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Optimization of culture conditions for production of L-glutaminase enzyme from *Klebsiella pneumoniae*

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ABSTRACT

The main objective of this study was to investigate the production and activity of glutaminase by some clinical bacterial species. Two marine specimens, saltwater and sediment gathered from the Red Sea coast of Hurghada, Egypt, yielded 100 marine bacterial isolates altogether. Based on cultural and biochemical testes, there were 60 isolates of Gram-negative bacteria belong to the Escherichia coli, including 10, isolates of *Pseudomonas aeruginosa*, 25 isolates of *Klebsiella pneumoniae* (*K. pneumonia*) and 2 *Acinetobacter junii*. Whereas the remaining isolates were identified as Gram-positive bacteria were distributed as 3 *Enterococcus faecalis*. All the bacterial isolates were screened for L-glutaminase enzyme activity using rapid plate assay. The isolate showing the highest production of L-glutaminase was identified by 16S rDNA gene sequence analysis. Then L-glutaminase production was optimized by various process parameters such as: The effect of incorporation of additional carbon source, nitrogen source, different concentrations of sodium chloride (0.2-1.2 %), initial pH values (4-9), incubation temperatures (25-50°C), and incubation periods (24-120 h.). Fifty isolates were found to be L-glutaminase producers. The zone index was calculated for all L-glutaminase producing samples which are ranged from 5.0 to 1.0. The maximum zone index was given by *K. pneumonia*. The enzymatic activity was ranged from (82.75±4.71-15.7±0.86) IU/ml. The maximum activity was recorded by *K. pneumonia*. Various parameters that enhance the yield of L-glutaminase by *K. pneumonia* was investigated and resulted in fructose was the best carbon source, beef extract was the best nitrogen source, 1% of NaCl concentration was the optimum for L-glutaminase production from *K. pneumonia*, the optimum L-glutaminase production was recorded at pH 8.0, The maximum enzyme productivity was obtained at 96 h., Maximum L-glutaminase production was noticed at a temperature of 40°C. In conclusion, the current study showed microbial source of glutaminase enzyme production from *K. pneumonia* pure culture. Nutritional parameters, such as carbon and nitrogen sources also played an important role in enhancing the yield.

Introduction

Enzymes produced by microbes play an important role in the diagnosis, biochemical investigation, curing, and monitoring of some diseases. Microorganisms are an important source of many therapeutic enzymes because of their biochemical diversity and their susceptibility to genetic manipulation. The production of enzymes for use as drugs is an important tool for today's pharmaceutical industry (Saptarshi and Lele, 2011). Amidohydrolase group of enzymes has a wide application due to their diverseness and peculiarities. However, since the anticancer activity of L-glutaminase enzyme in the microbial system was reported, there is a wide scope for various new microbial sources (Patel *et al*., 2020). L-glutaminase has been cited as the most potent molecule that inhibits proliferation of cancer cell, which significantly that raises the possible applicability for cancer therapy, and the possibility of its application as an alternative drug to chemotherapy (El-Gendy *et al*., 2017). L-glutaminase is an amide enzyme which has an important contributory role in all living cells in cellular nitrogen metabolism (Kiruthika and Saraswathy, 2013). L-glutamine amidohydrolase (EC 3.5.1.2) is the most unequalled enzyme that is adherent to β-lactamases family (serine dependent) and penicillin-binding proteins due to its higher affinity for polymerizing and modifying peptidoglycan biosynthesis that is essential for bacterial cell wall synthesis (Irajie *et al*., 2016). Furthermore, the enzyme is categorized based on catalytic efficiency as a type of proteolytic endopeptidase enzyme that causes cleavage of peptide linkage and produces ammonia and glutamate as a by-product. The L-glutaminase enzyme plays an important role in the assimilation of nitrogen molecules and their related compounds (Binod *et al*., 2017). Additionally, the L-glutaminase enzyme was known for its application in the food industry as a flavour enhancer (Rastogi and Bhatia, 2019). There are many sources of L-glutaminase enzyme including plant, animal and microbial sources. From these sources, microbial sources are the most preferred sources due to their ease in cultivation and economic large-scale production. Microbial sources mainly involve, bacteria (Kiruthika *et al*., 2018; Ramli *et al*., 2020), fungi (Bazaraa *et al*., 2016) and actinomycetes (Aly *et al*., 2017). L- glutaminase commercial production is carried out using submerged fermentation (SmF) technique. Also, solid state fermentation (SSF) has emerged as a promising technology for the development of several bioprocesses which include a large-scale production of industrial enzymes (Athira *et al*., 2014).

A recent trend at a large scale is shifting fermentation by submerged technique for all such enzymes. Although there are many advantages of using solid-state fermentation compared to submerged fermentation. Mainly lower water content and low chances of contamination, substrates are cheap and easily available (Astolfi *et al*., 2019). There are few reports showing a higher yield of the product by solid-state fermentation (Chahande *et al*., 2018). The main objective of this study was to investigate the production and activity of L-glutaminase by some clinical bacterial species.

Materials and methods

Collection, isolation, and identification of isolates

Two marine specimens, saltwater and sediment gathered from the Red Sea coast of Hurghada, Egypt, yielded 100 marine bacterial isolates altogether. The samples were collected, and transferred immediately to

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the laboratory (virology lab, faculty of science, Zagazig University) for culturing on Nutrient agar and MacConkey agar, then incubated at 37ºC for 24 h. Colonies were purified and used for identification tests. All bacterial isolates were examined by biochemical tests according to Bergey's manual of determinative bacteriology (Holt *et al*., 1994).

Biochemical identification

By using VITEK technique for identification, the culture was maintained on Nutrient agar medium slants. Inoculated slants were grown in an incubator at 37ºC for 24 h. After that the slants were stored at 4ºC in a refrigerator for short term preservation and sub cultured every 15 days on the abovementioned medium.

Qualitative production of L-glutaminase Enzyme (Screening Test and Rapid Plate Assay)

The minimal agar media (g/l of distilled water) contains: NaCl, 0.5; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 1; $FeSO_47H_2O$, 0.1; $ZnSO_47H_2O$, 1; L-glutamine, 0.5: as nitrogen source, and supplemented with 2.5% phenol red dye (prepared in ethanol and the pH was adjusted to 7.0). Control plate was prepared without glutamine (containing NaNO3 instead of glutamine as a nitrogen source). After autoclaving, the prepared media were inoculated with 24 h, old bacterial colonies then incubated at 37ºC for 24 h. The pink zone around bacterial colonies were observed, and the zone index was calculated (Gulati *et al*., 1997).

Quantitative screening of the different bacterial isolates for their glutaminase activity

The main goal of this experiment was to evaluate the potential activity of the isolated bacterial species collected during this study for production of glutaminase in their culture media. The bacterial species isolated were grown on synthetic liquid media containing 1%glutamine as sole nitrogen source. 24 hours old bacterial cultures were used as standard inoculum for each of the tested bacterial isolates. At the end of the incubation period (4 days), the bacterial cultures were filtered, and their filtrates were used for assay of glutaminase activity.

Molecular identification of selected Klebsiella pneumoniae isolate

The isolate showing the highest production of L-glutaminase was identified by 16SrDNA gene sequence analysis using BLASTN software for sequence alignment.

Extraction of DNA according to QIA amp DNA mini kit instructions

QIAamp DNA Mini Kit

The QIAamp DNA Mini Kit (Catalogue no.51304) provides silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization, so total hands-on preparation time is only 20 minutes.

Polymerase chain reaction (PCR)

Emerald Amp GT PCR master mix (Takara)Code No. RR310A, Contained Emerald Amp GT PCR master mix (2x premix), and PCR grade water.

Oligonucleotide primers used in cPCR

They have specific sequence and amplify a specific product (130 bp) and were designed according to Turton *et al*. (2010).

Cycling conditions of the primers during cPCR

Temperature and time conditions of the primers during PCR were according to Turton *et al.* (2010), and Emerald Amp GT PCR master mix (Takara) kit.

DNA molecular weight marker

Gene ruler 100 bp DNA ladder (cat. no. SM0243) supplied from Ferments (Number of bands: 10; Size range: 100-1000 bp). The ladder was mixed gently by pipetting up and down. 6 µl of the required ladder were directly loaded.

Agarose gel electrophoreses (Sambrook et al.,1989) with modification.

Twenty µl of each uniplex PCR product samples, and 40 µl of each duplex PCR product samples negative control and positive control were loaded to agarose gel (1.5 %). The power supply was 1-5 volts/cm of the tank length. The gel was photographed by a gel documentation system and the data was analysed through computer software.

Inoculum preparation of L-glutaminase

The inoculum of L-glutaminase producing isolates was prepared in 250 ml Erlenmeyer flasks which contain 100 ml of the abovementioned media at pH 7.0. The medium was autoclaved at 121 ºC for 15 min., then the medium inoculated with the bacterial isolate. The inoculated flasks were kept on a shaker at 150 rpm for 24hrs., then used as an inoculum.

Optimization of L-glutaminase production

Various process parameters that enhance the yield of L-glutaminase by *K. pneumonia* was investigated. The effect of incorporation of additional carbon sources (glucose, fructose, sucrose, lactose, maltose, and soluble starch at 1% w/v) and nitrogen sources (peptone, yeast extract, beef extract, urea, and sodium nitrate and amino acids (l-glutamine at 1% w/v) were investigated. Moreover, the effect of different concentrations of sodium chloride (0.2-1.2%), initial pH values (4–9), incubation temperatures (25–50°C), and incubation periods (24–120 h.) was studied. During this optimization process, when a particular parameter was optimized, the optimum condition of that specific parameter was used in the subsequent studies where another parameter is to be optimized. L-glutaminase activity for all the extracts obtained from the above-mentioned parameters was studied. All experiments conducted in triplicate, and the mean values were calculated.

Detection of L-glutaminase activity in culture filtrates

The release of ammonia from glutamine substrate was used as a standard assay for detection of enzyme activity.

Crude enzyme preparation

At the end of the incubation period the bacterial cells are harvested in refrigerated centrifuge at 8000 rpm for 20 min at 4°C. The supernatant was the source of crud enzyme and used for further enzymatic assay procedures.

Determination of enzyme activity

L-glutaminase activity was assayed according to Imada *et al*., (1973). The reaction mixture, containing; 0.5ml of an enzyme preparation,0.5 ml of L-glutamine (0.04 M), 0.5 ml of phosphate buffer 0.1 M (pH 8.0), and 0.5 ml of distilled water to reach a total volume of 2ml solution. Then this

solution was incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5 ml of 1.5 M Trichloro acetic acid. Then, we add 0.1 ml of the above mixture to 3.7 ml of distilled water and 0.2 ml of Nessler's reagent was added then, the colour developed was read after the mixture was kept for 20 min at 20°C at 450 nm in a spectrophotometer. Enzyme and substrate blanks were used as controls. One unit of L-glutaminase activity was defined as the amount of enzyme that liberate 1 µmol of ammonia per one minute under optimum assay conditions. Assays were done in triplicate and the mean enzyme activity was expressed as International Unit per ml (IU/ml).

Protein estimation

Protein content in the crude enzyme source was estimated by Lowry's method (Lowry *et al*., 1951) using bovine serum albumin as the standard, and the values were expressed as mg/ml.

Statistical analysis

Data were edited in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). A Shapiro-Wilk test was conducted in order to check for normality as described by Razali and Wah Data (2011). The significant effects of the treatments were examined according to the One-way a nova (PROC ANOVA; SAS Institute Inc., 2012) with the level of significance set at $\alpha = 0.05$. Results were expressed as means ±SE. Tukey's' test was used to perform pairwise comparisons between means in case of a significant effect was detected. Statistical significance between means was set at p-value less than 0.05. Figures were fitted by the GraphPad Prism software 9.0 (GraphPad, USA). Data were expressed as means ± SD. The mean values were calculated based on the data taken from at least three independent experiments (n= 3). Statistical analysis was performed by using student's t-test. Differences were considered significant at $P > 0$.

Results

Identification of Isolates

In the present study, the rapid plate method was used to identify strains of the 50 marine bacteria that produce L-glutaminase. The bacterial isolates produced measurable pink colour zone around the colony on minimal glutamine agar (MGA) medium that was proportional to their ability to produce L-glutaminase. The quantitative screening for L-glutaminase production revealed that these isolates produced L-glutaminase with a range activity of 82.75±4.71-15.7±0.86IU/ml.

Biochemical identification

The bacterial isolates were identified by Vitec technique giving that the isolate belongs to genus *Klebsiella pneumoniae*, *Acinetobacter junii* and Escherichia coli.

Qualitative production of L-glutaminase Enzyme (Screening Test and Rapid Plate Assay)

Fifty isolates were found to be L-glutaminase producers. The pink zone around bacterial colonies were observed, and the zone index was calculated for all L-glutaminase producing samples which are ranged from 5.0 to 1.0.

Quantitative screening of the different bacterial isolates for their glutaminase activity

Under experimental conditions it can be demonstrated that all experimental bacterial species grew on the tested media. The results obtained showed that the glutaminase activity varied not only among the different bacterial genera but also between different species of the same genus. The highest activity of glutaminase enzyme was obtained from the culture filtrate of *Klebsiella pneumoniae* (82.75±4.71 U/ml crude enzyme) followed by *E. coli* then *Acinetobacter junii* came in the last rank of glutaminase activity (21.5±1.02 U/ml crud enzyme); in descending order after *Klebsiella pneumoniae* and *E. coli*.

Molecular identification of selected K. pneumonia isolate.

The isolate that showed the highest production of L-glutaminase (18.5 IU/ml) was selected for further study. To confirm the identification of the selected isolate, 16SrDNA gene sequence analysis was performed. The sequence alignment using BLASTN software for the comparison of up to 1500 bp indicated that the 16S rDNA gene sequence of the selected strain shows that GenBank accession number of *Klebsiella* (OQ703039) show a similarity of 98.9%. Therefore, our isolate was identified as *Klebsiella pneumoniae* and named *K. pneumonia* AS KP 23.

Optimization of L-glutaminase production

Effect of carbon sources

The results illustrated in Figure 1a showed that the incorporation of additional carbon sources enhanced the enzyme yield from 49 to 247.93 IU/ml by the tested bacterium. Among the various sugars tested for their effects on the L-glutaminase production, fructose was found to be the best carbon source, yielding maximum L-glutaminase production (247.93 IU/ml) followed by glucose (162.6 IU/ml).

Effect of different fructose concentrations on productivity of L-glutaminase by *K. pneumonia*

The result represented in Fig. 1b revealed that, the utilization of fructose by *K. pneumonia* for glutaminase production was found to be concentration dependent. The highest yield of L-glutaminase was determined in culture filtrates of *K. pneumonia* (637.6 u/mg protein) at 10 g/l fructose. By increasing fructose concentration there is no significant increase in glutaminase production by *K. pneumonia*.

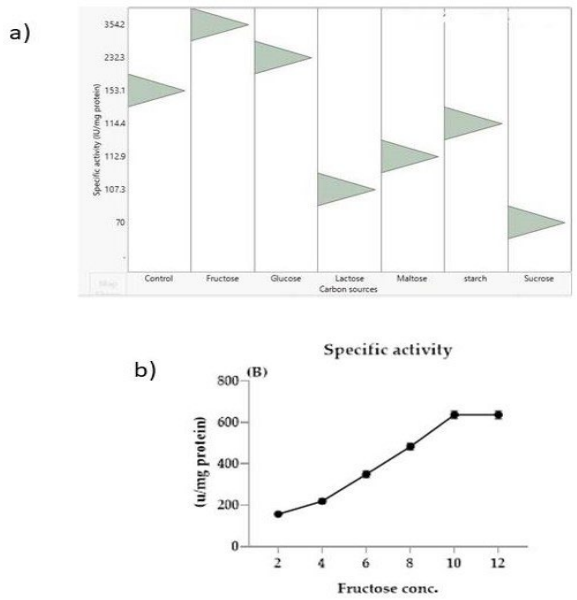

Fig. 1. a) The effect of carbon sources on L-glutaminase production from *K. pneumonia* indicating that fructose is the most favourable carbon source, b) Effect of different fructose concentrations on productivity of L-glutaminase by *K. pneumonia*.

Effect of nitrogen sources

The results in Figure 2a showed that the effect of the addition of different nitrogen sources on enzyme production level. These results revealed that the enzyme yield enhanced from 61.9 to 194.6 IU/ml. Among the various nitrogen sources tested for their effects on the L-glutaminase production, beef extract was found to be the best nitrogen source, yielding maximum L-glutaminase production (194.6 IU/ml) followed by peptone (131.5 IU/ml).

Effect of different Beef extract concentrations on productivity of L-glutaminase by *K. pneumonia*

The obtained results in Figure 2b indicated that the level of beef extract in the tested fermentation media exerted a marked effect on glutaminase enzyme productivity by *K. pneumonia*. The data also showed that the biosynthesis of L-glutaminase is concentration dependent. L-glutaminase production by *K. pneumonia* increased gradually with increasing the level of beef extract in the culture media to 1% followed by decreasing in glutaminase production by increasing concentration of beef extract. Therefore, 1% of beef extract will be used in the subsequent optimization processes.

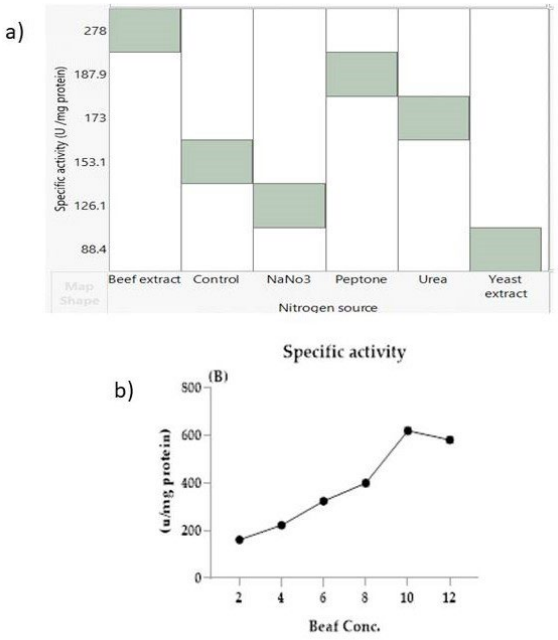

Fig. 2. a) The effect of nitrogen sources on L-glutaminase production from *K. pneumonia*e indicating that beef extract is the most favourable nitrogen source. b) Effect of different Beef extract concentrations on productivity of L-glutaminase by *K. pneumonia*.

Effect of sodium chloride concentrations

The results presented in Figure 3a showed that the activity of L-glutaminase was increased with increasing NaCl concentration up to 1% as maximum as 262.5 IU/ml and it was low in 0.2% and 0.8% of NaCl concentrations. Yield was decreased, when the concentration was increased above 1%. Hence, 1% of NaCl concentration was the optimum for L-glutaminase production from *K. pneumonia*.

Effect of initial pH

In the present study, the optimum L-glutaminase production reached 114.7 IU/ml was recorded at pH 8.0 and any further alteration either increase or decrease of the medium pH negatively influenced the enzyme production. Critical analyses of the L-glutaminase productivity values suggest that alkaline conditions are more supportive compared to the acidic environment during fermentation by this isolate (Fig. 3b).

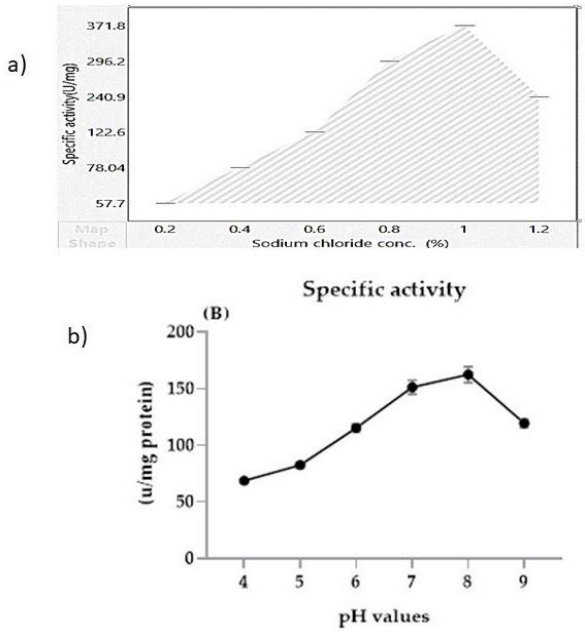

Fig. 3. a) The effect of sodium chloride concentrations on L-glutaminase production from *K. pneumonia*e indicating that the concentration of 1% is the most favourable NaCl concentration. b) The effect of initial pH on L-glutaminase production from *K. pneumonia* indicating that pH 8 is the most favourable pH for L-glutaminase production.

Effect of temperature

Growth temperature is another critical parameter that needs to be controlled. Maximum L-glutaminase production of 158.3 IU/ml was noticed at a temperature of 40°C. Variation of the temperature on either side of this resulted in decrease in L-glutaminase production (Fig. 4a).

Effect of incubation period

The maximum enzyme productivity (544.9 IU/ml) by *K. pneumonia* was obtained at 96 h. of cultivation period. After that, the enzyme production decreased, suggesting its association with growth parameters. It is noteworthy to state that by optimizing the above-mentioned components and culture conditions, the production of L-glutaminase has been increased (Fig. 4b).

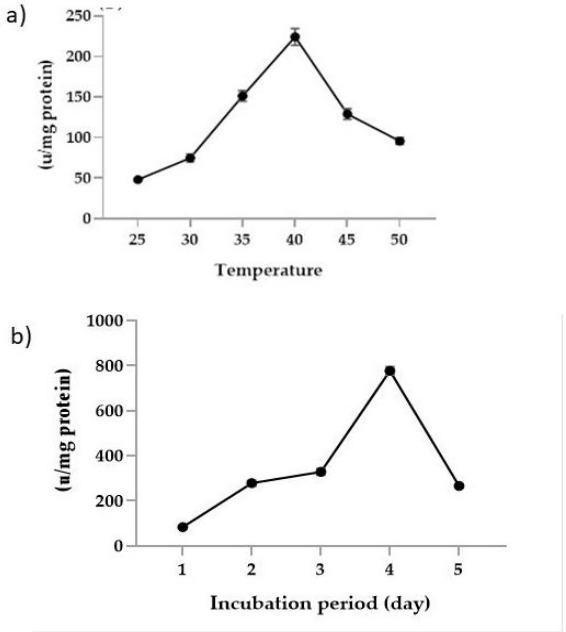

Fig. 4. a) The effect of temperature on L-glutaminase production from *K. pneumonia* indicating that 40°C is the most favourable temperature for L-glutaminase production. b) The effect of incubation period on L-glutaminase production from *K. pneumonia* indicating those 4 days is the most favourable period for L-glutaminase production.

Effect of different concentrations of Zinc sulphat on productivity of L-glutaminase by *K. pneumonia*

The obtained results in (Fig. 5a) indicated that the level of Zinc sulphat in the tested fermentation media exerted a marked effect on glutaminase enzyme productivity by *K. pneumonia*. The data also showed that the biosynthesis of L-glutaminase is concentration dependent. L-glutaminase production by *K. pneumonia* increased gradually with increasing the level of Zinc sulphat in the culture media to (1 g/l) followed by decreasing in glutaminase production by increasing concentration of Zinc sulphat. Therefore, (1 g/l) of Zinc sulphat will be used in the subsequent optimization processes.

Effect of different concentrations of magnesium sulphat on productivity of L-glutaminase by *K. pneumonia*

The obtained results in (Fig. 5b) indicated that the level of magnesium sulphat in the tested fermentation media exerted a marked effect on glutaminase enzyme productivity by *K. pneumonia*. The data also showed that the biosynthesis of L-glutaminase is concentration dependent. L-glutaminase production by *K. pneumonia* increased gradually with increasing the level of magnesium sulphat in the culture media to (0.5 g/l) followed by decreasing in glutaminase production by increasing concentration of magnesium sulphat. Therefore, (0.5 g/l) of magnesium sulphat will be used in the subsequent optimization processes.

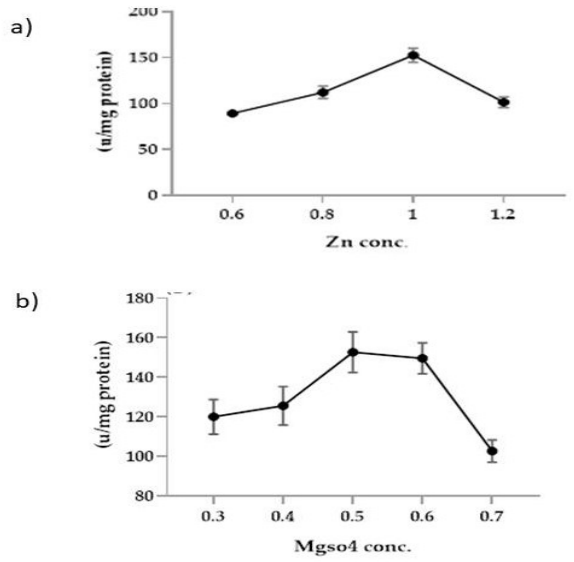

Fig. 5. a) Effect of different concentrations of Zinc sulphat on productivity of L-glutaminase by *K. pneumonia*. b) Effect of different concentrations of magnesium sulphat on productivity of L-glutaminase by *K. pneumonia*.

Effect of different concentrations of potassium chloride on productivity of L-glutaminase by *K. pneumonia*

The obtained results in Fig. 6a indicated that the level of potassium chloride in the tested fermentation media exerted a marked effect on glutaminase enzyme productivity by *K. pneumonia*. The data also showed that the biosynthesis of L-glutaminase is concentration dependent. L-glutaminase production by *K. pneumonia* increased gradually with increasing the level of potassium chloride in the culture media to (0.5 g/l) followed by decreasing in glutaminase production by increasing concentration of potassium chloride. Therefore, (0.5 g/l) of potassium chloride will be used in the subsequent optimization processes.

Effect of different concentrations of Feso4 on productivity of L-glutaminase by *K. pneumonia*

The obtained results in Fig. 6b indicated that the level of Feso4 in the tested fermentation media exerted a marked effect on glutaminase enzyme productivity by *K. pneumonia*. The data also showed that the biosynthesis of L-glutaminase is concentration dependent. L-glutaminase production by *K. pneumonia* increased gradually with increasing the level of Feso4 in the culture media to (0.2 g/l) followed by decreasing in glutaminase production by increasing concentration of Feso4. Therefore, (0.2 g/l) of Feso4 will be used in the subsequent optimization processes.

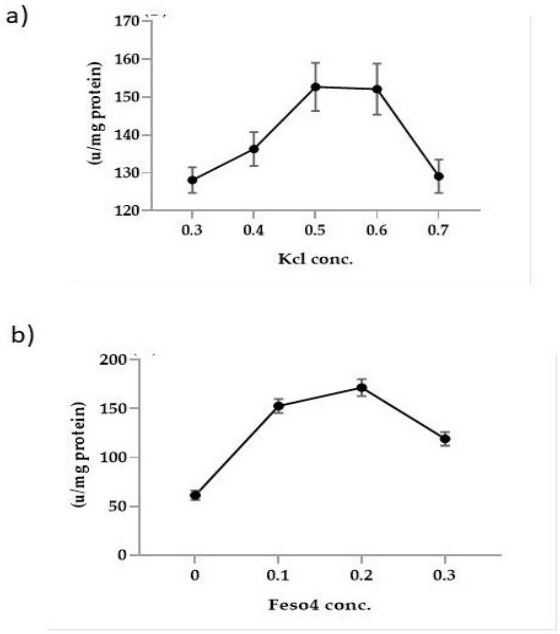

Fig. 6. a) Effect of different concentrations of potassium chloride on productivity of L-glutaminase by *K. pneumonia*, b) Effect of different concentrations of $Feso_4$ on productivity of L-glutaminase by *K. pneumonia*.

Discussion

In recent years, bacteria play an important role in the fields of health, medicine, and industry. In the present study, upon screening of the bacterial strains isolated from marine environment, the strain showed the highest production of L-glutaminase was selected and identified as *K. pneumonia*. L- Glutaminase exhibits its anticancer activity by depleting L-glutamine from the tumor cells, prompting their death as they are dependent on this amino acid. L-Glutaminase is a vital ordinary antioxidant that helps in avoiding human infections. Moreover, L-Glutaminase is not related to lethal and cancer-causing effects like those of artificial antioxidants (Mousumi and Dayanand, 2013; Unissa *et al*., 2014).

Selective isolation of L-glutaminase producing bacteria is of great interest for discovering new antitumor agents. During this study, among the isolated 100 bacterial isolates, 50 isolates (50%) produced L-glutaminase at significant level and the most active isolate was *K. pneumonia*.

After screening of the samples, the bacterial colonies further identified with the help of different biochemical tests. The bacterial strain producing the appropriate type and amount of enzyme was then selected for L-glutaminase production on large scale.

The production of a zone with pink colour around the bacterial colony that grown on minimal glutamine agar (MGA) medium give an indication of L-glutaminase production (Hymavathi *et al*., 2009). Other method involves the isolation of microorganisms by routine isolation procedures from certain environments, then screened for enzymatic activity. However, the use of selective media and the presence of antibiotics, NaCl, and pH indicators make MGA medium suitable for both direct and selective isolation of L-glutaminase producing organisms (Balagurunathan *et al*., 2010).

Based on the sequence obtained from the 16S rRNA, the bacterium isolated was similar to the genus *K. pneumonia*. The sequences of the following organisms. (GenBank accession number OQ703039), *Klebsiella* showed a similarity of 98.9%. Therefore, our isolate was identified as *Klebsiella pneumoniae* and named *K. pneumonia* AS KP 23.

The energy source that required for the growth of microorganisms is the carbon source. The favourable carbon sources for many types of microbes are carbohydrates and related compounds. The enhancement of L-glutaminase production by incorporation of carbon sources may be due to the positive effect of additional carbon sources together with glutamine for enhancing the biosynthesis of the enzyme (Chitanand and

Shete, 2012). In the present study, fructose was used as an additional carbon source for yielding the maximum L-glutaminase by *K. pneumonia*. On the other hand, glucose was the best carbon source for glutaminase production by *Pseudomonas aeruginosa* (Al-Zahrani *et al*., 2020), rhamnose cause the highest L-glutaminase production of the marine bacterial isolate Bacillus subtilis OHEM11 (Orabi *et al*., 2020) and glucose was used as an additional carbon source for producing the maximum L-glutaminase by Bacillus sp. DV2-37(Gomaa, 2022).

Nitrogen source has an important influence on the glutaminase enzyme production because it is the ultimate precursor for biosynthesis of protein. Additionally, the nitrogen source can affect the medium pH, which in turn may influence the stability and activity of the enzyme. The results of the present study revealed that beef extract enhance the enzyme yield. On the contrary, Kiruthika and Nachimuthu (2014) reported that the production of glutaminase by marine Bacillus subtilis JK-79 was enhanced by using peptone and yeast extract which enhance the enzyme production by Bacillus sp. DV2-37(Gomaa, 2022). In the present study, L-glutamine was found to increase L-glutaminase production by *K. pneumonia*. This observation may suggest that L-glutamine acts as an inducer for the production of L-glutaminase enzyme. Similar results were reported by Prakash *et al*. (2010) who indicated that L-glutamine resulted in ahigh production of L-glutaminase. Moreover, Al-Zahrani *et al*. (2020) showed that the maximum glutaminase activity by *Pseudomonas aeruginosa* was achieved with glutamine out of various nitrogen sources.

It is well-known that the pH of the culture medium affects, the availability of certain metabolic ions and the permeability of bacterial cell membranes, which in turn provide cell growth and production of the enzyme (Krishna-kumar *et al*., 2011). In general, the pH range 6.0 to 8.0 was reported to be the most favourable range by the majority of microbial organisms for the production of L-glutaminase (Sathish and Prakasham, 2010). In the present study, it was noticed that pH 8.0 is optimum for the L-glutaminase production by *K. pneumonia*. In the same line, marine Vibrio azureus JK-79 bacterial strain produces the maximum glutaminase yield at pH 8 (Kiruthika, 2013). However, Lakshmi and Jaya (2012) stated that the optimum pH for L-glutaminase production by Aspergillus oryzae NCIM 1212 was at pH7, it was noticed that pH 7.0 is the optimum pH for the L-glutaminase production by Bacillus sp. DV2-37(Gomaa, 2022). In addition to that, the best production of L-glutaminase by the forest soil isolated bacterial strain of *Bacillus* sp. was observed at pH 7 (Nagaraju and Raghu Ram, 2018).

Furthermore, the growth temperature has an influence on the microbial metabolism with respect to the enzymatic reactions and the rates of cellular processes. Sivakumar *et al*., (2006) reported that any temperature beyond the optimum range is found to have some adverse effects on the metabolic activities of the microorganisms. In the present study, the highest L-glutaminase production by *K. pneumonia* was noticed at a temperature of 40°C. On the other side, Kiruthika, (2013) found that the maximum glutaminase activity by Vibrio azureus JK-79 isolated from marine environment was at 37 °C and Al-Zahrani *et al*., (2020) found that the temperature of 35 °C is the optimum one for glutaminase production by *Pseudomonas* NS16.

Furthermore, it was reported that the maximum enzyme production could be obtained after a certain incubation period, that allows the culture to grow, after that it decreases. This could be due to the nutrients depletion in the medium that stressed the bacterial physiology. This nutrients reduction resulted in the inactivation of the enzymes secretary machinery (Alexandra *et al*., 2003). In the present study, the maximum enzyme productivity by *Klebsiella pneumoniae* was obtained at 96 h. of incubation period. In the same manner, Krishna-kumar *et al*. (2011) mentioned that the highest L-glutaminase production by the marine alkalophilic Streptomyces sp. SBU1 was at 96 h. of incubation period. However, maximum production of L-glutaminase was achieved at 18 h. of incubation time by marine bacterial strain Bacillus subtilis (Zhang *et al*., 2019) and at 72 h of incubation period for *Pseudomonas* VJ-6 (Hiremath, 2011).

The isolates producing L-glutaminase enzyme catalyze the hydrolysis of L-glutamine to L- glutamic acid and ammonia which react with Nessler's reagent, producing deeper yellow color or brown precipitate. Determination of the released ammonia is an indirect indicator of L-glutaminase enzyme, produced by the tested bacterial isolates that giving positive result (Imada *et al*., 1973).

Conclusion

Nutritional parameters, such as carbon and nitrogen sources also played an important role in enhancing glutaminase yield. The specific activity of glutaminase enzyme from *K. pneumonia* under optimum conditions (587.9 U/mg) is higher than many species that were used in glutaminase production such as production of L-Glutaminase enzyme from Alcaligenes faecalis. Further research will be made on this enzyme so that the optimum benefit of it can be reached.

Conflict of interest

The authors declare that they have no conflict of interest.

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