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Screening L-Glutaminase producing some *Pseudomonas* sp. isolated from contact lenses by Rapid Plate Assay

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ABSTRACT

L-glutaminase is enzyme led to decomposition glutamine to glutamic and ammonia in the presence of water. Twenty isolates were isolated from the eyes wearers' lenses in Jeddah city-Saudi Arabia. The bacterial ability to produce L-glutaminase was detected by rapid plate method and assayed by agar well diffusion method. Based on16S rDNA sequencing, the highest L-glutaminase producer strain was submitted under accession numbers as *(pseudomonas aeruginosa* B17 KX963365.1). The maximum enzyme production was performed at 35 °C in a shaking incubator, at 7pH after 24 hours by *pseudomonas aeruginosa*B17, and was partially purified. The enzyme was precipitable at 80% ammonium sulphate sedimentation; it was thermally stable at 30 °C and 7.5 pH. SDS-PAGE of purify L-glutaminase given (1) band with molecular weight (138.761kb).

Key words: *pseudomonas aeruginosa*, L-glutaminase, 16S rDNA.

1. INTRODUCTION

L-glutaminase stimulates hydrolysis L-glutamine to L-glutamic and ammonia. Microbial enzymes have been identified to play central roles as metabolic catalysts, led to using in many applications. End-user market for industrially enzyme is very widespread with frequent lucrative industrially productions (Adrio et al., 2005). As a result, today enzymes became one of mainly commodities, and its manufactures and productions in recent past consider as one major industry (Tiwari et al., 2015). L-Glutamine has demonstrated its abilities to significant inhabitation of spearing certain cancer cells, thereby raised possibilities of their applications such new alternative for chemotherapy (El-Gendy et al., 2017). Microbial L-glutaminase productions are easier to obtained, inexpensive, and fast, make it very suitable substitute for enzyme from high organism, throughout industrially application (Jesuraj et al., 2017). Many of the original producers of L-glutaminase are microorganisms as bacteria, yeast and filamentous fungi (Binod et al., 2017). L-glutaminase has been isolated from many microorganisms such as bacteria (Jersuraj et al., 2017 and Kumar et al., 2011), fungi (Bulbul and karakus, 2013), yeasts (Aryuman et al., 2015) and actinomycetes (Desai et al., 2016). These L-glutaminase microbes have higher stability than those in higher organisms (Dutt et al., 2014). *Pseudomonas aeruginosa* is ranked among the best bacteria producing good activity of L-glutaminase (Binod et al., 2017).

Our study aims were to conduct qualitative studies of L-glutaminase activity of some types of *Pseudomonas* sp producing L-glutaminase, quantifying the L-glutaminase activity by (agar well diffusion method) and determining the highest *Pseudomonas* sp. production of L-glutaminase.

2. MATERIALS AND METHODS

Ten samples were obtained from wearing different lenses and from used eye lenses in Jeddah, Saudi Arabia. Samples were obtained by sterile swabs to isolate *Pseudomonas* sp.

Isolation of *Pseudomonas* sp.:

For isolation and purification of *Pseudomonas* sp., the selective Cetrimide agar medium was used (Gelatinepeptone: 20.0, $MgCl_2$:1.4, K_2SO_4:10.0, Cetrimide: 0.3). Serial dilutions were performed for the samples. 0.1 ml of each diluent was transferred to the solid selective medium and incubated at 35 ° C. After 24 h of incubation, sub isolates were cultured on nutrient agar medium at 4 ° C for further studies (Ashdown, 1979).

Qualitative screening of L-glutaminase producing *Pseudomonas* sp. isolates by rapid plate method:

The bacterial isolates test for L-glutaminase producing using (rapid plate method) RPA on solid modified M9 medium (Na_2HPO_4. $2H_2O$: 6.0, KH_2PO_4: 3.0, NaCl: 0.5, L-Glutamine: 10.0, $MgSO_4$. $7H_2O$: 0.5, CaCl2: 0.5, Glucose: 20, PH: 7±0.2), Phenol red (2 % w/v in ethanol, 1 ml L-1) was added as pH indicator. After 24h of incubation at 35˚C, the cultures were examined for changing of the colour to pink around the colonies, it was identified as L-glutaminase producers (Gulati *et al.*,, 1997).

Quantitative estimation of L-glutaminase producing *Pseudomonas* isolates by agar well diffusion method:

The pure cultures of bacteria were used to inoculate in Glucose nitrate medium broth that supplemented with L-glutamine, the cultures were incubate on 35°C /24 h. Free-Cell filtrate was collected by centrifugate the culture filtrate at (4000 rpm) /thirty min. Quantitative assay for L-glutaminase in Free-Cell filtrate (CFF) was done by agar well diffusion method with glutamine as sole energy source. Glutamine agar media plates prepare by pouring 20 ml molten media in sterile petri dish. After solidify 8mm wells were punching using sterilized corkborer, a 100µ of cell-free filtrate was loaded in wells, the plates kept in upright position at 35˚C for 24-48 hrs the colour change to pink was measured. The highest L-glutaminase productivity isolates were chosen for further studies (Jain et al., 2012).

Partial Purification of L-glutaminase producing by *Pseudomonas* B17 isolate:

L-glutaminase purified according to Bilimoria, (1969). A solid (NH4) 2SO4 added to a supernatant liquid to achieve 20% saturation. Suspension centrifuging and solid (NH4)2SO4 add to supernatant till 40 and 80% saturations. Sediment is collected (0-20, 20-40 and 70-80%) by centrifugation. precipitates dissolve into Tris-HCL buffer, pH 8.0 and dialyzed overnight. Post exclusion of ammonium sulphate, enzymatic activities in every dialyzed solution was determined by nesslerization. Fragment showing above the activities of the enzyme designated as the partially purified extract.

Partially purified L-glutaminase Characterizations:

Temperature effects of on L-glutaminase activities

Enzyme keeps at several temperatures 10, 20, 30, 37,40,50,60 and 121° C before being added to the reaction mixer for assay. Specific enzyme activities calculated by measuring emitted ammonia, based on Nessler's reaction, using the Mashburn and Wriston (1963) method.

PH effects on L-glutamine activities

Tris-HCl buffer pH add in reaction mix of enzyme activities were adjust to 4-9 and optima pH determine through detected enzyme activities at every level.

Morphological, Physiological and Biochemical characterization of L-glutaminase producing *Pseudomonas* B17isolate:

Cultural characteristics and morphological properties were studied. Various microbial determination tests were performed according to the Bergey's Manual of Systematic Bacteriology (van Belkum, 2006).

Molecular characterization of bacterial isolate:

The JET-Thermo Fisher Scientific Genomic DNA Purification Group used to isolate genomic DNA. Amplikon of 1500 bp fragments representing the full length of the 16S rDNA gene amplify by highly preserved Universal primers as a flow (Edwards et al 1989). *Primers designed*: for PCR amplification: Universal primers (forward: (27F) 5'AGAGTTTGATCCTGGCTCAG-3 'and vice versa: (1492R 5'TACGGYTACCTTGTTACGACTT-3'. *PCR mixture:* 3µL DNA, 1µL per primer, 12.5µL master mix and 9.5µL dH2O sterile. *PCR amplification conditions:* Initial: 5 minutes at 94 ° C, denaturation: 1 minutes at 94° C, Annealing: 1 minute at 55 ° C and span: 2 minutes at 72 ° C, *Number of cycles:* 35. Final extension: 10 minutes at 72 ° C. Genetic sequence of DNA sequences and their comparison with GenBank to verify proximal evolutionary relatives using the BLAST algorithm and RDP database. In order to describe the strain, the nucleotide sequences for 16S rRNA and the phylogenetic tree created by Neighbour-Joining (N-J) method, based on the 16S rRNA sequences.

Purification of protein and SDS-Page

Ammonium sulfate add for culture filtrate which grow on glycerol glutaminase broth medium at 30ºC and 200 rpm, at 4°C to precipitate the L-glutaminase (Amena et al., 2010). Precipitates were collected by centrifugating (10,000 rpm / 20 min) and dissolve in 50 mM Tris-HCl buffer pH 8.6 and dialyze against same buffer. The concentrated enzyme solution was applied to the column of Sephadex G200 (1.5×45 cm) calibrated in advance, using 50 mM of Tris-HCl solution with a pH 8.6.Rinse protein was performed in the same solution at a flow rate regulator 3 ml / 30 minutes. Fraction activities were collect, dialysis and concentrating. Protein estimate (Lowry, 1951) use bovine serum albumin.

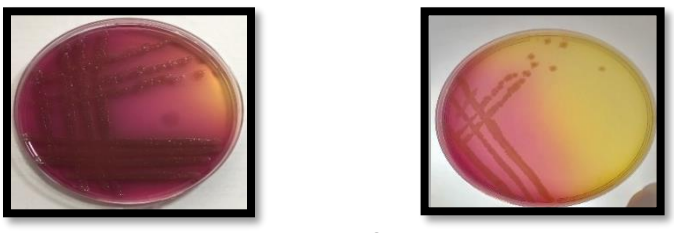
3. RESULTS

Isolation of *Pseudomonas* sp.:

From ten samples, twenty isolates from eyes lenses wearers and eleven from lenses, at Jeddah in Saudi Arabia. *Pseudomonas* sp. was isolated by using selective medium, Cetrimide agar.

Qualitative screening of L-glutaminase producing *Pseudomonas* sp. isolates by rapid plate method:

Results in (Fig1) showed that most of the bacterial isolates have ability to produce L-glutaminase, this result was observed for isolates *Pseudomonas* (B2, B4, B5, B8, B12, B16 and B17).



B17 **B2**

Figure 1 Qualitative screening of L-glutaminase producing *Pseudomonas* sp. isolates by rapid plate method

Quantitative estimation of L-glutaminase producing *Pseudomonas* isolates by rapid plate method (agar well diffusion method of CFF):

The quantitative assay of extracellular L-glutaminase was examined by agar well diffusion method of Cell-Free filtrate culture CFF provided of cultures supplemented with L-glutamine as a sole energy source in absence of carbon source, and other cultures with L-glutamine were used as nitrogen source and glucose as carbon source. Results in Figure (2) showed, the highest amount of L-glutaminase produced appeared after 12 hrs. of incubation and after 24 hrs. of incubation in CCF at 35±2C°, in absence and in presence of glucose as a carbon source. Figure (2-3) clear that sex isolates produced L-glutaminase in cultures supplemented with L-glutamine as the sole organic nitrogen source in presence of glucose better than without glucose. The biggest diameter of L-glutaminase zone of cell-free filtrate (CFF) was 27 mm of isolate *Pseudomonas*B17. The formation of red color around the well confirms that, extracellular L-glutaminase produced by all selective isolates.

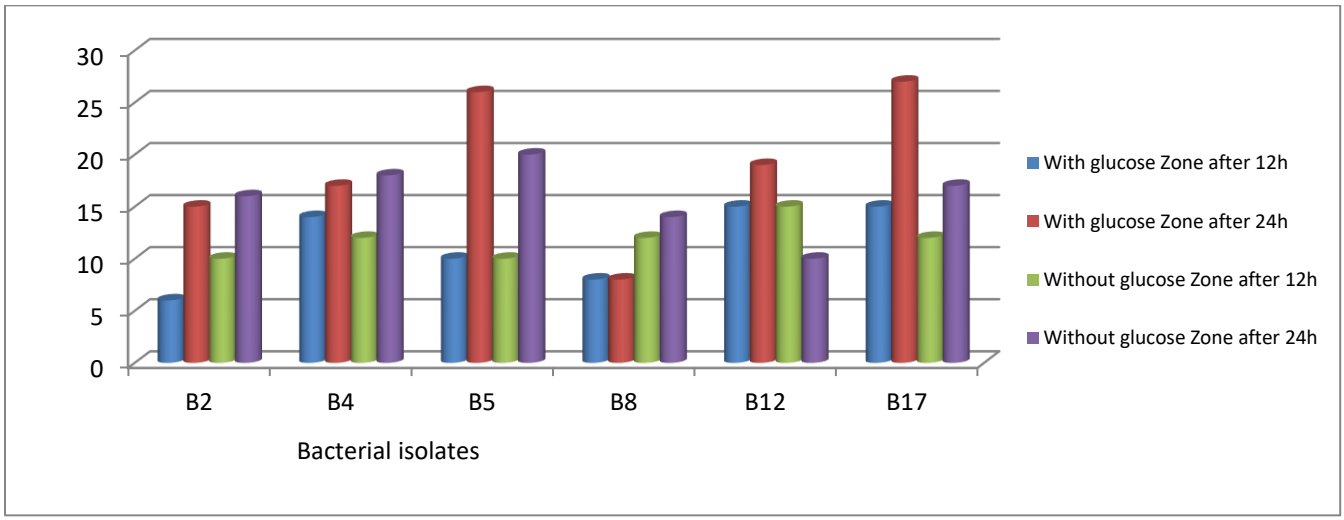


Figure 2 Quantitative estimation of L-glutaminase producingPseudomonas isolates by rapid plate method

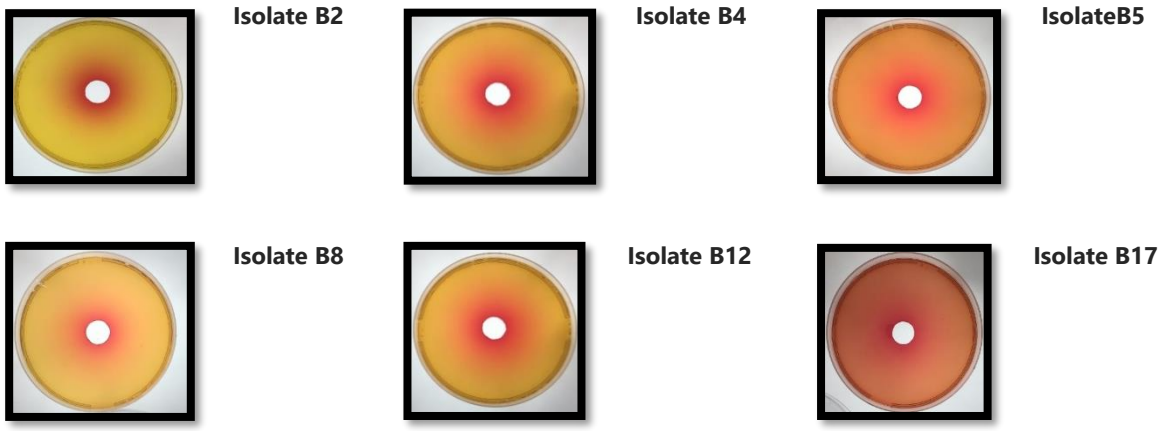


Figure 3 Quantitative estimation of L-glutaminase producing *Pseudomonas* isolates by rapid plate method

Characterization Partial Purification of L-glutaminase producing by *Pseudomonas* B17 Isolate:

The CFF of the isolate was used to purify the enzyme using ammonium sulfate. The fractions obtained by 80% of ammonium sulfate of CFF of the bacterial isolate.

Temperature and pH effects on L-glutaminase activities:

Characteristic of the partial purified L-glutaminase was conducted by studding its stability at different degrees of temperatures and several pH values. Results of the activity of the partial purified enzyme under various temperature ranges in Figure (4) shows enzyme activity of *Pseudomonas aeruginosa*B17 was slightly decreased by increasing temperature from 4 to 121°C. Highest enzyme activities

observed at 4°C 0.599 U\min\ml and the lowest at 121°C 0.302 U\min\ml. Results in Figure (5) show the effect of pH values 4, 5, 6, 7, 8 and 9 on L-glutaminase enzyme activities. Enzyme activities are increased by increasing pH values. Max enzyme activities at pH8 and decreased at above and lower.

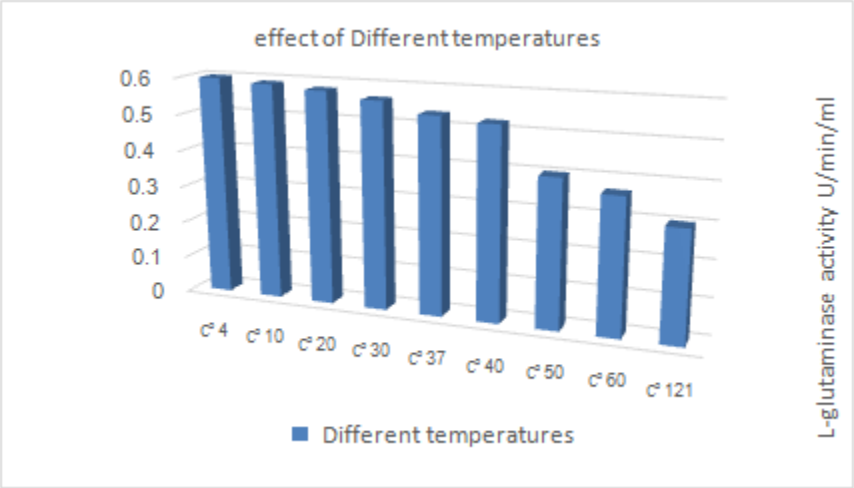


Figure 4 Temperature effects on L-glutaminase activities.

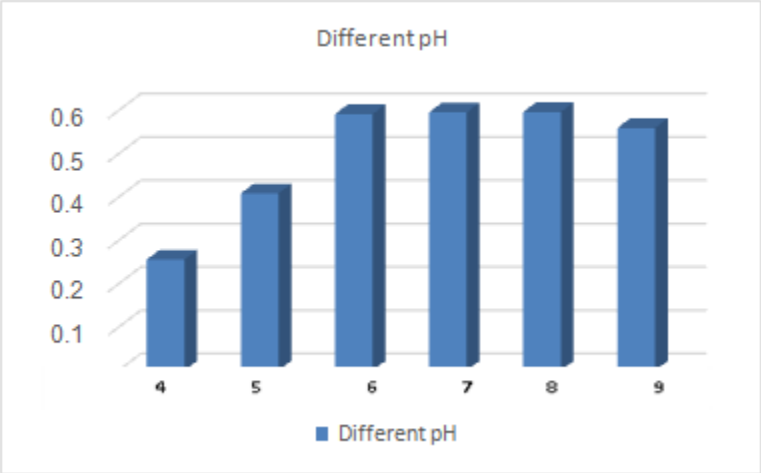


Figure 5 pH effect on L-glutaminase activities

Morphological, Physiological and Biochemical characterization of L-glutaminase producing *Pseudomonas* B17isolate:
Gram-negative, rod shaped, Non-spore-forming, Motile, Produce water soluble pigments. Tests for citrate, catalase and oxidase are (+). Indole and VP was (-) it does not ferment lactose or other carbohydrate but oxidizes glucose and xylose. Colonies are smooth, circular, umbonate, undulate pigmented colonies growth on nutrient agar at wide rang 6-42°C optimum 37°C.

Molecular identification of *Pseudomonas* sp. isolates based on 16S rRNA
Partial 16S rDNA sequences of the selected isolates were submitted into the Bacterial 16S ribosomal RNA sequence database as *Pseudomonas aeruginosa* (Table 1).

Purification of protein and SDS-Page:
The purified protein was downloaded on SDS-PAGE gel to confirm the molecular weight determination for four selected highest isolates *Pseudomonas aeruginosa*B4,*Pseudomonas aeruginosa*B8, *Pseudomonasaeruginosa*B12,and, *Pseudomonas aeruginosa*B17. The analysis of purified enzyme samples run on SDS-PAGE is presented in Figure (6) shows a separated band and, the comparison of this

separated bands with the protein marker (116 kD) revealed an apparent molecular weight of highest band by isolate *Pseudomonas aeruginosa*B12 (168kDa) of protein and so on the other isolates.

Table 1 Molecular identification of Pseudomonas sp. isolates based on 16S rRNA

Bacterial isolates	Name and Accession of No. of the most related strain in NCBI GenBank		Coverage (%)	Identity (%)
B17	KF413420.1	*Pseudomonas aeruginosa*	**100%**	**99%**

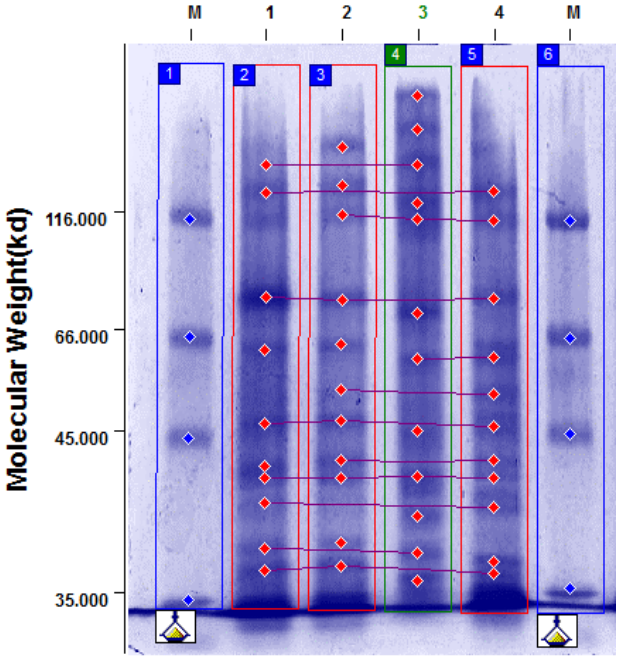


Figure 6 Purification of protein and SDS-Page
Figure shows: no.1 *Pseudomonas aeruginosa*B8, NO.2 *Pseudomonas aeruginosa*B4, NO.3 *Pseudomonas aeruginosa*B12 and, NO4.*Pseudomonas aeruginosa*B17.

4. DISCUSSION

L–glutaminase, has essential role in therapeutic enzyme and in food industry as a flavor agent (Binod *et al.*,, 2017). *Pseudomonas aeruginosa* was ranked one of the best bacteria producing L-glutaminase activity even though many of the original L-glutaminase from microorganisms such as bacteria, yeast, and fungi were isolated, purified, and differentiated (Binod et al., 2017).

According to (Karim and Thalij, 2016), L-glutamine production was assayed using the rapid plate method. The red phenol in the acid pH (yellow) and in alkaline pH (pink), pink color surrounds colonies that produce L-glutaminase. Our outcome was consistent with different researchers such as (Gulati et al., 1997 and Jyothi, 2011), and our results revealed that Pseudomonas B17 isolated from eye lenses was a potentially extracellular L-glutamine bacterium with high enzyme yield.

partial Purified L-glutaminase revealed a precipitate by ammonium sulphate maximum fold purification at 80% saturation in agreement with found by Jyothi et al., (2011). However, fractionation can very effective method of partially purifying enzymes (Chaplin and Bucke, 1990), precipitation of enzymes by ammonium sulphate is one of the best known and used methods of purifying and enzyme concentration. Enzyme given high stables on 30°C and pH 7.5 (Sinsuwan et al., 2012).

Most microorganisms have strong dependence on the extracellular pH of medium (Abu-Tahon and Isaac, 2016). Neutral pH gave maxenzyme yields (45.8 U/ml) comparing to remain pH. Max. L-Glutaminase activities obtained on pH 7.0 (Rashmi *et al.*,, 2012).

Based on morphological, Physiological, Biochemical and molecular characteristics, *Pseudomonas* sp.B17 isolate was identified as *Pseudomonas aeruginosa* with 100% identity percentages with our isolates (B17). The partial 16S rDNA sequence of the selected isolates were submitted into the Bacterial or Archaeal 16S ribosomal DNA sequences database under the accession numbers: MF777034.1

The post dialysis enzyme extract was subjected for SDS-PAGE to confirm molecular weight. L-glutaminase analyzed by SDS–PAGE. The molecular weight of the purified enzyme to *pseudomonas aeruginosa*B17 was found to be 138.761 kDa and it had a specific activities of 59.3 % IU/mg analysis of different step purified enzyme sample run on SDS-PAGE is presented. A separated band uniformly positioned in all purified enzyme samples. The comparison of this separated band with the protein marker Phosphorylase b (116 kD) by using SDS-PAGE a comparison study between *pseudomonas aeruginosa*B16, *pseudomonas aeruginosa*B4, *pseudomonas aeruginosa*B12, and *pseudomonas aeruginosa*B17 so *pseudomonas aeruginosa*B12 was found begets molecular Wight. L-glutaminase molecular weight estimate about 42KDa and 145KDa (Chasanah et al., 2013). Jyothi et al., (2011) found molecular weight for purify enzyme to pseudomonas-VJ7 is 37 kDa.

5. CONCLUSION

In this study, some *Pseudomonas* sp. Strains were obtained from eye wearers' lenses in Jeddah, Saudi Arabia, and examined for L-glutaminase production using a semi qualitative method. The highest L-Glutaminase producing *Pseudomonas* sp was selected and identified using morphological, biochemical and molecular characteristics. Moreover, the quantitative assay for extracellular L-glutaminase by the agar diffusion method was well investigated for CFF culture and used to partially purify the enzyme with ammonium sulfate. The effect of variants on the activity of partial purified enzyme was studied. L-Glutaminase was purified by a series of purification steps that included hemodialysis, SDS-PAGE to confirm the molecular weight.

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Ethical Approval

Ethical approval cleared by ethic committee of Department of Biology Sciences, Faculty of Science, University of Jeddah, Jeddah.

Conflict of Interest

The authors declare that there are no conflicts of interests.

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Data and materials availability

All data associated with this study are present in the paper.

Peer-review

External peer-review was done through double-blind method.

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