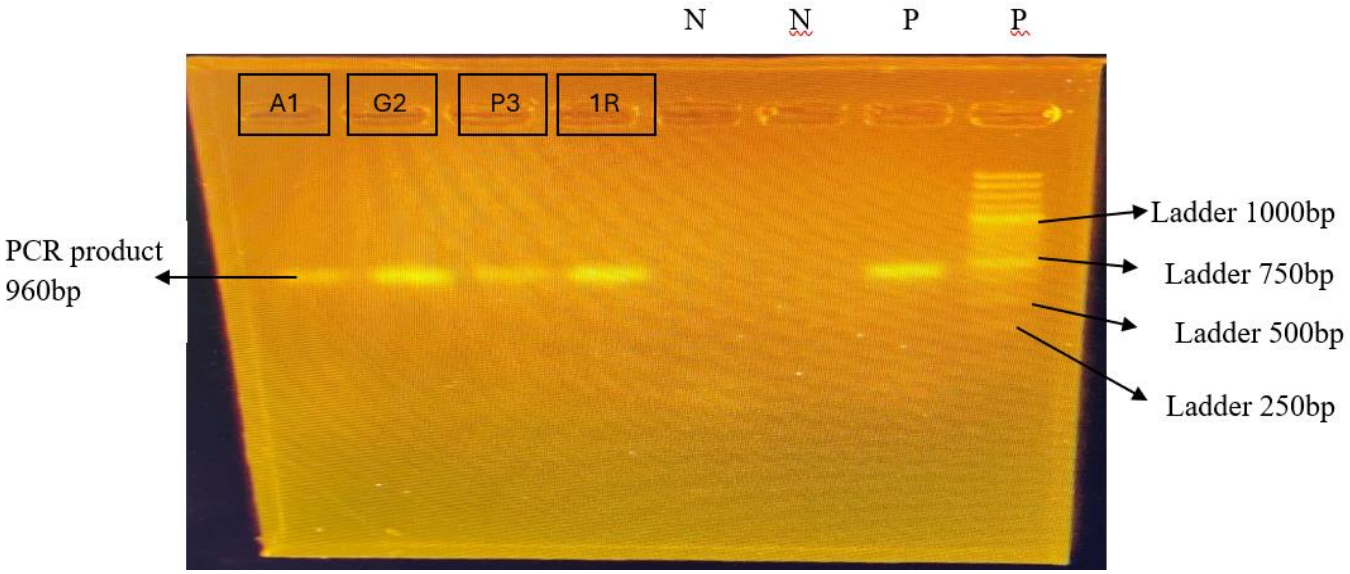

Figure 5. Confirmation of PCR products (selected isolates) using 2 % agarose gel. N stands for negative, and P stands for positive.

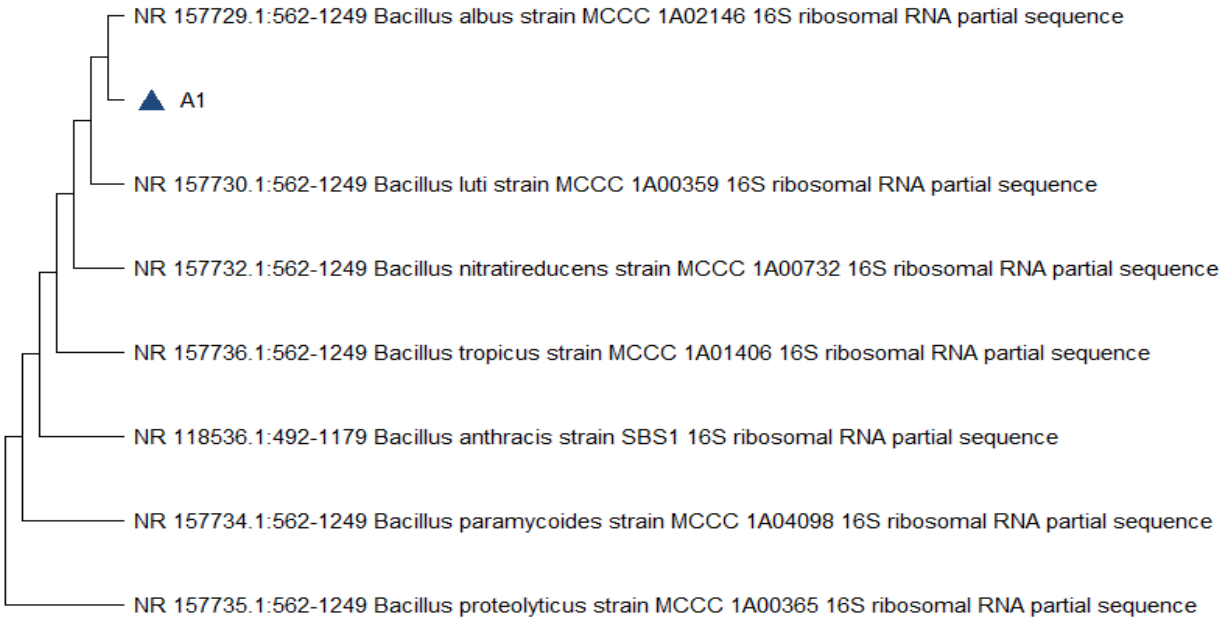

Figure 6. Phylogenetic tree of A1 isolate is similar to *Bacillus albus*

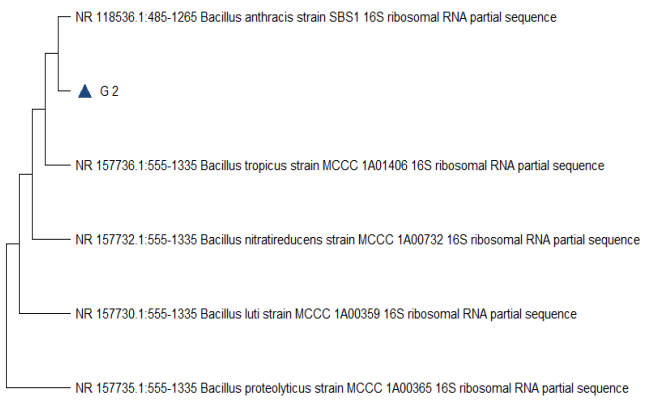

Figure 7. Phylogenetic tree of G2 isolate is similar to *Bacillus anthracis*

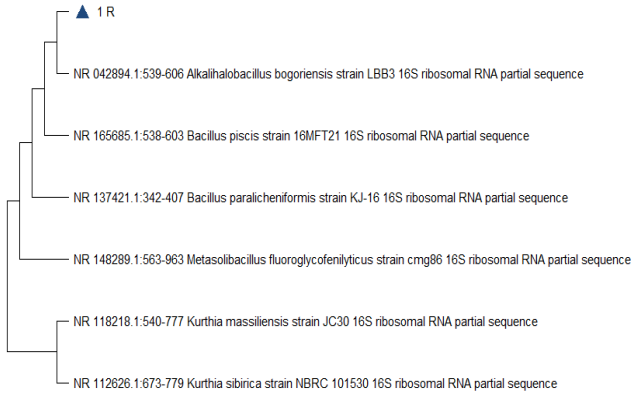

Figure 8. Phylogenetic tree of IR isolate is similar to *alkilobacullus youbogonensis*

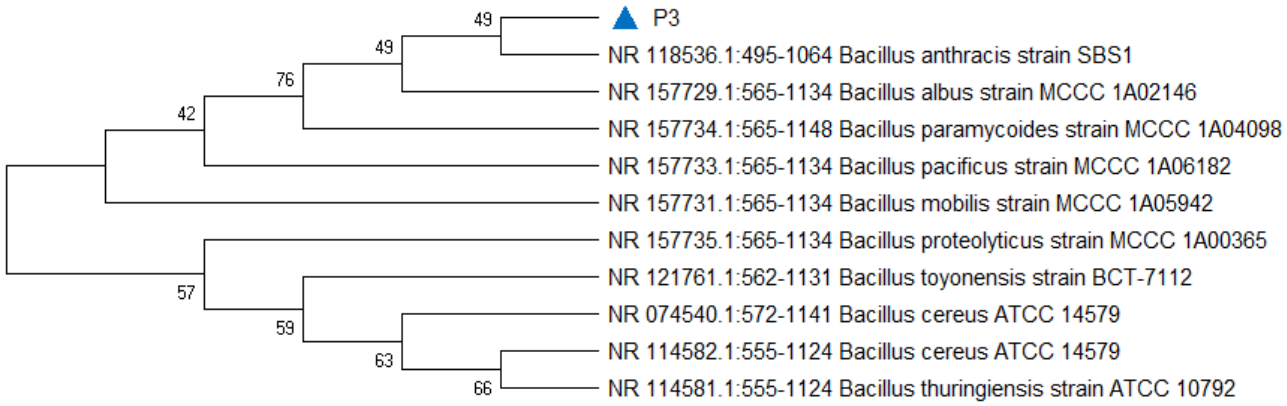

Figure 9. Phylogenetic tree of P3 isolate is similar to *Bacillus anthracis.*

DISCUSSION

The glutaminase is an enzyme that catalyses the hydrolytic degradation of glutamine and ammonia. Glutaminase enzyme has been shown to have anticancer properties, as well as being used as a flavour enhancer, an antioxidant, and a biosensor to differentiate between glutamine and glutamate. The glutaminase enzyme isolates from bacteria, fungi, plant and animal sources. Microorganism remains the best choice due to stability and ease of culturing.

The primary goal of this current research work was to extract glutaminase producing bacteria from agricultural waste polluted soil and to determine their identification and examine glutaminase development and production at various pH, temperature, and incubation conditions. In the previous studies, it is reported that the glutaminase enzyme is widespread in nature since it is found in both macro-and microorganisms, including bacteria, fungus, and yeast [29]. Animals, plants, bacteria, actinomycetes, yeast, and fungus are among the possible sources. Numerous bacteria, including *Bacillus sp.*, *Pseudomonas*, *Actinobacterium sp.*, and *E. coli*, are involved in the manufacture of extracellular and intracellular glutaminases as reported by Saleem & Ahmed [11]. *Aspergillus sp.* and *Trichoderma sp*. are the primary sources of fungal glutaminases. There are several species of actinomycetes that produce glutaminase. Plant tissue is also used in the manufacturing process; however, there is little evidence of the extraction of glutaminases from plants owing to the limited feasibility of certain extraction methods. Animals are not often studied in the area of enzyme separation from their tissues because of their complicated organisational structures [11].

A total of 34 bacterial isolates were recovered from soil samples, with 10 of them displaying a pink color zone surrounding the colony on a medium containing phenol red

as a pH indicator. Previously reported glutaminase generating bacterial isolates were screened using specific medium. Furthermore, the isolates that change the color of the media from yellow to pink were tested for glutaminase activity [30]. It was discovered that all isolates generated a clean zone. Glutaminase producers were identified using the same phenol red media described in the literature [30]. As a result, these isolates were chosen as the top glutaminase producers for further investigation [31].

In the study about morphology, biochemistry, and culture characteristics, it was said that all of the characteristics of strains that make glutaminase match those in BERGEY'S manual of systemic bacteriology. All the bacteria had different kinds of pigmentation and different phenotypic traits. In this study, the glutaminase producers that were chosen from soil polluted by agricultural waste all had different phenotypic traits. So, the first step in identifying the selected isolates showed that they were all Gram-positive rods that made spores.

Molecular weight was confirmed by SDS-PAGE after partial refinement of this product. Four different strains of *Bacillus* with different molecular weight were *alkilobacillus bogoriensis* 1R 34 KDa, *Bacillus anthracis* P3 38 KDa, *Bacillus albus* A1 33 KDa and *Bacillus anthracis* G2 37 KDa. A similar investigation by Reda [32] on *Bacillus anthracis* revealed the molecular weight of 38 kDa for this organism. Findings by Kumar *et al.* [27] and Nandakumar *et al*. [1] reported that glutaminase produced by many *Bacillus* species ranges between 19 to 50 kDa. There was a wide molecular weight range of the glutaminases synthesized by different organisms including *B. subtilis* and *B. coagulans* [32].

It is necessary to choose strains according to their glutaminase activity and to keep up the reaction of the best parameters of pH and temperature as indicators. In our study the enzyme activity was measured over a range of pH (4-11) and temperature (20 ºC to 50 ºC) for all four strains, including the detected peaks. The factors like control of nutrition, environment and physiological functions for better enzyme production in selected organism is very crucial while enzyme production in regulated by temperature and PH as well. In the present study, growth and viability assay of the isolates producing glutaminase at different pH and temperature were investigated. The optimal pH value for the cultivation of *Bacillus albus* strain A1 is pH 8. It is the first time that it had been identified that *Bacillus anthracis* prefers a pH of 8 to grow, and no data is available regarding the optimal growth conditions of this particular strain to produce the maximal amount of glutaminase enzyme. Growth and productivity of *Bacillus albus* strain maximum amount of glutaminase enzyme was produced at pH 8 as that only was the phase of maximum growth. In this study, it has been demonstrated that there is no growth associated with manufacture of glutaminase and suggest that the glutaminase production might require an alkaline condition. It is widely known that *Bacillus* species improve from pH ranging between 5 to 8 for reproduction and growth by previous researcher studies.

Based on previous studies it is known that *Bacillus* species thrive best in a pH range of 5 to 8. Specifically, *Bacillus* sp. B12 shows peak glutaminase production at pH 8 with stability observed between pH 6 and 9.5 which corresponds to our results seen in *Bacillus anthracis*. For *Pseudomonas aeruginosa* the optimal pH for glutaminase production falls within the range of 7.5 to 9 [33]. A study conducted by ZH Wardah *et al*. [34], underscores how enzyme production levels fluctuate with varying pH levels pinpointing the favourable pH for l glutaminase production, by *S. Pseudogriseolus* ZHG20. A study by Saleem & Ahmed [11] that for Bacillus species, the optimal glutaminase activity found at a neutral to slightly alkaline pH. For example, a study conducted on L- glutaminase production by *Bacillus* species found that the best pH for glutaminase enzyme activity is around 7.0 to 9.0.

During solubilization we also measured the growth and productivity of each of the selected isolates to determine how temperature affected the isolates. The identities of the strains most active for glutaminase enzyme at 45°C were *Alkilobacillus bogoriensis* 1R. While for *Bacillus anthracis* P3 and *Bacillus anthracis* G2, the optimal temperature was 40°C. However, *Bacillus albus* A1 showed its maximum activity at slighter lower temperature which was 30°C. The optimal temperature for each one of the four isolated strains using glutamine as substrate was observed. The temperature of 40°C seemed to be the best one for the four samples that showed maximum glutaminase activity. Studies from the previous literature have shown that different types of *Bacillus* bacteria work best in terms of enzyme activity between 25°C and 45°C [35]. In a study conducted by Brown *et al* [36], they identified the glutaminase enzyme from *Bacillus subtilis* highlighting its significant effectiveness at temperatures close, to 37°C, which aligns with the higher temperature values observed in our own research.

In this research we confirmed glutaminase enzyme production through the amplification of the 16S rRNA gene via PCR. The resulting PCR products were visualized on a 2% agarose gel, alongside a 1000bp ladder for size comparison. In Figure 4, the gel image displays distinct bands corresponding to PCR products of approximately 965bp, indicative of successful amplification of the targeted

gene fragment. This confirms the presence of the desired genetic material in the isolated DNA of the bacterial isolates. In literature the study on Halomonas meridiana's production of L-glutaminase [4]. L-glutaminase synthesis by marine Halomonas meridiana isolated from the red sea and its efficiency against colorectal cancer cell lines. A study on optimizing L-glutaminase production by *Streptomyces pseudogriseolus* [34] employed 16S rRNA gene amplification to identify the bacterium. When the PCR results were subjected to gel electrophoresis analysis, discrete bands approximately 965 bp were seen which is consistent with our findings. In contrast the study conducted on regulation of mammalian glutaminase. Although 16S rRNA gene amplification was also used in this investigation with a range of varying band widths on the agarose gel [37].

Based on phylogenetic tree, our isolates were classified in the *Bacillus* species. The 16S rRNA gene sequence-analysis of bacteria is the most cost-effective tool for recognition as well as differentiation of *Bacillus* and related species [38]. *Bacillus subtilis* Evolutionary Tree Created by Kubo *et al.* [39]. They got the bacteria from dry rice straw (for the beginning of the natto fermentation). Fachrial *et al.* [40] also isolated *Bacillus* species from the south Sulawesi hot spring.

Analysis of the 16S rRNA gene sequence was used to place bacteria in their taxonomic group and Figure out what species they were. Comparing the 16S rRNA gene sequences showed that isolate A1 is most similar to *Bacillus albus*, isolate G2 is most similar to *Bacillus anthracis*, isolate P3 is most similar *to Bacillus anthracis*, and isolate 1R is most similar to *alkilobacillus bogoriensis*. According to some prior research, the *Bacillus* genus may not be monophyletic, so whole-genome sequencing or additional particular genetic markers may be required for an accurate classification. When compared to our results based exclusively on 16S rRNA gene analysis, this may result in alternative classifications or more precise distinctions within the Bacillus group [41].

However, there is ample evidence in previous studies supporting the classification of *Bacillus* species, such as *Bacillus albus* and *Bacillus anthracis*, using 16S rRNA gene sequencing. High-resolution phylogenetic connections between *Bacillus* species are provided by this approach [42], which validates our findings.

In research different types of soil bacteria like those from the *Streptomyces* genus as mentioned by Desai *et al.* [43] have been recognized for their ability to produce glutaminase. The discovery of *Bacillus* species, such as *Bacillus anthracis* and *Bacillus albus* aligns with studies that have also identified soil bacteria capable of producing significant enzymes, like glutaminase [44]. A study by Saleem & Ahmed [11] also isolated and characterized L-glutaminase producing bacteria from soil sample. They performed Subordinate screening which involved purification, morphological character observation, biochemical testing of bacterial strains, and 16S rRNA sequence homology testing. They chose Five bacterial strains based on their performance in enzymatic and protein assays, fermentation-based enzyme synthesis, and glutaminase positive screening tests. The taxonomical identification of these five isolates were *Bacillus subtilis* U1, *Achromobacter xylosoxidans* G1, *Bacillus subtilis* Q2, *Stenotrophomonas maltophilia* U3, and *Alcaligenes faecalis* S3.

CONCLUSION AND RECOMMENDATIONS

The outcome of this study indicates that the *Bacillus* species has the potential to produce secondary metabolites (Glutaminase) which can be useful in different industries to enhance the flavour of fermented food. Total 34 soil samples were collected from different areas of district Kohat. 10 samples are positive for the glutaminase production and total, 30% samples show the activity for glutaminase. Bacteria were isolated from soil using standard microbiological techniques. For glutaminase enzyme activity of bacteria phenol red was added as an indicator. Selected isolates were identified using various biochemical tests. Among 10 samples of soils, total four types of bacteria were identified in this study mostly are *Bacilli*. Among all isolates, *Bacillus albus* showed best glutaminase activity. 12% SDS-PAGE for visualization of protein bands. Four different strains of *Bacillus* with different molecular weight were *alkilobacillus bogoriensis* 1R 34 KDa, *Bacillus anthracis* P3 38 KDa, *Bacillus albus* A1 33 KDa and *Bacillus anthracis* G2 37 KDa. The selected isolates were grown in a flask containing liquid media and maintained pH between (4-11) then incubated at 37ºC overnight. The best activity was showed in alkaline pH. The outcome of temperature on glutaminase producing bacteria was tested at different temperature ranges from 20 ºC-50 ºC. The optimal temperature of these isolates was 40 ºC. The phylogenetic tree evaluation revealed that isolate A1 is most similar to *Bacillus albus*, isolate G2 is most similar to *Bacillus anthracis*, isolate P3 is most similar *to Bacillus anthracis*, and isolate 1R is most similar to *alkilobacillus bogoriensis*. Based on the phylogenetic analysis, it is concluded that *Alkilobacillus bogoriensis* (1R) is distinct from the other *Bacillus* species identified. Glutaminase enzyme should be commercialized on industrial scale and should be cloned to produce large quantities for application in various fields.

DECLARATION

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Authorship Contributions

This paper is a part of the M.Phil research work of Mr Hafiz Ullah. Dr. Waheed Ullah conceptualized and designed the study, while Faryal Khan and Muhammad Saeed Khan contributed significantly to data collection, analysis, and interpretation. We appreciate the efforts of Dr. Imran Ali and Dr. Niamat Khan for their role in statistical analysis and manuscript writing. Special thanks to Dr. Yar Muhammad and Dr. Faran Durrani for their critical revisions and insightful suggestions that improved the quality of this paper

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Ethics approval and consent to participate

The study was conducted without human beings and animals. Ethical approval is not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declared that there is no conflict of interest.

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