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Microbial genetics studies on L-glutaminase producer *Pseudomonas* NS16 isolated from eye contact lenses.

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Abstract: L-glutaminase is a significant enzyme which recognized chance application in biotechnology, industrial food, and therapeutic applications. L-glutaminase is an enzyme that prompts the decompose of glutamine into glutamic acid and ammonia within the presence of water. The bacterial isolates ability for L-glutaminase production tested on the modified mineral salt M9 (L-glutamine agar medium) and with phenol red added as the pH indicator. Colonies with pink-red zone around selected as L-glutamine degrading bacteria. Eleven bacterial isolates obtained from eye contact lenses of some persons in Jeddah city-Saudi Arabia; seven isolates produced good amounts of L-glutaminase. By Nesslerization assay, the highest L-glutaminase producer was *Pseudomonas* NS16 (50.4U/ml). Considering to the biochemical tests and identification by 16S rDNA sequencing, the most top L-glutaminase production strain was *Pseudomonas aeruginosa* CP012001.1. The incubation conditions and nutrition for the highest amounts of L-glutaminase were studied. The highest amount of the enzyme (52.5U/min/ml) created in media enhanced by.....gm/l glucose as carbon source and.....of the best nitrogen source glutamine, pH7.0 at 035°C after 24 hr of incubation.

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Keywords: L-glutaminase, glutamine, *Pseudomonas aeruginosa*.

1. Introduction

Microbial enzymes have distinguished to assume an essential role as metabolic catalysts, resulting in their utilization in different applications and production; industrial enzymes in the end-user market very popular with frequent lucrative industrial applications (Adrio *et al.,* 2005). therefore, enzymes today have gotten one of the significant wares, and their production and applications have shown up, in the ongoing past, as one of the significant industries (Tiwari *et al.,* 2015). L-glutaminase has appeared to inhibit the spread of some censer cell lines fundamentally, in this manner, expanding its chance utilized as an option in contrast to chemotherapy (El-Gendy *et al.,* 2017).

The enzyme has appeared to show a critical inhibit of activity against HIV replication in a recombinant form in vivo (Roberts *et al.,* 2001). L-glutaminase has a central role in the food industry, filling in food fermented as a flavour enhancer. Its catalytic product, glutamic acid, defines umami taste (Rastogi and Bhatia, 2019; Amobonye *et al.,* 2019). Microbial enzymes most of them take after the production of L-glutamines produces by microbial generally simpler, economically, and quickly, making them appropriate substitutes compared to enzymes taken from higher organisms, through various industrial applications (Jesuraj *et al.,* 2017). a few microorganisms like filamentous fungi, yeast, and bacteria have native L-glutaminase. Consequently, isolated, purified, and characterization, all things considered, the purification, heterologous expression, and characterization of recombinant L-glutaminase not many reports about it (Binod *et al.*, 2017). *Pseudomonas aeruginosa* classified from the best bacteria that produce high activity of the L-glutaminase enzyme (Binod *et al.,* 2017).

The aim of this study: qualitative Studies and quantitative assay of L-glutaminase activity by Nesslerization and molecular identification of the highest *Pseudomonas* strains productive of L-glutaminase.

2. Materials and Methods:

Twenty samples were obtained from different eyes contact lenses at Jeddah city, Saudi Arabia. The samples were obtained by sterilized swabs to isolate *pseudomonas* sp. Isolation of *Pseudomonas* sp. For isolation and purification of *Pseudomonas* sp., the selective Cetrimide agar medium used. Serial dilutions of the samples done. 0.1ml of each dilute transferred to the solid selective medium after that incubated at 35°C after 24h of incubation; the isolates were sub-cultured on nutrient agar medium for further

studies (Ashdown, 1979). The cultural and morphological properties of the isolates examined (Marteinsson *et al.,* 1997). The isolates obtained were preserved on nutrient agar in a slant at 4°C.

Qualitative screening of L-glutaminase producing *Pseudomonas* sp. bacterial isolates was tested for L-glutaminase production by (rapid plate method) RPA on solid M9 medium, Phenol red (2 % w/v in ethanol, 1 ml L-1) added as pH indicator. After 24h of incubation at 35˚C, the cultures examined for changing the colour to pink around the colonies; it identified as L-glutaminase producers (Gulati *et al.,* 1997). Quantitative assay of L-glutaminase by Nessler reaction L-glutaminase determined in the cell-free filtrate by Nesslerization (Imada *et al.*, 1973).

The identification of the highest L-glutaminase produced by *Pseudomonas* sp. the cultural characteristics, morphological, physiological, and biochemical properties investigated. Different microbial identification tests performed according to Bergey's Manual of Determinative Bacteriology (Brown, 1939)

Molecular characterization of bacterial isolate genomic DNA isolation using Gene JET Genomic DNA Purification Kit - Thermo Fisher Scientific. An amplicon of 1500 bp fragments representing the full length of the 16S rRNA gene amplified using highly conserved universal primers as flowing (Edwards *et al*., 1989) Primers designed: for PCR amplification: Universal primers (forward: (27F) 5'AGAGTTTGATCCTGGCTCAG-3' and reverse: (1492R) 5'TACGGYTACCTTGTTACGACTT-3'. PCR mixture: 3µL DNA, 1µL of each primer, 12.5µL Master mix, and 9.5µL sterile dH_2O. PCR amplification conditions: *Initial*: 5 min at 94°C, Denaturation: 1 min at 94°C, Annealing: 1 min at 55°C and Extension: 2 min at 72°C, Number of cycles: 35. Final extension: 10 min at 72°C. The obtained partial nucleotide sequences of the genes 16S rRNA were aligned using codon code aligner software (Barth *et al.,* 2007). The sequences of DNA phylogenetically analyzed and compared with those of GenBank to check for close evolutionary relatives using the BLAST algorithm and RDP database. To characterize the selected strain, the nucleotide sequence of the 16S rRNA was determined.

Preparation of inoculum of selected isolate, the colonies of a 24h culture of the bacterial isolate was inoculated in 10ml of a sterile seed medium (glucose nitrate medium) and incubated at 35±2ºC in shaking incubator at 150rpm for 24h. The number of bacterial cells in each ml of inoculums calculated by a dish method. The best nutrition and environment parameters L-glutaminase production by selected isolate, both environment and nutrition parameters were optimized (Keerthi *et al.,* 1999). The enzyme was assayed in CFF by Nesslerization. Studying the effect of nitrogen source on the production of L-glutaminase, various nitrogen sources yeast extract, peptone, glutamine, asparagine was added as 10g/L with glucose as the carbon source and without. The effect of carbine source, various carbon sources (20 g/L) were used, fructose, sucrose, lactose, maltose, glucose. The cultures incubated in an orbital shaking incubator (rpm 150) for 48 hours.

The best environmental parameters for L-glutaminase production were studied, like the effect of incubation type (shaking and static) were studied: two sets of each isolate of fermentation medium was inoculated with each isolate of bacteria, one of them was incubated in a static incubator, another was kept in a shaking incubator (150) rpm for 72h at 35˚C±2. The effect of incubation temperature on the production of L-glutaminase was studied. The cultures of the selected isolates were incubated at different temperatures (25- 30- 35- 40°C. The influence of pH on the L-glutaminase production, the selected isolate was grown in the fermentation medium at varying pH 6, 6.5,7, 7.5 and 8. After 48 hours of incubation at 35±2ºC. L-glutaminase was assayed by Nessler's reaction.

3. Results:

From twenty samples, eleven bacterial isolates of *Pseudomonas* sp. were obtained from eye contact lenses of some persons, in Jeddah Saudi Arabia. *Pseudomonas* sp. was isolated by using the selective medium, cetrimide agar. Qualitative screening of L-glutaminase producing of isolated *Pseudomonas* sp. by rapid plate method**.** Results showed that most of the bacterial isolates have L-glutaminase productive, these isolates were *Pseudomonas* (NS2, NS4, NS5, NS8, NS12, NS16and NS17). *Pseudomonas* NS16 and *Pseudomonas* NS17 isolates were showed higher activity than other isolates. Figure 1. obtained from used eye lenses.

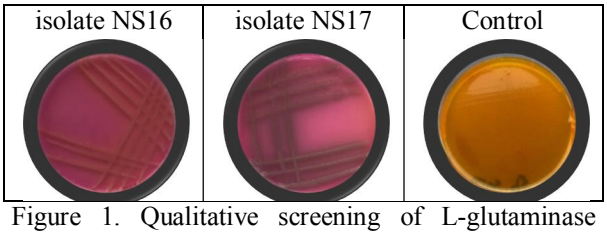

Figure 1. Qualitative screening of L-glutaminase producing *Pseudomonas* sp. isolates by rapid plate method.

Quantitative of L-glutaminase produced by bacterial isolates, with and without glucose as a carbon source was estimated by Nessler reaction.

Pseudomonas NS16 produced the highest amounts of L-glutaminase after incubation. Figure 2., for that this isolate selected for further studies.

Identification of the highest L-glutaminase producing *isolate Pseudomonas* NS16. Cultural, Morphological, and Physiological properties of *Pseudomonas* NS16 isolate shown in Table 1.

Molecular identification of *Pseudomonas* NS16 isolates based on 16S rRNA. The partial 16S rRNA gene sequence of the selected isolates was submitted into the Bacterial 16S ribosomal RNA sequences database under the accession numbers CP012001.1 for strain NS16 eyewear lenses as *Pseudomonas aeruginosa* Table 2.

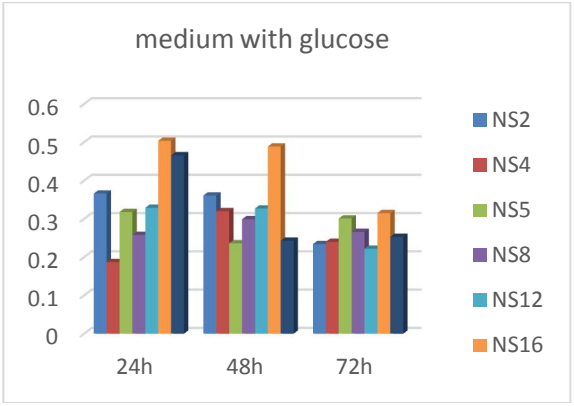

Figure 2. Assay L-glutaminase was producing *Pseudomonas sp.* Isolates by Nessler reaction after 24h.

Table 1. Morphological, Physiological, and Biochemical characterization.

Bacterial Isolate	Morphological Characterization	Physiological and Biochemical Characterization	Gram stain	Classification
Pseudomonas NS16 isolate	- rod-shaped -Motile -Produce water soluble pigments -Non-spore-forming	Colonies are smooth, circular, umbonate, undulate pigmented colonies growth on nutrient agar at wide rang 6-42°C optimum 37°C. Tests for citrate, catalase, and oxidase are (+). Indole and VP were (-) It does not ferment lactose or other carbohydrate but oxidizes glucose and xylose.	Gram-negative	Kingdom: Bacteria Phylum: Proteobacteria Class: Gammaproteobacteria Order: Pseudomonadales Family: Pseudomonadaceae Genus: *Pseudomonas aeruginosa* Species: *Pseudomonas aeruginosa* group

Molecular identification of *Pseudomonas* NS16 isolate based on 16S rRNA. The partial 16S rRNA gene sequence of the selected isolate was submitted into the Bacterial 16S ribosomal RNA sequences database under the accession numbers CP012001.1 for strain NS16 eyewear lenses as *Pseudomonas aeruginosa* Table 2.

Table 2. 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 (%)
NS16	CP012001.1	Pseudomonas aeruginosa	99%	99%

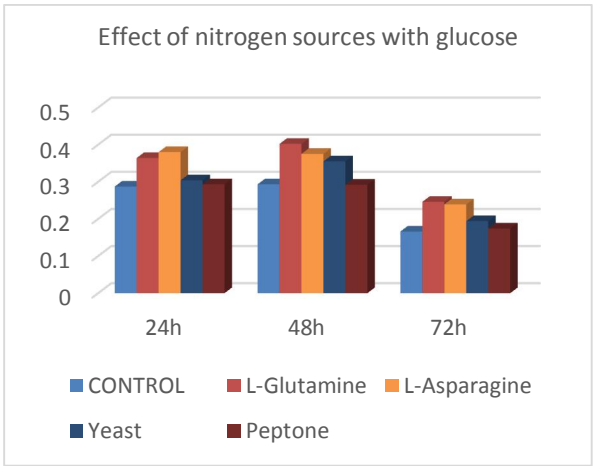

Figure 3. Effect of nitrogen sources on the production of L-glutaminase by *Pseudomonas aeruginosa*NS16 isolate.

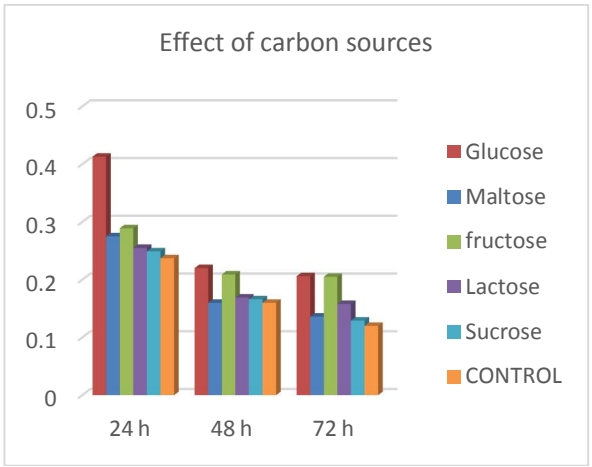

Figure 4. Effect of carbon sources on the production of L-glutaminase by *Pseudomonas aeruginosa* NS16 isolate.

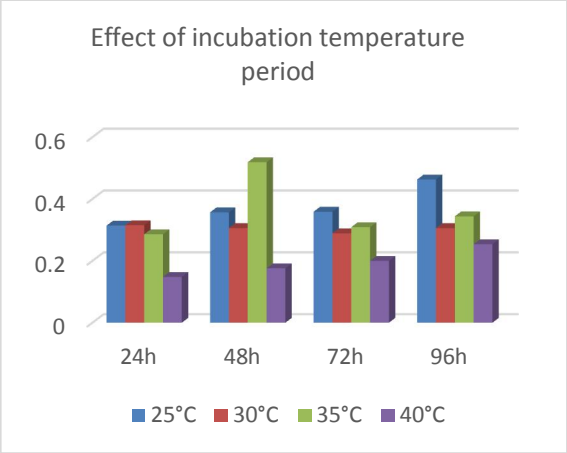

Figure 5. Effect of incubation temperature on the production of L-glutaminase by *Pseudomonas aeruginosa* NS16 isolate.

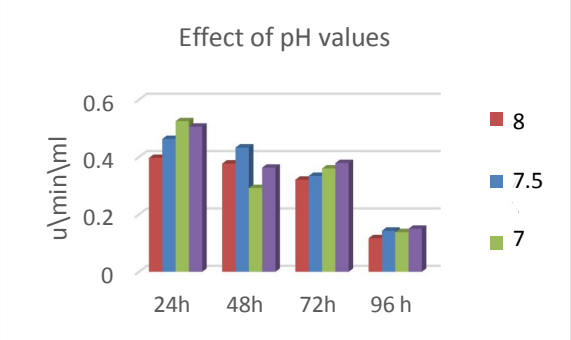

Figure 6. Effect of initial pH on the production of L-glutaminase *Pseudomonas aeruginosa* NS16.

The best environment and nutrition parameters for L-glutaminase production by *Pseudomonas aeruginosa* NS16, the result of studying the effect of nitrogen sources on the production of L-glutaminase by *Pseudomonas aeruginosa* NS16 isolate revealed, L- glutamine was the best nitrogen source for the production of the maximum amounts of L-glutaminase 0.412 U/min/ml with glucose as carbon sources and followed by L-Asparagine 0.380 U/min/ml results in Figure 3. The result in Figure 4. shows the highest amount of L-glutaminase produced by *Pseudomonas aeruginosa* NS16 isolate, when glucose used as carbon source, it was 0.413U/min/ml followed by fructose 0.289 U/min/ml after 24h at 35±2°C.

Results in Figure 5. show the highest production of the enzyme by selected isolate in cultures that incubated at 35±2C^{o}; it was 0.515 U\min\ml; this amount decreased at above or lowered this degree. results of the effect of pH on the production of L-glutaminase results in Figure 6. revealed, the productivity of L-glutaminase by selected isolate increased by increasing the alkalinity of the medium. The maximum L-glutaminase production in shaking incubation was 0.525 U/min/ml at pH7, the production of the enzyme decreased at above or lower than pH7.

4. Discussion

L–glutaminase is an amidohydrolase (3.5.1.2) enzyme, biologically Plays essential roles in the food industry as a procures, its significance as a flavour-enhancing agent and a therapeutic enzyme (Binod *et al.*, 2017). Hence, there is a potential requirement for bacteria producing extracellular L-glutaminase. Microorganisms were the best source to obtained all enzymes, fungi, actinomycetes and bacteria utilized for the production of L-glutaminase (Pandian, 2015). According to Karim and Thalij, (2016), L-glutaminase production assayed by using a rapid plate method. Phenol red appeared yellow at acidic pH and turns pink at basic pH. Subsequently, a pink zone conforms to produce L-glutaminase by microbial colonies. The results in agreement with different specialists (Gulati *et al.*, 1997) and (Jyothi *et al.,* 2011). Our results revealed that *Pseudomonas* NS16, isolated from eye lenses wearer was a potential extracellular L-glutaminase producing bacterium with a high yield of the enzyme.

In light of the morphological properties, cultural biochemical characteristics and Molecular analysis and identification using DNA sequencing of 16S rDNA showed that *Pseudomonas* NS16 isolate was like the variety of genus *Pseudomonas aeruginosa* NS16. similarity of 99% of our isolate, sequences with *Pseudomonas aeruginosa* CP012001.1 submitted to GenBank by other workers. Hence, the isolate was recognized as *Pseudomonas aeruginosa* and named as NS16. These results agreement with (Jyothi *et al.*, 2011).

The effect of various nitrogen sources on production of L-glutaminase by this isolate, our results revealed that the optimum nitrogen source L-glutamine between all tested nitrogen sources for the maximum amount of the L-glutaminase enzyme by *Pseudomonas aeruginosa*. Quite comparable results found it by other researchers (Banik and Singh, 2011; Sathish et al., 2008 and Mousumi and Dayanand, 2013) a range of biosynthetic procedures, and additionally repeatedly involved with binding sites or protein active. Also agreed with Desai et al., (2016), who reported production of L-glutaminase by *Streptomyces* sp. was seen to be optimum with Dayanand, (2013) portrays glutamine has amide nitrogen is a source of amino groups in wide rang the glutamine as a nitrogen source. *Pseudomonas* sp. requires the addition of amino acid to the medium for synthesizing extracellular L-glutaminase. Probably

amino acids added to the fermentation medium plays the role of inducer for L-glutaminase synthesis for these bacteria (Jyothi *et al*., 2011), our result agrees with this result.

Glucose was the better Carbone source for the highest production of the enzyme. Also, (Jyothi *et al.*, 2011) and (Desai *et al.*, 2016) discovered that glucose was the best Carbone source to amplify L-glutaminase production. Results found by about the best carbon source was glucose. *Pseudomonas aeruginosa* NS16 produced the most elevated L-glutaminase in cultures enhanced with glucose and incubated in shaking incubator 0.515 U/min/ml than that incubated in the static incubator. Our results agree with that found by (Jyothi *et al*., 2011, Durthi *et al.*, 2019, Sathish *et al.,* 2018). (Jyothi *et al.*, 2011) reported that cultures in broth should always shake to ensure ventilation, oxygen, and nutrient availability as well as to avoid bacterial dominion at the bottom of the flask. It leads to cell death due to a lack of nutrient availability and prevents bacteria clumps or the formation of biofilms, ensuring prolific bacterial multiplication.

Different incubation periods at 35°C showed maximum productivity of the enzyme after 48 h of incubation and decreased by increasing incubation period. our result disagrees with (Prakash *et al.*, 2009)and (Jyothi *et al.*, 2011) who found the best incubation period for the highest amount of the enzyme by *Pseudomonas* sp. after 72h, while (Durthi *et al.,* 2019) found the maximum amount of the enzyme produced after 32 h of incubation. (Nagaraju and Ram, 2019 and Prakash *et al.,* 2009) find the most extreme enzyme production observed at 72 hrs. of incubation.

Most of the microorganisms have an important characteristic that is a strong dependence on the extracellular of medium pH (Abu-Tahon and Isaac, 2016). the maximal enzyme yield was produced in Neutral pH (45.8 U/ml) while contrasted with the rest of pH values. L-Glutaminase activity maximum was gained at pH 7.0 by (Rashmi *et al.,* 2012, Van Elsas *et al.,* 2006).

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