OPTIMIZATION OF PROCESS PARAMETERS FOR L-GLUTAMINASE PRODUCTION BY *Pseudomonas* species ALG3

ABSTRACT

The present study reports the optimization of process parameters for L-glutaminase production by *Pseudomonas* species ALG3. The L-glutaminase – producing bacterium had already been isolated from soil at a location within Chukwumemeka Odumegwu Ojukwu University, Uli in Anambra state. It was identified using cultural, biochemical and molecular characteristics. Optimization of L-glutaminase production was done by assessing the effect of various parameters using a 50 ml mineral salt medium in 250 ml flasks. The parameters included various bivalent metals, pH, surfactants, growth promoters, amino acids and fermentation time. The Results showed that the addition of FeSO4 stimulated optimum glutaminase yield of 38.36 U/ml by *Pseudomonas* species ALG3 , while the least production was observed with MnSO4. Enhanced glutaminase yield of 30.73 U/ml was recorded at pH 8.0, while the lowest enzyme accumulation was observed at pH 9.0. The highest glutaminase accumulation of 82.25 U/ml was achieved with Tween 80, while the lowest yield was recorded with stearic acid. Urea, stimulated maximum Glutaminase yield of 43.82 U/ml, while minimum accumulation was observed with peptone. Proline stimulated highest glutaminase yield of 42.63U/ml, while the lowest yield was observed with tyrosine. Highest glutaminase yield (91.88 U/ml) was observed after 72h, the yield decreased thereafter. The results obtained in the study illustrated that the optimization of process parameters, increased appreciably the glutaminase yield of *Pseudomonas* species ALG3. This suggest that *Pseudomonas* species can be used for large scale production of glutaminase, which can offer great potential for applications in Medicine and food industry.

Keywords: L-glutaminase, *Pseudomonas* species ALG3, optimization**,** process parameters

1. INTRODUCTION

L-Glutaminase (L-glutamine amidohydrolase) catalyses the hydrolysis of L-glutamine to glutamic acid and ammonia [1,2,3]. The enzyme is important in nitrogen metabolism and has a wide distribution in cells of microorganisms, plants and animals [4]. Several microorganisms which include bacteria, fungi and yeast have been reported to secrete L-glutaminase into fermentation media. The use of microbes as the enzyme producer is more preferable due to their

simple growth requirements, easy processing and handling as well as cheaper production [5]. L-glutaminase production has been reported from *E. coli* [6], *Bacillus subtilis* [7], *Proteus morganni, P. vulgaris, Xanthomonas juglandis, Erwinia carotovora, E. aroideae, Serratia marcescens, Enterobacter coacae, Klebsiella aerogenes* and *Aerobacter aerogenes* [8]. Also, L-glutaminase synthesis has been reported from *Streptomyces rimosus* [9], *Streptomyces* sp.-SBU1 [10] and *Streptomyces avermitilis* [11]. Different methods of fermentation technology can be applied for the production of L-glutaminase. Commercial production of L-glutaminase had been carried out using submerged fermentation (SmF) and solid state fermentation (SSF) techniques[12,13,14]. L-glutaminase enzyme has attracted significant attention owing to its potential application in medicine as an anticancer agent, anti-retroviral agent and could be of significance in enzyme therapy of acute lymphocytic leukaemia [15]. The enzyme causes selective death of glutamine dependent tumor cells by depriving these cells of glutamine. The use of enzymes to deprive neoplasms of essential nutrients helps in the treatment of malignancies [16]. Another most promising application of glutaminase is in biosensors for monitoring glutamine levels in mammalian and hybridoma cell cultures without the need of separate measurement of glutamic acid [17]. Glutaminase is also taking an important role that controls the delicious taste of fermented foods such as soy sauce and in general food products by increasing the glutamic acid content therefore, this enzyme has attracted a great attention in food industries [18, 19].
Commercial demand of this enzyme urges the researchers to develop an economically viable bioprocessing technology for large scale production. Manipulating the physicochemical parameters of bioprocess plays an imperative role to attain higher metabolite production [20].
Since the sources for L-glutaminase are limited, the search for potential microbial strains that hyper produce the enzyme with novel properties for their industrial production is being pursued all over the world [21].

In our previous study(published), it was possible to isolate a L- glutaminase producing bacterium *Pseudomonas* species ALG3 from the soil [22]. The present study is a continuation of the research and the aim was to carry out optimization of process parameters for L- glutaminase production by *Pseudomonas* species ALG3 .

2. MATERIALS AND METHODS

2.1 Preparation of inoculum

Two loopfuls (24 h) of *Pseudomonas* species ALG3 were inoculated into 100ml Erlenmeyer flask containing 30ml of seed medium, which was sterilized at

121°C for 15 min. The seed medium was composed of peptone, 10.0g; yeast extract, 10.0g; NaCl, 5.0g; water, 1.0 L; pH adjusted to 7.2. The flasks were incubated for 24 h on a rotary shaker (150 rpm) at 30^0C.

2.2 Fermentation medium

Various 250ml Erlenmeyer flasks containing 50ml of mineral salt glutamine medium that consisted of the following (g/l): glutamine, 10.0; K_2HPO_4, 1.0; KH_2PO_4, 0.1; $MgSO_4$, 1.0; NaCl, 0.5; yeast extract, 0.5, pH 7.0 were used for the experiment.

2.3 Optimization of process parameters for L-glutaminase production

2.3.1 Effect of bivalent metals

The effect of different bivalent metals ($FeSO_4$, $MnSO_4$, combination of $FeSO_4$ +$CuSO_4$, combination of $FeSO_4$ + $MnSO_4$, combination of $CuSO_4$+ $MnSO_4$, combination of $FeSO_4$ + $CuSO_4$ + $MnSO_4$) on L-glutaminase production by *Pseudomonas* species ALG3 was studied. The mineral salt medium as previously described, was supplemented with 0.1% (w/v) of the bivalent metals and sterilized at 121 ^{0}C for 15 min. Thereafter, the medium was inoculated with 2 ml (4.2 x 10^6 cfu/ml) of 24 h seed inoculum. The flasks were placed in a rotary shaker (150 rpm) at 30^0C for 72 h. After incubation, 5ml of the fermentation broth was collected and used for the determination of L-glutaminase production and bacteria growth.

2.3.2 Effect of pH

The effect of pH values on l-glutaminase production by *Pseudomonas* species ALG3 was determined. The pH of the mineral salt medium was adjusted to various values of 6 to 10 and thereafter sterilized at 121 ^{0}C for 15 min. Afterward, the various flasks containing the medium were inoculated with 2 ml of a 24 h seed inoculum. The flasks were placed on a rotary shaker (150rpm) at 30^0C for 72 h. After incubation, 5ml of the fermentation broth was collected and used for the determination of L-glutaminase production and bacteria growth.

2.3.3 Effect of Surfactant

The effect of surfactants (palmitic acid, stearic acid, oleic acid and tween 80) on l-glutaminase production by *Pseudomonas* species ALG3 was investigated. The mineral salt medium as previously described, was supplemented with different concentrations of surfactants (0.1 to 0.3 % w/v of palmitic and stearic acid and 0.1 to 0.3 % v/v of oleic acid and tween 80) and sterilized at 121^0C for 15 min. Afterward, the various flasks containing the medium were inoculated with 2 ml

of a 24 h seed inoculum. The flasks were placed on a rotary shaker (150rpm) at 30^0C for 72 h. After incubation, 5ml of the fermentation broth was collected and used for the determination of l-glutaminase production and bacteria growth.

2.3.4 Effect of growth promoters

The effect of different growth promoters (malt extract, beef extract, peptone, tryptone, urea and casein) on l-glutaminase production by *Pseudomonas* species ALG3 was studied. The mineral salt medium as previously described, was supplemented with 0.1% w/v of the different growth promoters and sterilized at 121 ^{0}C for 15 min. Afterward, the various flasks containing the medium were inoculated with 2 ml of a 24 h seed inoculum. The flasks were placed on a rotary shaker (150rpm) at 30^0C for 72 h. The flasks were placed in a rotary shaker (150rpm) at 30^0C for 72 h. After incubation, 5ml of the fermentation broth was collected and used for the determination of l-glutaminase production and bacteria growth.

2.3.5 Effect of amino acid

The effect of amino acids (glutamic acid, alanine, glycine, threonine, proline and tyrosine) on l-glutaminase production by *Pseudomonas* species ALG3 was investigated. The mineral salt medium as previously described, was supplemented with 0.1 % (w/v) of different amino acids and sterilized at 121^0C for 15 min. Afterward, the various flasks containing the medium were inoculated with 2 ml of a 24 h seed inoculum. The flasks were placed on a rotary shaker (150rpm) at 30^0C for 72 h. After incubation, 5ml of the fermentation broth was collected and used for the determination of L-glutaminase production and bacteria growth.

2.3.6 Effect of fermentation time on growth and glutaminase production

The effect of fermentation time on growth and glutaminase production by *Pseudomonas* species ALG3 was studied. The mineral salt medium as previously described, was sterilized at 121 ^{0}C for 15 min and thereafter, inoculated with 2 ml of 24 h seed inoculum. The flasks containing the medium were placed on a rotary shaker (150rpm) at 30 ^{0}C for 168 h. At interval of 24 h, 5ml of the fermentation broth was collected and used for the determination of bacteria growth, pH and L-glutaminase production.

2.4 Estimation L-glutaminase activity

L-glutaminase was assayed according to the method described by [23]. An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04 ML-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml of phosphate buffer (0.1 M, pH 8). The mixture was incubated at 37°C for 30 min and the reaction was arrested by the addition of 0.5 ml of 1.5 M trichloroacetic acid. From this mixture, 0.1 ml of the mixture was taken out and mixed with 3.7 ml of distilled water and 0.2 ml of Nessler's
reagent and A450nm was detected. Activity of the enzyme was determined in Unit/ml (U/ml) and L-glutaminase unit is the amount of the enzyme that liberates one μMol of ammonia.

2.5 Determination of Growth

It was determined turbidmetrically from the culture broth in spectrophotometer at 660nm.

2.6 Statistical analysis

The data obtained were analyzed by covariance matrix analysis using Microsoft excel 2013.

3. RESULTS

The result of the effect of bivalent metals on l-glutaminase production by *Pseudomonas* species ALG3 is shown in Figure 1. The result showed that maximum glutaminase yield (38.36 U/ml) was achieved with $FeSO_4$, while the least was recorded in $MnSO_4$. The bacteria growth was highest with the combination of FeSO4 and $CuSO_4$ and lowest with lowest with $CuSO_4$.

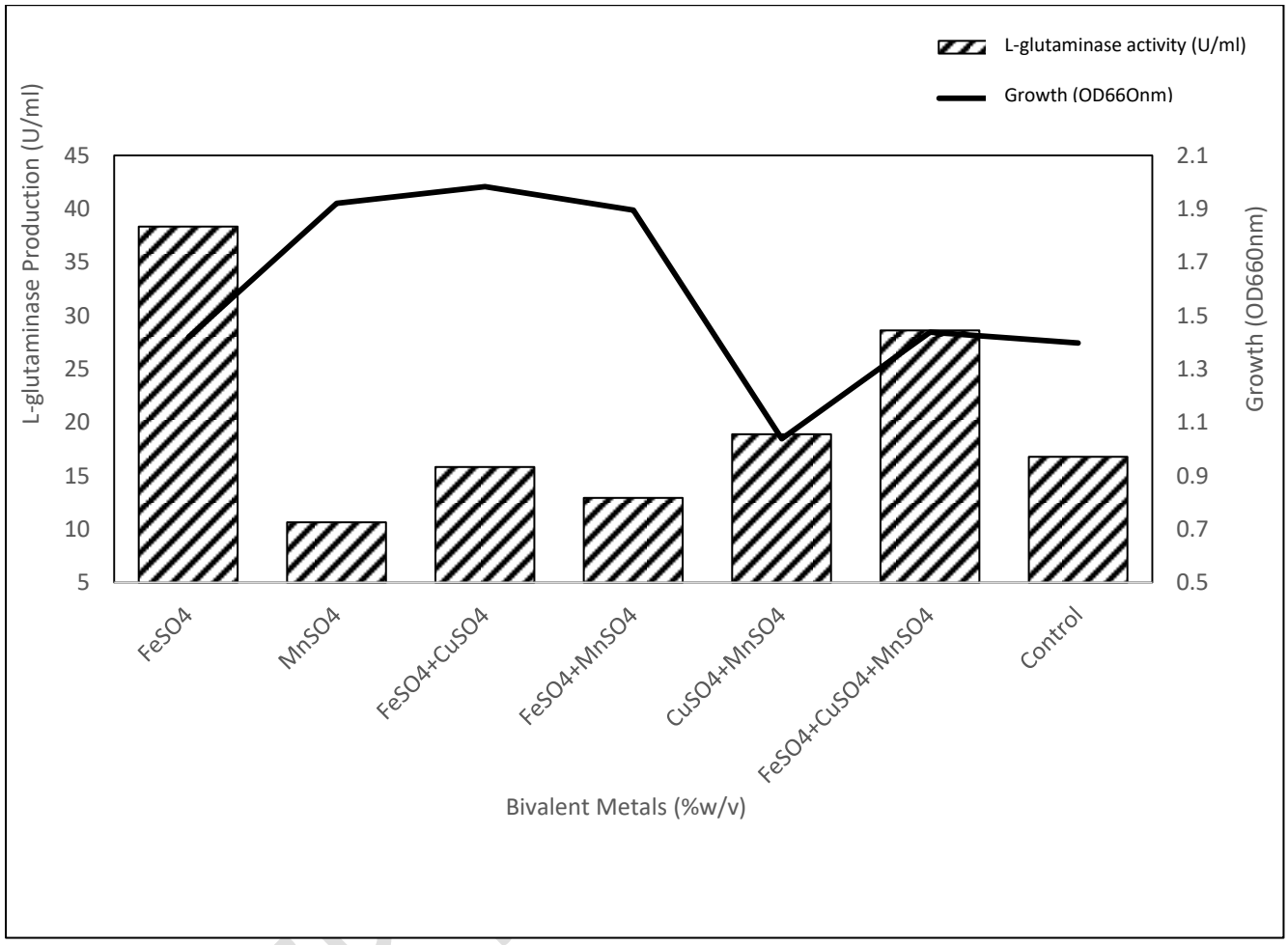

Fig. 1: The Effect of Bivalent Metals on L-glutaminase production by *Pseudomonas* species ALG3

The result of the effect of pH on l-glutaminase production by *Pseudomonas* species ALG3 is shown in Figure 2. The result showed that highest glutaminase yield (30.73 U/ml) was observed at pH of 8.0, while the lowest was recorded at pH of 9. Maximum bacteria growth was observed at pH of 7, while the lowest was observed at pH of 9 and 10. The covariance matrix analysis showed that there was a significant high value of effect at pH of 8.0 for glutaminase production.

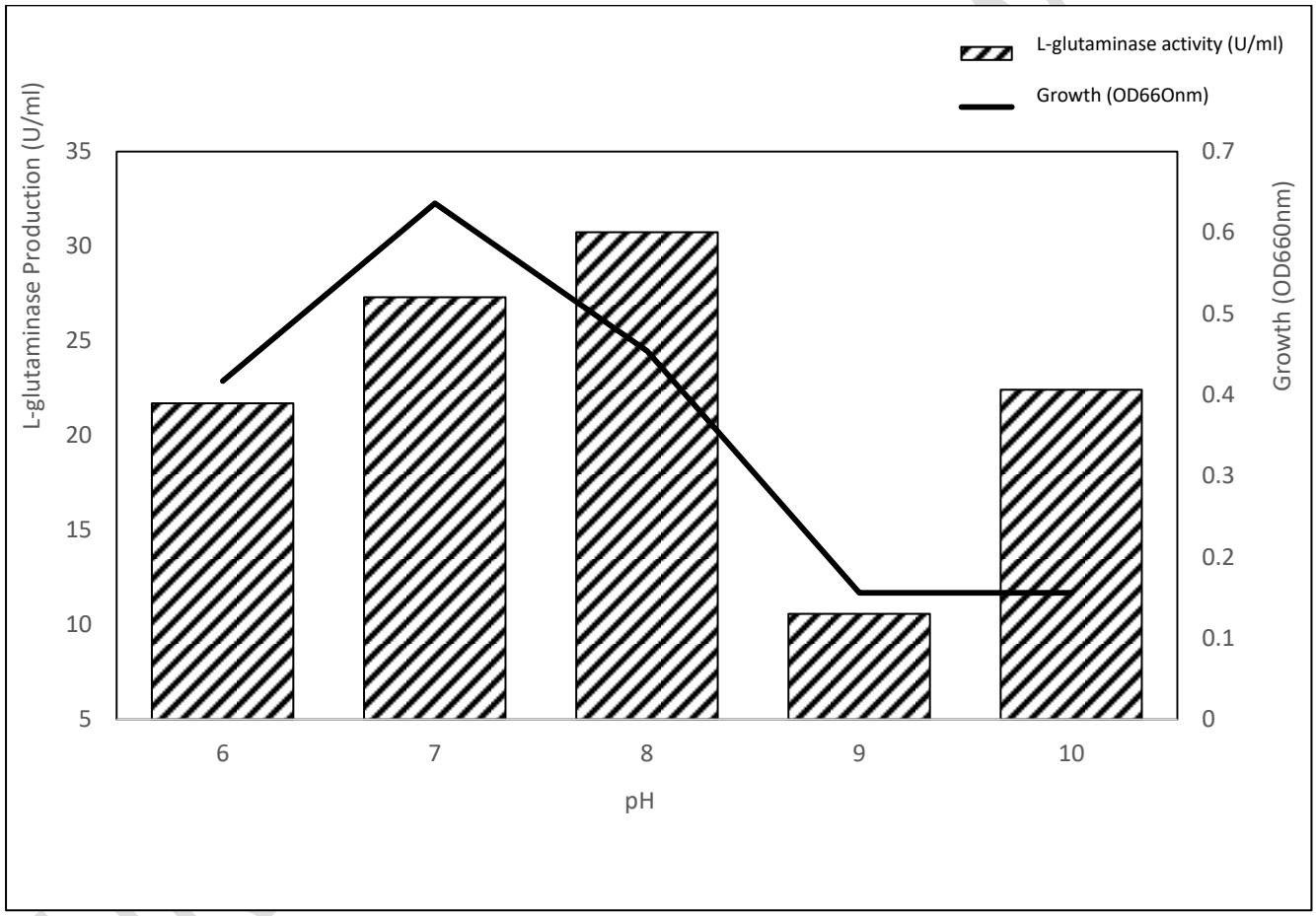

Fig. 2: The Effect of pH on L-glutaminase production by *Pseudomonas* species ALG3

The result of the effect of surfactants on l-glutaminase production by *Pseudomonas* species ALG3 is shown in Figure 3. The result showed that highest

glutaminase yield (82.25 U/ml) was achieved at 0.2% (v/v) Tween 80, while the least was observed at 0.2% (w/v) stearic acid. The bacteria growth was highest at 0.3% (v/v) oleic and lowest with 0.3% (w/v) palmitic acid.

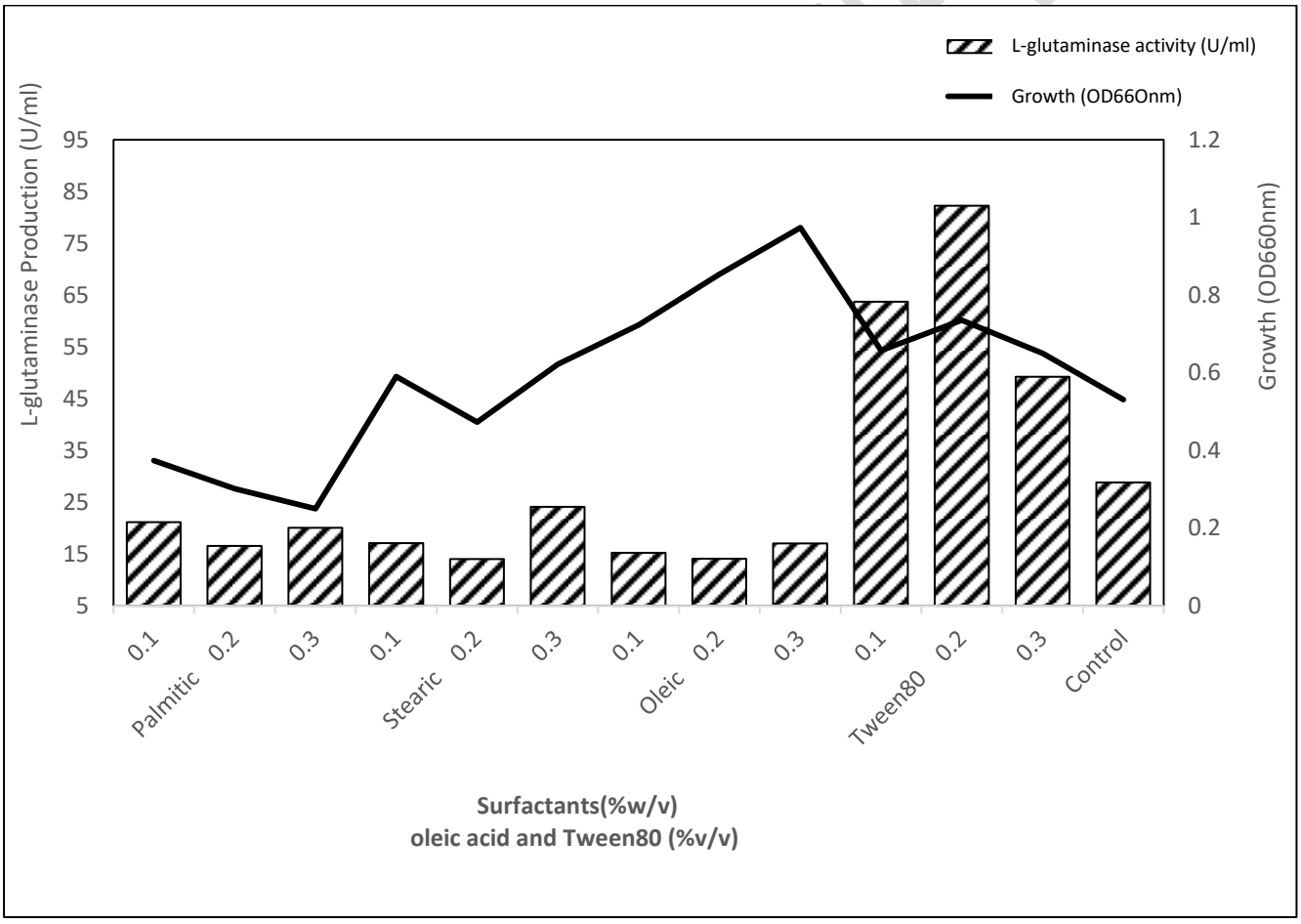

Fig. 3: The Effect of Surfactants on L-glutaminase production by *Pseudomonas* species ALG 3

The result of the effect of growth promoters on l-glutaminase production by *Pseudomonas* species ALG3 is shown in Figure 4. The result showed that the highest glutaminase yield (43.82 U/ml) was observed in urea, while the least was recorded in peptone. Maximum bacteria growth was observed in peptone, while the lowest was recorded in urea. The covariance matrix analysis shows that there was a significant high value of effect in urea for glutaminase production.

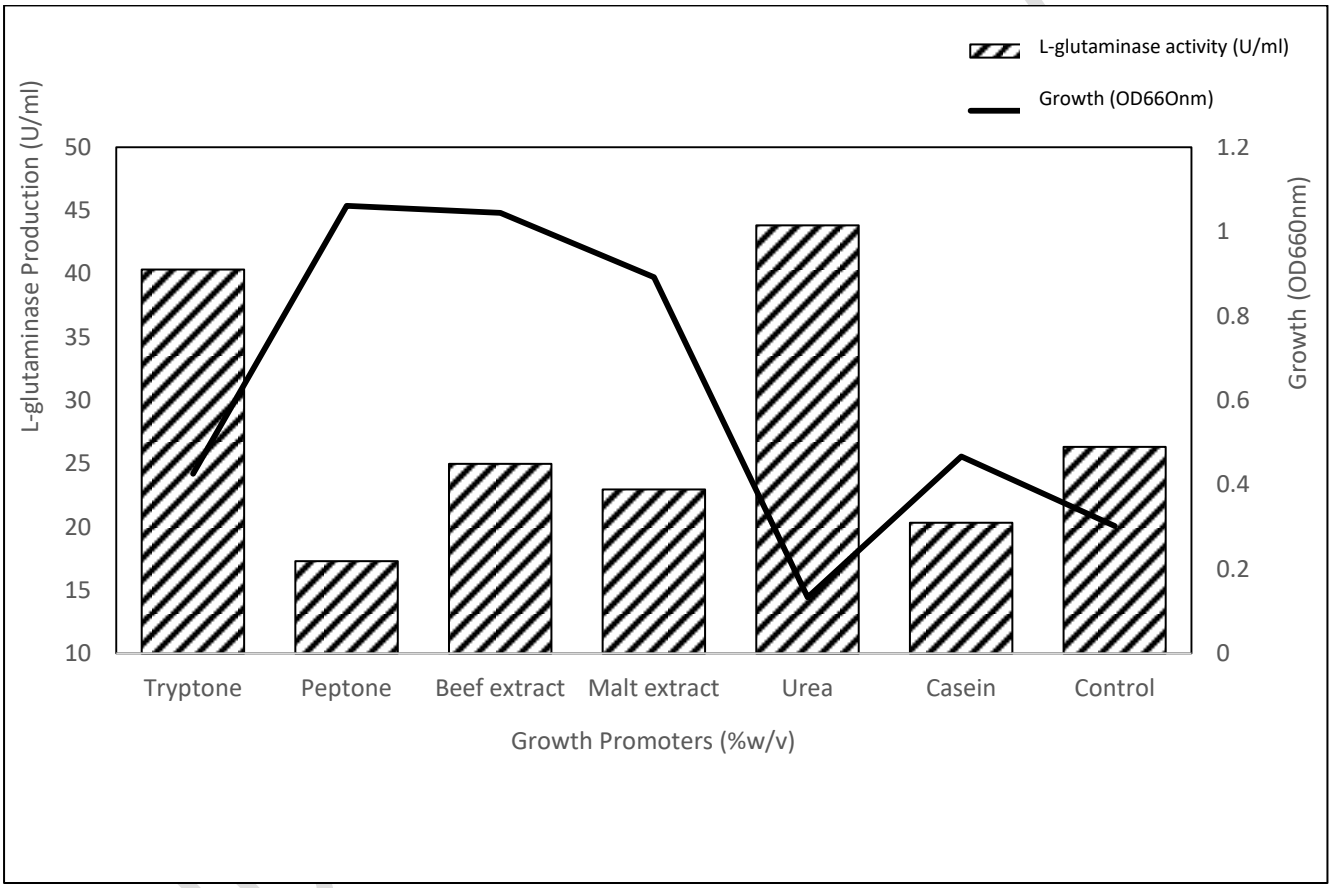

Fig. 4: The Effect of Growth Promoters on L-glutaminase production by *Pseudomonas* species ALG3

The result of the effect of amino acids on glutaminase production by *Pseudomonas* species ALG3 is shown in Figure 5. The result showed that highest glutaminase yield (42.63U/ml) was observed with proline, while the least was recorded with tyrosine. The bacteria growth was also highest with proline and lowest with threonine.

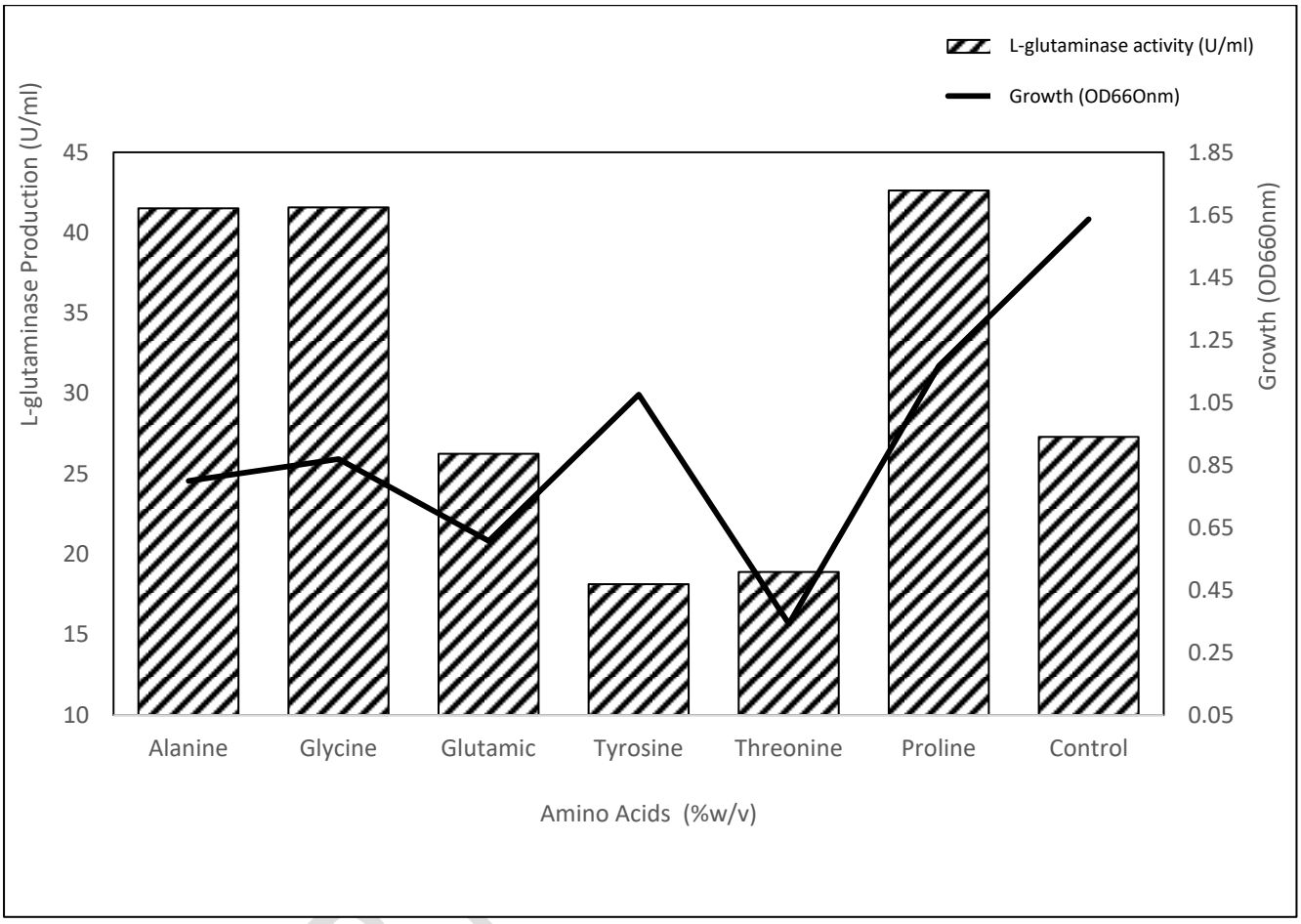

Fig. 5: The Effect of Amino Acids on L-glutaminase production by *Pseudomonas* species ALG3

The result of the effect of fermentation time on growth and glutaminase production by *Pseudomonas* species ALG3 is shown in figure 6. The result shows that highest glutaminase yield (91.88 U/ml) was observed in 72h, while the least was recorded in 144 h. *Pseudomonas* growth was also highest within 72h and lowest within 96h.

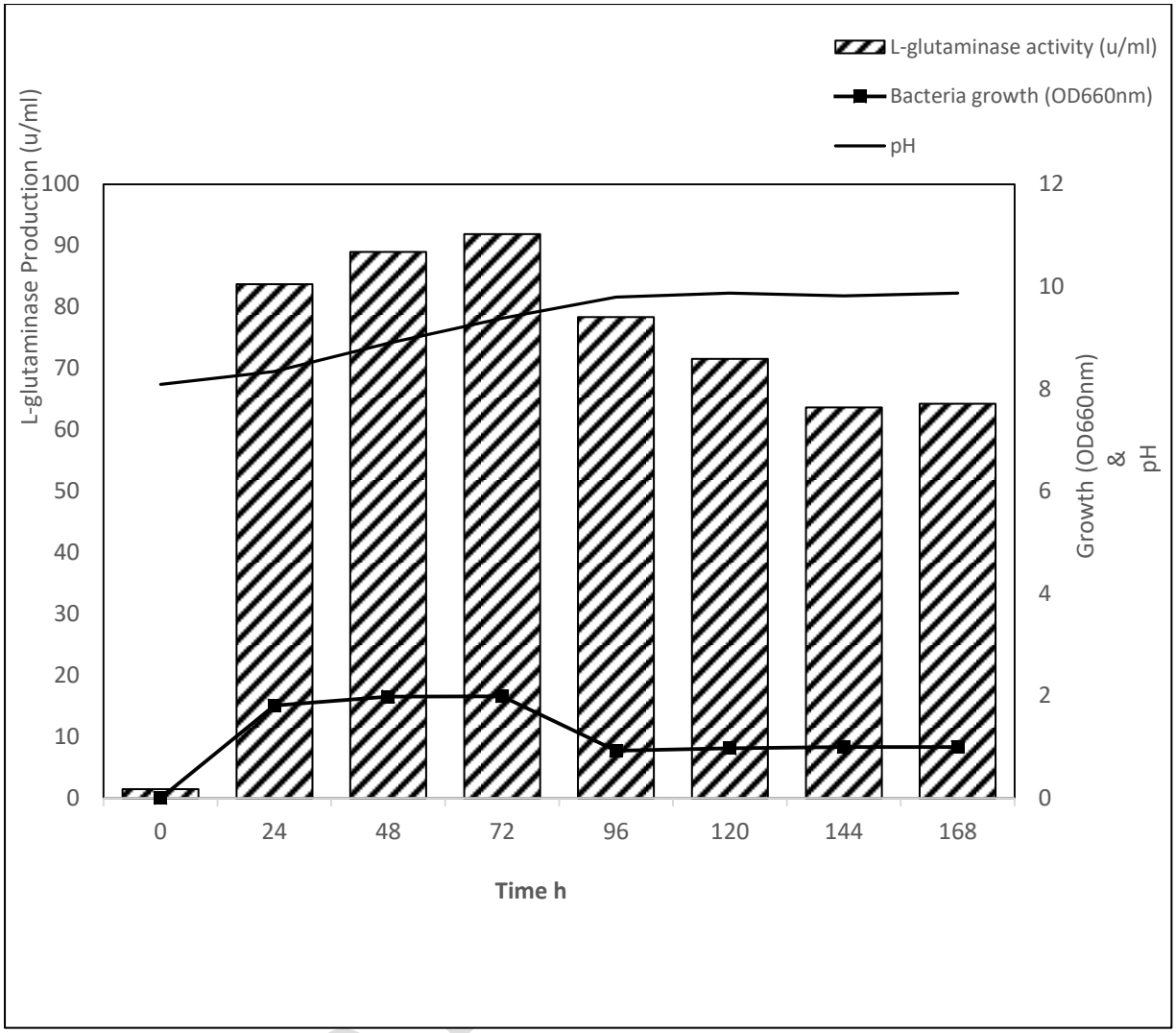

Fig. 6: The Effect of ferrmentation Time on L-glutaminase production by *Pseudomonas* species ALG3

4. DISCUSSION

The result obtained from this study indicated that the addition of $FeSO_4$ to the medium generated the highest production of l-glutaminase. This contradicted the report of [24], who studied the effect of four bivalent metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+}) supplementation on L-glutaminase yield from three bacteria isolates and found only Mg^{2+} and Mn^{2+} ions to slightly improve enzyme yield, while both Zn^{2+} and Fe^{2+} ions had negative impact on enzyme yield. Metal ions play a vital role in fermentation as they are co-factors for various enzymes and also facilitate the transport of materials across cell membrane.

[25] and [26] highlighted that the metal ions probably acted as activators or inhibitors of enzymes involved in the synthetic steps of metabolites. It has been reported that Fe^{2+} and Mn^{2+} are the most important of the trace elements as they play a role in the excretion of primary metabolites.

It was observed that at pH 8 the amount of l-glutaminase produced was highest. recorded in the study. This is not in accordance with the reports of [6, 12, 27, 15], who observed maximum l-glutaminase production at pH 7 for *Streptomyces* sp , *Trichoderma koningii*, *Bacillus* species and *Vibrio costicola* respectively. [28, 29, 30] observed that some microbial species were known to produce L-glutaminase in the neutral or slightly alkaline pH under submerged fermentation conditions. In other reports, L-glutaminase production at pH 6.0 was reported in *Cryptococcus nodaensis* [31] and *Pseudomonas* sp. [32]. Nathiya *et al.* [33], observed higher glutaminase production by *Aspergillus flavus* at acidic pH 4 , while the activity decreased up to 50% at neutral pH 7. Abdallah *et al.*, [34] reported that the optimum pH was 7.0 to 8.0 for *Streptomyces avemitilis*.

The surfactant Tween 80, was observed to stimulate maximum l-glutaminase production in the work. Surfactants decrease surface tension and increase the air supply of the medium. Various kinds of surface active agents are known to affect permeability in microorganisms [35, 36]. Reese *et al.* [37] revealed that addition of Tween 80 increased yield of enzymes like cellulase, amylase and sucrase.

In the study the amino acid proline stimulated increased production of L-glutaminase enzyme. This finding is contrary to reports of other workers. Kiruthika *et al.* [38] observed that Among the different amino acids, L-glutamine which is the actual substrate for L-glutaminase showed a significant increase in the production of the enzyme. Another observation was made by [12] who reported maximal L-glutaminase production at 2% L-glutamine in wheat bran. However, Renu [39], has reported that L-glutamic acid and lysine at 1% (w/v) were found to induce maximum L-glutaminase production in *V. cholera* ACMR 347 and *P. fluorescens* ACMR 171. [40], observed that among the amino acids utilized 0.1% cysteine supported the highest L-glutaminase production of 135.98 U/ml.

The fermentation time of the modified fermentation medium indicated maximum yield at 72hrs and further increase in incubation period showed low yield of l-glutaminase. This is in line with the report of [41] who observed maximum yield at 72 h of fermentation in optimization of submerged fermentation process for L-glutaminase production by *Pseudomonas aeroginosa* BGNAS-5.

5. CONCLUSION

It is concluded from this study that under optimal conditions, the glutaminase production by *Pseudomonas* species was enhanced. This indicated that *Pseudomonas* species ALG3 which was isolated from soil has immense potential as an industrial organism for the production of L-glutaminase using submerged fermentation. Thus, this will reduce the cost of industrial enzymes imported from other countries, offer sustainability of local enzymes production and the economy of the nation. Further research is needed to study the optimization of other parameters for optimum glutaminase accumulation by *Pseudomonas* species

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