Purification of L-Glutaminase from *Bacillus* sp. *B12* and study its properties

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Abstract

L-glutaminase is an enzyme that catalyse the conversion L-glutamine to glutamic acid and ammonia. The important application of the L-glutaminase is a chemotherapy agent. In this study a novel strain, *Bacillus B12*. that isolated from Diyala soil was explored for production of extracellular L-glutaminase. The enzyme has been purified 23.9-fold from cell-free extract with 45.4% recovery (specific activity 76.6 U/mg protein). Enzyme has molecular weight of 199 kDa, and pH 8 and stable in pH range 6-9.5. Temperature optimum is 40 ºC and completely stable between 25-45 ºC. The kinetics studies revealed that the km and V_{max} of purified L-glutaminase were estimated in 0.4mmol/L and 0.133mmol/min, respectively. The result showed that, the enzyme was active when incubated with 10 mM of Mn^{2+}, Mg^{2+},Ca^{+2} and Na^{+}. Whereas, K^{+}, Co^{2+}, and Ni^{2+} show no effect. However, Hg^{2+}, Cu^{2+},Fe^{2+} and Zn^{2+} decreased L-glutaminase activity . The 10mM of DEAE, PMSF, and sodium azide did not show a clear effect against the enzyme activity. Higher decrease in enzyme activity was observed by using Cysteine and 2-Mercaptoethanol (30 and 25%, respectively).

Keywords: *Bacillus sp.B12,* L- glutaminase, purification, Ion exchange , pH, and Thermostability.

Introduction

Microbial communities are an important biological component of soil function, and all organisms in an ecosystem rely on the activity of microorganisms for their role in improving soil quality and regulating nutrient availability [1]. Producing extracellular enzymes which are needed to break down organic material for sustainable soil productivity. L-glutaminase plays a major role in the nitrogen metabolism of both prokaryotes and eukaryotes[2]. It is an important enzyme therapy for a variety disease of the lymphatic system especially acute lymphocytic leukemia [3].

The enzyme has also been applied in the treatment of Hodgkin 's disease, lymphosarcoma, and acute myelocytic leukemia [4]. L-glutamine is not an essential amino acid for the animal cell, and the neoplastic cells are not able to induce the synthesis of glutamine synthetize. However, they are dependent on the extracellular level of L-glutamine to protein synthesis[5]. As L-glutamine is withdrawn from plasma by L-glutaminase, this amino acid leads to inhibit the growth in neoplastic cells [6]. There is a great demand to produce chemotherapy, a variety of nutritional and physiochemical factors influences the production of enzymes. For this reason, this enzyme has been developed to use as therapy to tumor cells [7]. Nowadays optimization of different production parameter would enhance the quality and quantity of enzyme production, and turn in turn influence enzyme synthesis and cell yield [8]. The microbial source is very common for L-glutaminase, because of easily cultured, extraction, and purification for the industrial scale production. The most used microorganisms to produce L-glutaminase *E.coli*, *Actinomycetes*, *Pseudomonas sp, Erwinia carotovora sp.*[9]. Because of the continuous need to screen newer strains that capable to produce high yield of L-glutaminase, this paper deals with the purification, kinetics properties, and biochemical characterization of L-glutaminase from *Bacillus B12* .

Method

Bacterial strain, culture condition and enzyme purification

Bacterial strain *Bacillus* sp.B12 (Genbank accession

no.:SAMN12069785) was used . The strain was grown on nutrient agar at 30 ºC for 24 h.

L-glutaminase purification

All purification steps were carried out at 4ºC. *Bacillus sp. B12* L-glutaminase was purified by a modification of the method of Boyd and Philips [10].

L-glutaminase assay

The enzyme was assayed by direct nesslerization method according to Imada et.al [11].

Estimation of protein content

The protein concentration was determined using Lawry method [12]. A calibration curve was made by using bovine serum albumin (BSA) as standard.

Molecular weight determination

Molecular weight of the purified L-glutaminase was estimated by Sephadx G-100 performed as described above using standard proteins (BSA, trypsin, pepsin, and catalase) and proteins standard curve [13].

Effect of pH on enzyme activity and stability

The optimum pH for enzyme activity and stability was determined. The enzyme was incubated for 1 hr at various pH values (4-11).

Effect of temperature on enzyme activity and stability

The optimum temperature for enzyme activity and stability were determined. The enzyme was incubated in different temperature values (10°C to 60ºC).

Influence of different effectors on L-glutaminase activity

The enzyme activity was determining in the presence of different metal salts and other compounds (10mM). Under the standard assay conditions, the enzyme was incubated for 60-min at 35ºC with the each effector. The relative activities were determined by considering 100% activity of enzyme in the absence of additives.

Kinetics parameters

Kinetic parameters were determined using the hydrolysis the substrate L-glutamine (0-20mmol/L) under standard assay conditions. The Michaels- Menten (km) constant and maximum velocity (Vmax). The reaction mixture was 0.02M tris-HCL buffer, pH 8.6. The values of Km and Vmax were then determining from line weaver-Burk plot [14].

Results and Discussion

Purification of L-glutaminase and molecular mass estimation

L-glutaminase from *Bacillus* sp.B12 was purified 23.9 folds, with yield of 45.4 (Table1) after sulfate fractionation (68%). Fast column chromatography was performed through the Ion exchange DEAE-Cellulose followed by Sephadex G-100 (Fig.1.) These results were in the agreement with L-glutaminase activity from *Bacillus* sp. LKG-01was purified to 49-fold with 25% recovery with specific activity 584.2 U/mg protein [15].

Table 1. sequential multi-steps process for purification of L-glutaminase from *Bacillus* sp. B12

Purification step	Volume (ml)		Total protein (mg/ml)	Specific a ctivity (U/ mg	Total activity (U)	Purification Fold	Recovery (%)
Culture supernant	45	3.6	1.1	3.2	162	1	100
Ammonium Sulphate (68%)	20	4.5	0.9	3.9	90	1.2	55.5
DEAE cellulose	13	6.2	0.6	10.3	80.6	3.2	49.7
Sephadex G-100	8.0	9.2	0.12	76.6	73.6	23.9	45.4

Characterization of purified L-glutaminase

Determination the molecular weight

The molecular homogeneity of purified L-glutaminase revealed a specific activity of 73.6 U/mg, and the molecular weight was estimated as 199 KDa (Fig.1). There has been wide variation in the molecular weight of L-glutaminase produced from different sources, for instance the strain *Pseudomonas aurantiaca* produced 148 kDa [16]. Also, Elshafei et al (2014) demonstrated that an intracellular L-glutaminase from *Penicillium breyicompactum* NRC829 showed molecular mass 71kDa [17].

While, the purified L-glutaminase from *Pseudomonas aeruginosa* 50071 presented the molecular weight 160kDa [18]. Similarly, the partially purified enzyme from *Bacillus cereus* MTCC 1305 showed an a proximal molecular weight 140 kDa [19]. Most of the L-glutaminase are monomers; however, the enzyme of *Erwinia carotovora* exists in the form dimmer with molecular weight 40.2kDa [20].

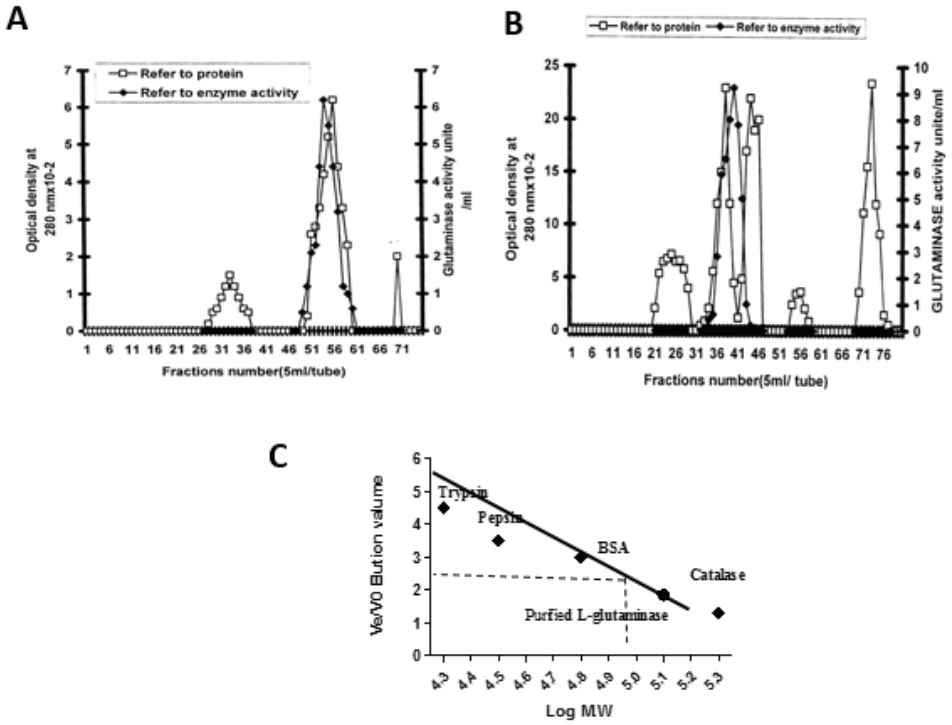

Figure 1. L-glutaminase purification and characterization.

A: Chromographic separation of L-glutaminase produced by *Bacillus* sp. B12. Elution diagram of L-glutaminase of *Bacillus* sp. B12 from DEAE-Cellulose. **B:** Chromographic separation of L-glutaminase produced by *Bacillus* sp. B12. Elution diagram of L-glutaminase of *Bacillus* sp. B12 from SephadexG-100. **C:** Native molecular mass estimation by gel filtration. Standard markers: Trypsin (23.3 kDa) Pepsin (34.5 kDa), BSA (66.4 kDa), Catalase (57.3 kDa). The molecular weight size marker (▲), molecular mass determination for enzyme (△).

Effect of pH and temperature on the activity and stability on the purified L-glutaminase

L-glutaminase is one of the amidases that are generally active and stabile at neutral and alkaline pH. Figure 4 revealed that pH. 8 was the optimal for L-glutaminase activity (3U/ml) (Fig.2) . In agreement with our results, Elshafei et al [17]reported that, pH. 8.5 to be the optimum pH for amidase activity. Continuously, an optimum pH range of 7.5 to 9 was found for L-glutaminase production from *Pseudomonas aeruginosa* [21].

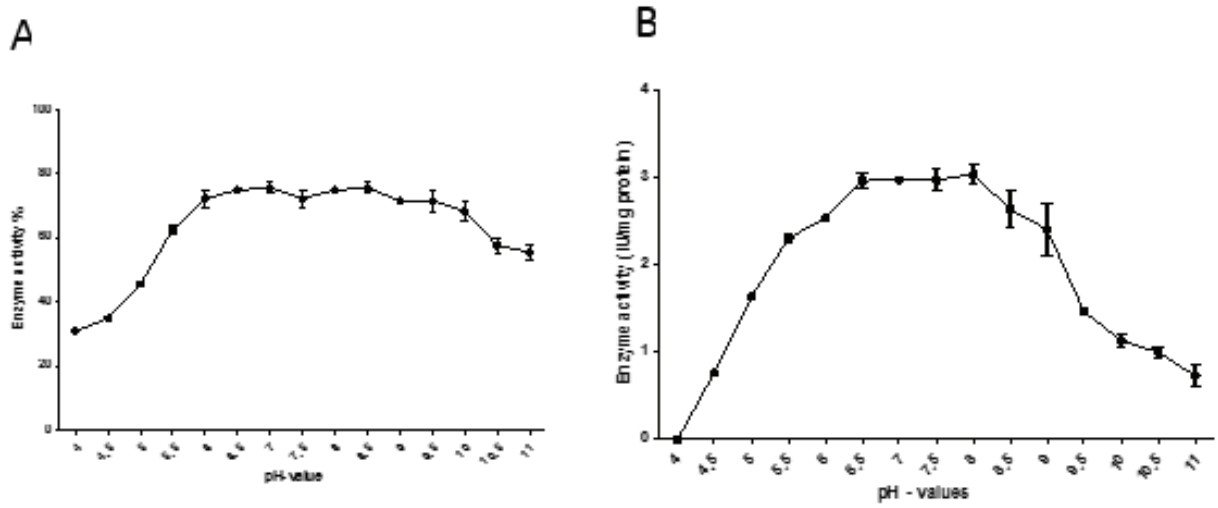

Figure 2. A: Determination of pH stability of purified L-glutaminase. B: Activity of purified L-glutaminase under different pH-values .

The purified L- glutaminase from *Bacillus* sp.B12 was more stable in alkaline pH more than acidic one; it returns more than 75% of the total activity in the pH range 6-10. Our results also demonstrated that L-glutaminase retained about 50 % of its activity after storage at pH 11 (Fig.2). Ohshima reported that, pH from 9.5 to 10 seems to be the most suitable pH for the storage this enzyme [22].

Effect of temperature and thermal stability behaviour

As far as the temperature dependence activity is concerned, many of the L-glutaminases reported to have both optimal and stable temperature of around 40°C to 50 °C. Based on the results, the purified L-glutaminase from *Bacillus* sp. B12 was active over a wide range temperature from 25°C to 50 ºC (Fig.3). L-glutaminase kept about 75% if initial activity after incubation at 40 °C. A notable decrease in enzyme activity was observed at higher temperature above this value, at 60ºC only 25% of L-glutaminase activity was still present. In this concern, Singh and Banik (2013) recorded that the purified L-glutaminase enzyme produced from *Bacillus cereus* MTCC 1305 showed greatest activity at 35C°.

The results of temperature effect on enzyme stability indicated that no significant enzyme activity was lost when it was pre-incubated at 55 °C for 60 min (Fig.3). Incontrast with our result, Elshafei et al (2014) reported that the purified L-glutaminase from *Penicillum brevicompactum* NRC829 showed stability in range temperature from 50°C to 60°C [17].

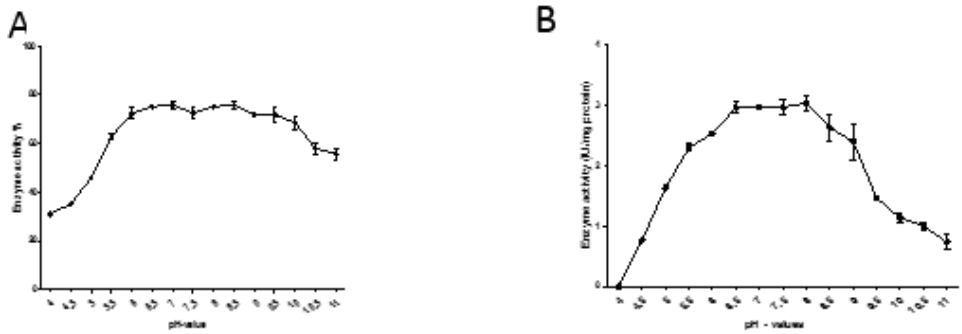

Figure 3. A: Thermal stability of purified L-glutaminase from *Bacillus* sp.B12. B: Determination of Temperature stability of purified L-glutaminase.

Determination of the kinetic parameters Km and Vmax

The kinetics parameters for L-glutaminase towards substrate was determined from the Lineweaver-Burk Plot. The affinity of the purified L-glutaminase to L-glutamine as substrate, with an apparent Km and Vmax values 0.4mmol/L and 0.133mmol/min, respectively (Fig.4). The low values of kinetic parameters (Km and Vmax) indicated high affinity of the enzyme to glutamine meaning that the rate will approach Vmax more quickly [23]. The results were in agreement with that reported by Singh and Banik (2013), they revealed that *Bacillus cereus* MTCC 1305 L-glutaminase has the high affinity to L-glutamine and presents a small Km (0.129 mmol)[19] . Also, Elshafei et al [17] recorded the highest activity of *Penicillum brevicompactum* NRC829 L-glutaminase towards L-glutaminase, with km value of 0.13 mmol .

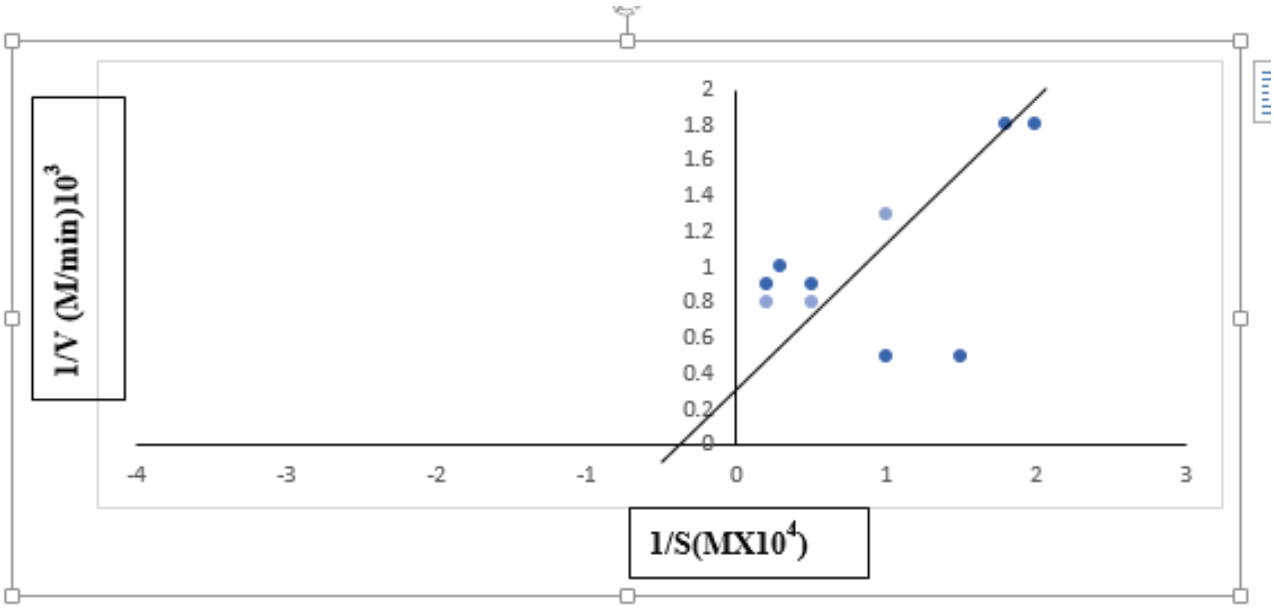

Figure 4. Lineweaver-Burk plot of L-glutaminase fraction of *Bacillus* sp.B12. The L-glutaminase fraction was incubated with different concentrations of L-glutamine (0-20mmol/L) as a substrate.

Effect of metal ions and inhibitors on the activity and stability on the purified L-glutaminase

The impact of various inhibitors and activators on prepare L-glutaminase was evaluated by pre incubation of the enzyme with each compound for 60 min without substrate. Results in Table 2 demonstrated a variation in enzyme activity, the presence of Ca^{2+}, Na^{1+}, Mn^{2+}, and Mg^{2+} enhanced the enzyme activity (Table 2). Also, the enzyme retained more than 90% of the total activity in presence of K^{+}, Ni^{2+}, and Co^{2+} . However, a considerable loss of activity was observed with the Sn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Fe^{2+} and they inhibit more than 50% of the purified L-glutaminase activity (Table 2). Constantly with our results, Singh and Banik (2013), reported that Na^{+}, K^{+} and phosphate ion activated the *Bacillus cereus* MTCC 1305 L-glutaminase; while, divalent cations Mn^{2+}, Mg^{2+}, Zn^{2+}, Pb^{2+}, Ca^{2+}, Co^{2+}, Hg^{2+}, Cd^{2+}, and Cu^{2+}inhibited its activity[19]. The Mn^{2+} (2mmol) acted as activator for L-asparaginase from *Rhizomucor miehei* [24].

Table 2. Effect of different metal cations salts & various compounds on L-glutaminase activity.

Metal salts	Concentration (mM)	Relative activity activity (%)
NiSO4	10	98
NaCl	10	106
MgCl2	10	112
CaCl2	10	104
MnCl2	10	109
KCl2	10	96
FeSO4	10	48
CuSO4	10	34
ZnSO4	10	33
HgCl	10	48
SnCl2	10	5
CoCl2	10	95
Cysteine	10	30
Sodium Azide	10	100
EDTA	10	95
PMSF	10	98
2-Mercaptoethanol	10	25

Moreover, the presence of 2-Mercaptoethanol and Cystein (10mM) acted as inhibitors of L-glutaminase activity, the activity was reduced to around 70% of the total activity. On the other hand, PMSF and EDTA kept more than 90% of the enzyme activity .While, Sodium azid has no effect on the enzyme activity, which indicates that L-glutaminase might not be metalloenzyme (Table 2). Inhibition in the presence of thiol group blocking reagents indicates that the enzyme has thiol group(s) in the catalytic site of the enzyme [25].

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Conflict of Interest: Nil

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