Characterization of a New L-Glutaminase Produced by *Achromobacter xylosoxidans* RSHG1, Isolated from an Expired Hydrolyzed L-Glutamine Sample

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Abstract: As significant biocatalyst, L-glutaminases find potential applications in various fields, from nourishment to the pharmaceutical industry. Anticancer activity and flavor enhancement are the most promising applications of L-glutaminases. In this study, L-glutaminase was isolated and purified from an old glutamine sample. A selected bacterial isolate was characterized taxonomically by morphological characters, biochemical testing and 16S rDNA sequence homology testing. The taxonomical characterization of the isolate identified it as *Achromobacter xylosoxidans* strain RSHG1. The isolate showed maximum enzyme production at 30 °C, pH 9, with Sorbitol as a carbon source and L-Glutamine as a nitrogen and inducer source. L-Glutaminasae was purified by using column chromatography on a Sephadex G-75. The enzyme has a molecular weight of 40 KDa, pH optimal 7 and is stable in the pH range of 6–8. The optimum temperature for the catalyst was 40 °C and stable at 35–50 °C. The kinetic studies of the purified L-glutaminase exhibited $K_m$ and $V_{max}$ of 0.236 mM and 443.8 U/mg, respectively. L-Glutaminase activity was increased when incubated with 20 mM CaCl$_2$, BaCl$_2$, ZnSO$_4$, KCl, MgSO$_4$ and NaCl, whereas EDTA, CoCl$_2$, HgCl, ZnSO$_4$ and FeSO$_4$ decreased the activity of the enzyme. The addition of 8% NaCl enhanced the glutaminase activity. L-Glutaminase immobilized on 3.6% agar was stable for up to 3 weeks.

Keywords: L-glutaminase production; optimization; characterization; molecular identification; *Achromobacter xylosoxidans* RSHG1

1. Introduction

Biocatalysts play a crucial role in maintaining and sustaining the food, chemical, agriculture and cosmetic industries as commercially economic products. Biocatalysts are involved in all the biochemical processes within the cells and living organisms. Moreover, they accelerate the rate of a chemical reaction without being a part of the end product [1–3]. Enzymatic processes are eco-friendly, which reduces the risk of byproducts that are toxic for the human health and the environment [4]. Due to these features, biocatalysts play a vital role in the pharmaceutical industry [2]. L-Glutaminases are amidohydrolases, belong to the hydrolase class of enzymes and cleave L-glutamine into L-glutamic acid and ammonia. This enzyme plays a significant role in nitrogen metabolism at the cellular level. This enzyme is ubiquitous and present in both microorganisms (bacteria, fungi and yeast) and macro-organisms (animals and plants) [5–7]. The probable sources may include animals, plants, bacteria, actinomycetes, yeast and fungi [7]. Numerous bacteria are involved in extracellular and intracellular glutaminases production, such as *Bacillus sp.*, *Pseudomonas, Actinobacterium sp* and *E. coli* [7,8]. Important fungal glutaminases are *Aspergillus sp.* and *Trichoderma sp.* [9]. Different species of actinomycetes also produce L-glutaminase. L-
Glutaminase production and the extraction from plants are not well studied. Due to complex organization, animals are not well known for L-glutaminase isolation from animal tissues [10–12]. Therefore, microbes are the chief source of enzymes, due to large-scale production in a short time, and can be genetically modified for increased enzyme production [13,14]. Microbial biocatalysts are significantly valuable from an industrial and biotechnological point of view [15]. The approaches towards enzymatic production involve mainly of two types of fermentation techniques, solid-state fermentation (SSF) and submerged fermentation (SMF) [16,17]. Catalysts represent 80% of the whole mechanical market. Desire to improve the procedures for chemicals utilized in ventures are increasing [18]. Biocatalysts are eco-friendly, low cost and encouraging for industrial use and bioremediation [2,19]. L-Glutaminase is an important enzyme in the food industry used for enhancing the taste and aroma of fermented food [20,21]. Glutaminase enhances the taste of fermented foods by hydrolyzing L-glutamine into glutamic acid, giving umami flavor [7]. L-Glutaminsae is a potential anticancer enzyme, flavor enhancer and antioxidant agent and is also used in biosensors [22]. Biosensors are prepared by the immobilization of L-glutaminase on a polyvinyl chloride (PVC) ammonium membrane electrode, constituting an ammonium-selective glutamine biosensor. These biosensors detect L-glutamine in biological samples such as healthy human serum, cerebrospinal fluid (CSF) and commercial glutamine capsules [23]. The hypoporia jecorina biosensor is prepared to check out the reproducibility and accuracy by immobilizing enzyme on a zinc oxide (ZnO) nanorod and chitosan [6]. L-Glutaminase, in the treatment of HIV, is a standout among the most encouraging remedial applications [24,25]. Microbial L-glutaminases have acquired importance due to their potential role in anticancer activity. L-Glutamine is the vital fuel for actively dividing cells of the intestine, immune cells and developing tumor cells [26]. When L-glutaminase converts L-glutamine into glutamate it helps inhibit cancerous cells by removing their principal fuel glutamine [27]. Due to the importance of this enzyme, the aim of the present research study is to isolate new indigenous bacterial strains with higher levels of L-glutaminase production, with high pH stability and salt tolerance that can be used in the food industry and as biosensors in the pharmaceutical industry.

2. Results

The present study shows the isolation, characterization and identification of bacterial isolates with optimization of L-glutaminase by selected bacterial strains isolated from old glutamine samples.

2.1. Screening and Isolation of Bacterial Isolates

Initially, ten strains were isolated from expired L-glutamine samples on glutamine salt media with phenol red as a pH indicator. Ten bacterial colonies gave a positive result in their purified form by changing the colour of the medium from yellow to pink (Figure 1).
The bacterial strain RSHG1 was selected based on secondary screening performed by point inoculation of the bacterial strain on glutamine salt medium. The pink coloured zones, produced after 24 h of incubation at 37 °C indicating substrate hydrolysed in the medium by L-glutaminase. Further, the bacterial isolate RSHG1 was grown in liquid media and the cell-free broth was added to the well for the agar diffusion assay measuring the activity of glutaminase in terms of hydrolyzing zones in screening media (Table 1). The results indicate that the zone of hydrolysis formed by point inoculation and agar diffusion assay was 26 mm and 30 mm, respectively, for the RSHG1 strain (Table 1).

Table 1. Screening of bacterial isolate for glutaminase production by zone of hydrolysis.

| Bacterial Isolate | Zone of Hydrolysis (mm) | Agar Diffusion Assay (mm) |
|-------------------|-------------------------|---------------------------|
| Achromobacter xylosoxidans RSHG1 | 26 | 30 |

2.2. Taxonomic Characterization of Bacterial Isolate

2.2.1. Cultural and Microscopic Characteristics

Features such as size, shape, margins, elevations, consistency, opacity and pigmentation showed that the isolate RSHG1 produced medium-sized colonies with a dome shape elevation. Regarding the shape of the colonies, the colonies of RSHG1 strains were rhizoid, opaque and moist and showed no pigmentation. The microscopic observations of the bacterial strain revealed that the bacterial isolate RSHG1 was a Gram-negative coccus with a single arrangement.

2.2.2. Biochemical Testing

To biochemically characterize the strain RSHG1, several biochemical tests were carried out. The results indicate that the bacterial strain was positive for oxidase, catalase, manitol salt agar, simon citrate, indole production, growth on MacConkey agar, arginine hydrolase test, urease, esculin ferric citrate test and gelatinase production, while other biochemical tests showed negative results (Table 2).

Table 2. Biochemical characterization of RSHG1 bacterial isolate.

| Ser # | Biochemical Test                               | Result |
|-------|-----------------------------------------------|--------|
| 1     | Oxidase                                      | +      |
| 2     | Catalase                                     | +      |
| 3     | Mannitol Salt Agar Test                       | +      |
| 4     | Simmon Citrate                               | +      |
| 5     | Indole Production                            | +      |
| 6     | MacConkey Agar                               | +      |
| 7     | Glucose Fermentation Test                    | -      |
| 8     | Arginine Hydrolase Test                      | +      |
| 9     | Urease Test                                  | +      |
| 10    | Esclulin Ferric Citrate Test                 | +      |
| 11    | Gelatin Hydrolysis Test                      | +      |
| 12    | β-Galactosidase Test                         | -      |
| 13    | D-Glucose Assimilation Test                  | -      |
| 14    | L-Arabinose Assimilation Test                | -      |
| 15    | D-Mannose Assimilation Test                  | -      |
| 16    | N-acetyl-glucosamine Assimilation Test       | -      |
| 17    | D-Maltose Assimilation Test                  | -      |
| 19    | Potassium Gluconate Assimilation Test        | -      |
| 20    | Capric Acid Assimilation Test                | -      |
| 21    | Potassium Gluconeate Assimilation Test       | -      |
| 22    | Capric Acid Assimilation Test                | -      |
| 23    | Adipic Acid Assimilation Test                | -      |
| 24    | Malic Acid Assimilation Test                 | -      |
| 25    | Trisodium Citrate Assimilation Test          | -      |
| 26    | Phenylacetic Acid Assimilation Test          | -      |
2.2.3. Identification of the Bacterial Isolate

The amplified product of the 16S rRNA gene of the bacterial isolate RSHG1 was 1500 bp and the sequence was BLAST on the NCBI database. Based on the homology between the sequence of the studied strain (>90%) with bacterial strains in the database, the bacterial isolate was identified as *Achromobacter xylosoxidans* RSHG1 (Table 3).

| Strain     | Size (bp) | Sequence Homology with          | Accession Number on NCBI | Identified As                  |
|------------|-----------|--------------------------------|---------------------------|--------------------------------|
| RSHG1      | 759       | *Achromobacter xylosoxidans*    | MZ868956                  | *Achromobacter xylosoxidans* RSHG1 |

2.2.4. Phylogenetic Analysis

The evolutionary history was studied using the neighbor joining method [28]. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed [29]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the maximum composite likelihood method [30] and are in the units of the number of base substitutions per site. This analysis involved seven nucleotide sequence ambiguous positions for each sequence pair removed by the pairwise deletion option. There were 759 positions in the final dataset phylogenetic tree conducted on the MEGA X software [31]; see Figure 2.

![Figure 2. Phylogenetic tree for the bacterial isolate RSHG1 showing relatedness with other organisms.](image)

2.3. *Microbial Production of L-Glutaminase*

Glutaminase production was performed from the bacterial isolate on Glutamine salt medium and optimized for various production parameters.

Optimization of Parameters for Glutaminase Production

1. **Effect of incubation time**

   Glutaminase production was performed by the selected strain RSHG1 on glutamine salt medium at 37 °C and pH 6.6. *A. xylosoxidans* RSHG1 produced the highest amount of enzyme, 1.3 U/mg, observed on the 2nd day of incubation (Figure 3).

2. **Effect of pH**

   *A. xylosoxidans* RSHG1 achieved maximum glutaminase production at pH 9. The highest activity was 3.26 U/mg at pH 9 on the 3rd day of incubation at 30 °C (Figure 4).

3. **Effect of Temperature**
Glutaminase production was optimized for temperature effect at 25°C, 30°C and 37°C by bacterial strains RSHG1. The highest Glutaminase activity was shown by *A. xylosoxidans* RSHG1 at 30°C on the 3rd day of incubation (3.23 U/mg); see Figure 5.

4. **Effect of Carbon source**

The enzyme from the strain *A. xylosoxidans* RSHG1 achieved maximum activity with sorbitol as carbon source (3.18 U/mg) on the 4th day of incubation at 30 °C, pH 9 (Figure 6).

5. **Effect of Nitrogen source**

The production of L-glutaminase by the strain *A. xylosoxidans* RSHG1 was at its maximum when L-glutamine was the nitrogen source, showing 3.1 U/mg activity on the 3rd day of incubation at 30°C, pH 9, with sorbitol as a carbon source (Figure 7).

6. **Effect of Inducers**

The effect of inducers on *A. xylosoxidans* RSHG1 showed the maximum activity of 6.8 U/mg with 1.5% L-glutamine as an inducer on the 3rd day of incubation Figure 8.

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**Figure 3.** Production of glutaminase by *A. xylosoxidans* RSHG1 bacteria in fermentation media at 37 °C, pH 6.8.
Figure 4. Effect of pH on glutaminase production by *A. xylosoxidans* RSHG1 on fermentation media at 30 °C.

Figure 5. Effect of temperature on Glutaminase production by *A. xylosoxidans* RSHG1, pH 9.
Figure 6. Effect of carbon source on Glutaminase production by *A. xylosoxidans* RSHG1 on fermentation medium at 30 °C, pH 9.

Figure 7. Effect of nitrogen sources on Glutaminase production by *A. xylosoxidans* on fermentation medium at 30 °C, pH 9.

Figure 8. Effect of inducers on L-glutaminase production by *A. xylosoxidans* RSHG1, on fermentation media at 30 °C, pH 9, with sorbitol as a carbon source.
2.4. Purification and Characterization of L-Glutaminase

2.4.1. Purification

The purification of L-glutaminase produced by *A. xylosoxidans* RSHG1 is summarized in Table 4. L-Glutaminase partially purified by 70% ammonium sulphate (fractional saturation) precipitation, followed by dialysis, showed increase in yield by up to 6.4%. The concentrated sample was run on a gel filtration column with Sephadex G-75; the best fractions were 5–11 (Figure 9), were showing the highest activity with enhanced yield by up to 3.73% purification of 16.6. folds.

| Method                          | Total Protein (mg) | Specific Activity (U/mg) | Purification (Fold) | Total Activity (IU) | Yield (%) |
|---------------------------------|--------------------|--------------------------|---------------------|---------------------|-----------|
| Crude extract                   | 75                 | 0.52                     | 1                   | 23,400              | 100       |
| Ammonium sulphate (70%)         | 35                 | 2.1                      | 4.1                 | 1500                | 6.4       |
| Gel filtration chromatography   | 1.04               | 35                       | 16.6                | 874                 | 3.73      |

![Figure 9. Purification of L-glutaminase produced by *A. xylosoxidans* RSHG1 by column chromatography using Sephadex G-75.](image)

2.4.2. Characterization

The purified enzyme L-glutaminase produced by *A. xylosoxidans* RSHG1 was characterized for kinetic parameters. The maximum activity was recorded to be 511 U/mg at 37 °C, pH 7, after 30 min of incubation (Figure 10).
Figure 10. Effect of incubation time on L-glutaminase activity produced by *A. xylosoxidans* RSHG1 at 37 °C, pH 7.

The maximum velocity (Vmax) and Michaelis–Menten constant (Km) of the purified enzyme were 443.8 U/mg and 0.236 mM, respectively, calculated with the Michaelis and Menten plot (Figure 11). The enzyme gained the highest activity of 505 U/mg at a pH of 7 (Figure 12) and was stable at a pH of 6.5–8.0. L-Glutaminase from *A. xylosoxidans* RSHG1 showed thermal stability between 40 and 50 °C, while maximum activity (520 U/mg) was observed at 40 °C (Figure 13). The effect of metal ions and inhibitors on L-glutaminase produced by *A. xylosoxidans* RSHG1 was checked (Figure 14). Enzyme activity was increased with the addition of CaCl₂, BaCl₂, KCl and NaCl and inhibited by EDTA, CoCl₂, HgCl₂, ZnSO₄, MgSO₄ and FeSO₄. The effect of different concentrations of NaCl on purified L-glutaminase produced by *A. xylosoxidans* RSHG1 resulted in the highest activity of 822 U/mg with 8 % NaCl; a further increase in the salt concentration decreased enzyme activity (Figure 15). After immobilization on agar medium, the enzyme was stable for 3 weeks. Its activity was almost similar up to 21 days. The immobilized enzyme retained good activity (23 U/mg.)

Figure 11. Effect of substrate concentration on L-glutaminase activity produced by *A. xylosoxidans* RSHG1 at 40 °C, pH 7.
Figure 12. Effect of pH on L-glutaminase activity produced by *A xylosoxidans* RSHG1 at 37 °C.

Figure 13. Effect of temperature on L-glutaminase activity produced by *A xylosoxidans* RSHG1 at a pH of 7.
Figure 14. Effect of metal ions on L-glutaminase activity produced by *A. xylosoxidans* RSHG1 at 40 °C, pH 7.

Figure 15. Effect of sodium chloride on L-glutaminase activity produced by *A. xylosoxidans* RSHG1 at 40 °C, pH 7.
3. Discussion

Since the discovery of L-glutaminase’s properties, such as anti-cancerous properties, different microbial sources has been the focal point of enthusiasm for the isolation of the enzyme. L-Glutaminase is present in most living organisms, such as plant tissues, and microorganisms, including bacteria, fungi and actinomycetes. Microbial L-glutaminases (L-glutamine aminohydrolase EC 3.5.1.2) are favored sources for biotechnological applications and effectiveness.

In the present study, one bacterial strain, A. xylosoxidans RSHG1, was isolated from an expired and hydrolyzed glutamine sample. L-Glutaminases from E. coli, Bacillus spp. [32] Pseudomonas spp., Citrobacter and Staphylococcus were isolated and studied attentively [33]. A total of 10 strains were initially isolated on a glutamine salt medium with a pH of 6.6, out of which we have selected one isolate that showed high activity in screening assays. The investigations carried out by Aly [34] indicated the presence of glutaminase in Streptomyces sp. Emelda 2016 [35] isolated bacteria from soil and an aquatic environment screened on minimal glutamine media with phenol red as an indicator and selected the colonies that produced a pink color due to the release of ammonia into the medium. In the bacterial screening, RSHG1 showed the largest zone of hydrolysis, of 26 mm, while, in the agar diffusion assay, a 30 mm zone was observed at a pH of 6.8. L-Glutaminases are isolated from different microbial sources for industrial applications [36,37].

The biochemical characterization of the selected bacterial isolate was performed and the isolate RSHG1 showed a 35.5% test positivity. L-Glutaminase was isolated from Bacillus subtilis and gave a 50% test positivity when 34 biochemical tests were performed [37]. The 16S rDNA sequencing of the L-glutaminase-producing strain RSHG1 showed a 90 percent similarity with the Achromobacter xylosoxidans strain RSHRSHG1. The sequence was deposited in the NCBI database with the accession number MZ868956. The molecular identification of 16S rDNA analysis of L-glutaminase-producing bacteria identified Bacillus subtilis JK-79, Alcaligenes faecalis KLU102 [38] and Stenotrophomonas maltophilia and Achromobacter species [39,40].

A. xylosoxidans RSHG1 was optimized for fermentation conditions, such as incubation time, pH, temperature, carbon source, nitrogen source and effects of inducers, for L-glutaminase production. L-Glutaminase produced by A. xylosoxidans RSHG1 achieved 1.3 U/mg activity on the 3rd day of incubation. Maximum L-glutaminase production was gained in a submerged fermentation after 18 h of incubation time by the marine isolated Bacillus subtilis [41] and, after 72 h of incubation, by Pseudomonas VJ-6 [42]. The effect of pH on A xylosoxidans RSHG1 L-glutaminase production was the best at a pH of 9. The highest L-glutaminase production was obtained at a pH of 7 from the forest soil-isolated bacterial strain of Bacillus sp. [43]. Vibrio azureus JK-79, isolated from a marine environment, showed maximum glutaminase production at a pH of 8 [44]. In the present study, A. xylosoxidans RSHG1 showed maximal activity at 30 °C. Kiruthika et al. (2018) [45] isolated Bacillus subtilis JK-79 from a marine environment and the isolate showed maximal glutaminase activity at 37 °C. Al-Zahra [46] found that 35 °C is the best temperature for glutaminase production by Pseudomonas NS16. In this study, Sorbitol was the best carbon source for glutaminase production by A. xylosoxidans RSHG1 among all the different carbon sources tested (Figure 6). In a study on the selective isolation of the potent L-glutaminase-producing soil bacteria, glucose was the best carbon source for glutaminase production by Pseudomonas aurignosa [46]. A xylosoxidans RSHG1 L-glutaminase showed maximum production with L-glutamine as a nitrogen source. Maximal glutaminase activity by Pseudomonas aurignosa was achieved with glutamine, out of various nitrogen sources [46]. Kiruthika and Nachimuthu [47] observed that glutaminase production by marine Bacillus subtilis JK-79 was enhanced by yeast extract. The best inducer for L-glutaminase production by A xylosoxidans RSHG1 was L-glutamine. Glutaminase production was induced by glutamine in the case of Bacillus subtilis JK-79 and Bacillus sp. [48,49].

L-glutaminase produced by A xylosoxidans RSHG1 was partially purified by using 70% ammonium sulphate saturation. Awad [22] partially purified glutaminase produced
by *Streptomyces rochei* SAH2_CWMSG using 75% ammonium sulphate. L-glutaminase produced by *Pseudomonas* VI-6 was partially purified by using 80% ammonium sulphate [42]. L-glutaminase produced by *A xylosoxidans* RSHG1 was purified on a gel filtration column with G-75. Awad [22] purified glutaminase from *Streptomyces rochei* SAH2_CWMSG using a gel filtration column G-100. The molecular weight of L-glutaminase produced by *A xylosoxidans* RSHG1 was 40 KDa. The molecular weight of L-Glutaminase from *Streptomyces avermitilis* was reported as 50 KDa [49]. The marine species *Halomonas meridian* produced L-glutaminase at a molecular weight of 57 kDa [50]. The activity of partially purified L-glutaminase produced by the endophytic isolate *Aspergillus* sp was tested for incubation times showed the highest peak after 30 min of incubation and a decrease in activity was observed with the increase in time [51].

*A xylosoxidans* RSHG1-produced L-glutaminase showed the maximum velocity of the enzyme as 443.8 U/mg and Km was 0.236 mM. Km and Vmax of L-glutaminase produced by *Bacillus* sp. B12 were 0.4 mmol/L and 0.133 mmol/min, respectively [52]. For L-glutaminase produced by *Bacillus* sp., Km and Vmax values were 66.83 mM and 0.755 mM/min, respectively [48]. *A xylosoxidans* RSHG1-produced L-glutaminase showed the best activity at a pH of 7. *Pseudomonas aeruginosa* isolated from Sangihe-Talaud Sea producing L-glutaminase also showed maximum activity at a pH of 7, while L-glutaminase produced by *Streptomyces* sp. achieved the highest activity at a pH of 8 [26]. L-Glutaminase from *vibrio* sp. M9 isolated from Mahabalipuram marine sediments showed maximum activity at a pH of 7 [53]. L-Glutaminase produced by *A xylosoxidans* RSHG1 achieved maximum activity at 40 °C. L-glutaminase produced by *B. cereus* glutaminase showed the highest activity at 30 °C [49]. L-Glutaminase produced by *A xylosoxidans* RSHG1 showed increased Vmax with addition of CaCl2, BaCl2, ZnSO4, KCl, MgSO4 and NaCl, and was inhibited by EDTA, CoCl2, HgCl, ZnSO4 and FeSO4. *B. cereus* glutaminase activity was enhanced by Mg2+, NaCl and Co2+, while it was inhibited by Ca2+, Ba2+, Fe3+, Zn2+, Cu2+, Hg2+ and Cd2+ [54]. When effect of metal ions was studied for L-glutaminase produced by *Bacillus* sp. B12 it was reported that enzyme was activated by Mn2+, Mg2+, Ca2+ and Na+, while no effect was observed with the addition of K+, Co2+ and Ni2+ and it was inhibited by Hg2+, Cu2+, Fe3+ and Zn2+ [52]. The results indicated that the enzyme is a metalloenzyme and requires activation by different metals, while it is inhibited by other metals. L-Glutaminase by *A xylosoxidans* RSHG1 showed increased activity by addition of NaCl—maximum activity of 822 U/mg with 8% NaCl. This indicates that the enzyme is stable in high salt concentrations. Its stability with NaCl makes this enzyme attractive for food-processing applications. L-Glutaminase-producing marine bacteria from Sangihe-Talaud Sea was halophilic, as the enzyme was stable up to 8% and began to decrease upon addition of a NaCl solution at 16% and 20% [55]. L-Glutaminase produced by *B. amyloliquefaciens* y-9 showed 68% increased activity at 20% NaCl [56]. L-glutaminase produced by *A xylosoxidans* RSHG1, after immobilization on agar, was stable for 3 weeks. Immobilization of the glutaminase enzyme produced by *Hypocrita jecorina* on polyacrylic acid was also reported [50].

4. Materials and Methods

4.1. Isolation and Screening of Bacteria

Bacteria were isolated from an expired and hydrolyzed L-glutamine bottle from the Lab, Quaid-i-Azam University Islamabad, Pakistan. For the screening, a glutamine salt medium with a pH of 6.6 was used. The chemical composition of the medium was NaCl (0.5 g/L), KCl (0.5 g/L), MgSO4·H2O (0.5 g/L), KH2PO4 (1 g/L), FeSO4·7H2O (0.1 g/L) and ZnSO4 (0.1 g/L), as well as L-Glutamine (5 g/L) as a nitrogen source and phenol red (0.012 g/L) as an indicator of bacterial glutaminase activity [57]. The glutamine sample from an expired bottle was diluted and were spread plated and incubated at 37 °C in a thermal
incubator. Bacteria that showed a change in colour around the colony due to basic pH were identified as glutaminase producers.

Further, the bacterial strain was point inoculated in the center of plates and the change in colour was observed and measured after 24 h of incubation at 37 °C. The isolated bacterial strain were cultivated on a production medium. The cell-free broth was poured in wells (50 µL) on the screening medium plates; it changed colour after 24 h and the zones were measured in millimeters.

Bacterial strains were grown on the same medium, maintained and stored for reuse in the form of glycerol stocks at −40 °C. Glutaminase production by RSHG1 was carried out at a pH of 9 at 30 °C with 1.5 % L-glutamine at 120 rpm, and then incubated for 3 days.

4.2. L-Glutaminase Assay

L-Glutaminase activity was quantified by using nesslerization of ammonia released by the hydrolysis of substrate L-glutamine. The reaction mixture of the enzyme assay contained 0.5 mL of 0.5 M phosphate buffer at a pH of 7.0, 0.5 mL of 40 mM L-glutamine, a substrate of L-glutaminase, 0.5 mL of the crude enzyme and 0.5 mL of distilled water. The reaction mixture was then incubated in a water bath at 37 °C for 30 min. After incubation, we added 0.5 mL of Trichloroacetic acid (TCA) for the termination of the reaction. In separate test tubes, 3.7 mL of distilled water, 0.1 mL of the reaction mixture and, finally, 0.2 mL of Nessler’s reagent were added [58]. The absorbance was measured at 450 nm and compared with the standard curve of ammonium sulfate to calculate the unit of enzyme activity.

4.3. Protein Estimation

The presence of extracellular protein in the crude enzyme, quantified by the Lowry method (1951) [59], was performed by using Bovine serum albumin (BSA) as a standard.

4.4. Taxonomic Characterization

The bacterial strain’s morphology and biochemical nature were examined by growing the bacteria on nutrient agar plates and incubated for 24 h at 37 °C. Colony morphology was monitored by checking size, pigmentation, shape, margin, elevation and opacity. A single colony was used for the Gram-staining procedure and observed under the microscope.

Biochemical characterization included indole production, urease, catalase, oxidase, starch hydrolysis, carbohydrates fermentation, mannitol salt agar and citrate utilization test, using a standard protocol [60,61]; the remaining biochemical testing was performed by using an API kit 20 Ne.

Molecular Characterization

Genomic DNA was isolated using the Solarbio bacterial DNA extraction kit. The 16S rRNA gene was amplified by PCR using MultiGene OptiMax (Labnet International Inc.). A 1500 bp fragment of the 16S rRNA gene was obtained by using universal primers, namely, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) forward primer and 1492 R (5′-TACGYTACCTTGTTACGACTT-3′) reverse primer [22]. The PCR reaction was prepared in a final volume of 25 µL containing 2.5 µL of 1× PCR buffer, 5 µL of dNTPs, 3 µL of 1 mM MgSO4, 0.75 µL (IU) of Taq DNA Polymerase, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse Primer, 2 µL of bacterial template DNA and 10.75 µL of double-distilled H2O. The amplification of DNA was performed on a PCR machine for 30 cycles, in 1 h and 40 min. The PCR cycle ran for an initial 10 s at 98°C, then the next 45 s at 94°C and, finally, 50 s at 52°C. Then, incubated at 72°C for 1 min and finished with a final extension of 10 min at 72°C. The sequencing of the the 16S rDNA amplified product was performed commercially. The sequence was identified using nucleotide BLAST on the NCBI site; Fasta aligned sequences were employed to make a phylogenetic tree by the neighbor joining
method that dissipated about the evolutionary basis of the strain on the basis of analyses using Mega software [22].

4.5. Optimization of Culture Conditions for L-Glutaminase Production

The strain RSHG1 was optimized for L-glutaminase production in 100 mL Erlenmeyer flasks containing 50 mL of fermentation medium. It was optimized for glutaminase production, on a glutamine salt medium with pH in the range of 6–9 at 30°C; for the examination of the effect of temperature on the fermentation medium, at 25 °C, 30 °C and 37 °C, and pH 9; for the examination of carbon source addition on the fermentation media, using glucose, sucrose, lactose, maltose, xylose and sorbitol (1%) at 30 °C, pH 7; for the examination of 0.1% nitrogen sources, specifically, tryptone, yeast extract, ammonium chloride, Sodium nitrate and L-glutamine, at 30 °C, pH 7. The effect of inducers such as Glycine, Glutamine and Lysine were monitored at a pH of 7, at 30 °C, with 1% sorbitol; L-glutaminase activity was measured after every 24 h of incubation.

4.6. L-Glutaminase Purification

L-Glutaminase was produced on a large scale, under optimal conditions. After centrifugation at 10,000 rpm for 12 min at 4 °C, 70 % ammonium sulphate was added slowly while stirring at the low temperature of 4 °C. Flasks were kept at 4 °C overnight. The mixture was centrifuged on the next day at 10,000 rpm in 50 mL falcon tubes. The pellet was dissolved in 15 mL of phosphate buffer, pH 7. The pellet dialyzed against a low ionic strength phosphate buffer of 0.01 M, pH 7. The dialyzed sample was lyophilized and further purified on a gel filtration chromatographic column with Sephadex G-75. The purified L-glutaminase molecular weight was determined by 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE).

4.7. Characterization of L-Glutaminase by Achromobacter xylosoxidans RSHG1

Purified L-glutaminase was used to monitor the effect of substrate concentration measured by adding the substrate concentration of 0.04 mM–0.6 mM at a pH of 7 with 0.5 M phosphate buffer at 40 °C. The influence of incubation time was verified after 15, 30, 45 and 60 min of incubation at a pH of 7 with 0.5 M phosphate buffer at 37 °C. The effect of pH was monitored on L-glutaminase activity by using 0.5 M buffer in the pH range of 4–9.5 at 37 °C. The influence of temperatures was observed on L-glutaminase activity at a pH of 7 with 0.5 M phosphate buffer at different temperatures (20, 30, 40, 50, 60 and 70 °C). The effect of metal ions, such as EDTA, cobalt chloride, calcium chloride, barium chloride, mercuric chloride, zinc sulfate, manganese sulfate, potassium chloride, magnesium sulfate, sodium chloride and without inhibitor, on L-glutaminase activity was examined at a pH of 7 with 0.5 M phosphate buffer at 40 °C. A partially purified enzyme was incubated with 20 mmol of different metal ions for one hour and then used for the enzyme assay. The effect of increasing the concentrations of sodium chloride (4%, 8%, 12%, 16% and 20%) on enzyme activity were examined at a pH of 7 with 0.5 M phosphate buffer at 40 °C.

4.8. Immobilization on Agar

Partially purified enzyme was subjected to immobilization on 3.6 % agar. Agar was prepared by adding agar in 15 mL of buffer (0.5 M Phosphate buffer, pH 5), autoclaved and cooled to after cooled to 60 °C. Then a volume of 5 mL of the partially purified enzyme was added, thoroughly mixed and poured in Petri plates for solidification, kept in the fridge at 4 °C for 2 h. The solidified material was cut into small cubes and washed with a 0.5 M phosphate buffer at a pH of 7, then assayed for enzyme activity. The immobilized enzyme was stored at 4 °C in a 0.2 M phosphate buffer at pH of 7. The enzyme was stable for 3 weeks.
4.9. Statistical Analysis

L-Glutaminase activities were measured in triplicate (n = 3); the graphs express mean values with error bars of the standard deviation of the means. For the kinetic studies, nonlinear regression and correlation were used.

5. Conclusions

The bacterial isolate *Achromobacter xylosoxidans* RSHG1 isolated from an expired glutamine sample proved to be a good source of L-glutaminase. The enzyme showed maximum activity at 30 °C, pH 9, with sorbitol as a carbon source and L-glutamine as a nitrogen source inducer. The molecular weight of purified L-glutaminase was 40 KDa and was stable at a pH range of 6–8, at a temperature of 35–50 °C. The purified L-glutaminase showed that Km and Vmax were 0.236 mM and 443.8 U/mg, respectively. Enzyme activity was enhanced by adding 20 mM CaCl2, BaCl2, ZnSO4, KCl, MgSO4 and NaCl, while it was decreased by EDTA, CoCl2, HgCl, ZnSO4 and FeSO4. Enzyme activity was increased by 8% NaCl. L-glutaminase was stable for up to 3 weeks when immobilized on 3.6% agar. L-Glutaminase produced by the local indigenous bacterial strain *A xylosoxidans* RSHG1 was stable in a wide range of pH and temperature conditions, having a high affinity for its substrate. Enzyme activity was enhanced by a number of metal ions, such as sodium chloride. Therefore, this enzyme can be used in the food industry for enhancing the taste of foods and in the pharmaceutical industry as an antileukemic agent, as well as in the development of biosensors.

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Conflicts of Interest: The 16S rRNA gene sequence was submitted to NCBI. “The authors declare that there is no conflict of interest.” We declare self-plagiarism because we previously submitted a paper on the isolation of glutaminase-producing bacteria, but later decided to drop the idea to publish; however, that manuscript is available on the Internet as open access.

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