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Production of *L-asparaginase* by *Alcaligenes faecalis* subspecies *phenolicus* Isolated from the Rhizosphere of *Zea mays L.*

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ABSTRACT

L-asparaginase is a naturally occurring enzyme that has been extracted from plant and animal tissues including microbial cells. The enzyme has been priced due to its industrial and biopharmaceutical importance. *L-asparaginase* has been used to eliminate tumour cells that rely on L-asparagine supplied by the serum for survival. The present study examined isolated bacteria from the rhizosphere environment of *Zea mays* in order to examine their ability to produce *L-asparaginase* through rapid plate assay technique**.** A medium containing L-asparagine as carbon and nitrogen source was used. Various conditions for the production of the enzyme through submerge fermentation technique were also optimized following One-Factor-At-A-Time technique. Promising bacterial isolate was picked and primed for identification using 16S ribosomal RNA (rRNA) sequencing technique. The molecular weight of *L-asparaginase* produced was determined by via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Stability of the enzyme was also examined at various pH and temperature and its ability to metabolise L-asparagine was also determined. Some of the bacteria screened for *L-asparaginase* production showed positive results. The highest zone of colour change (35.00±0.09mm) was produced by *Alcaligenes faecalis* subsp. *phenolicus*. Optimum production of *L-asparaginase* occurred at 84 hours (fermentation time), 40^{o}C (temperature), 200 rpm (agitation rate) and pH 7.5. The yield of *L-asparaginase* obtained using optimum fermentation conditions was 192±0.93 U/mL. The molecular weight of *L-asparaginase* was 39 kDa. The enzyme was stable between pH 7.5 and 8.0. The enzyme was also found to be thermostable between 25^{o}C and 55^{o}C. The enzyme metabolised L-asparagine and produced L-aspartic acid and ammonia in the process. This study concluded that *Alcaligenes faecalis* subsp. *phenolicus* isolated from the rhizosphere of *Zea mays* produced *L-asparaginase* and the study recommended that this bacterium can be explored in the industrial production of *L-asparaginase*.

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1.0 Introduction

Enzymes are defined as biological macromolecular catalysts that speed-up biochemical reactions. The substrate at the beginning of the process are changed into different products. Metabolic processes in cells need enzymes in order to proceed at rates sufficient to ensure propagation and survival (Stryer *et al*., 2002).

Various industrial procedures have used to produce enzymes from plants and animals including enzymes from microorganisms (Sindhu *et al.,* 2018). However, microbial enzymes have been promising in terms of commercial advantages (Rajendra *et al.,* 2016). Industrial enzymes were introduced to the West in the 1890s. During that time Jokichi Takamine a Japanese scientist was in USA to establish an enzyme manufacturing company with the aim of using the technology of Japan. The eventual end product was latter named Takadiastase (a mixture of proteolytic and amylolytic enzymes). It was produced by *Aspergillus oryzae* using rice or wheat bran as the medium. Takadiastase was thereafter sold in the USA for use in aiding digestion and prevent incomplete digestion of starch (dyspepsia) (Woodley, 2008).

The tumour-inhibitory activities of *L-asparaginase* (ASNase) were discovered in the 1950s and it was observed that guinea pig serum treated lymphoma-bearing mice underwent rapid regression. ASNases have been considered to be promising therapies for ALL since the 1970s. Pegylated *L-asparaginase* (ASNase or PEG-ASNase) has been designated as highly specific for the deamination of L-asparagine (Asn) to aspartic acid and ammonia. Elimination of Asn can give rise to a nutritional deficiency and inhibition of protein synthesis. The process can then give rise to apoptosis of T-lymphoblastic leukemias which require Asn for survival (Avramis and Tiwari, 2006).

L-asparaginase (EC3.5.1.1, L-asparagine amidohydrolase) has the ability to shutdown cancer cells that based their nutritional need on L-asparagine from serum (Alrumman *et al.*, 2019). *L-asparaginase* extracted from organisms such as *Escherichia coli* and *Erwinia* species has been used as antitumor and anti-leukemia agent. Just like other non-essential amino acids, some tumours are also auxotrophic for L-asparagine (Shirazian *et al.,* 2016). Therefore, asparagine-depleting enzymes can be promising biopharmaceutical agents against certain tumours (Shirazian *et al.,* 2016).

This current study examined bacteria that have the potential to produce *L-asparaginase*. The bacteria were isolated from the rhizosphere of *Zea mays.* Various conditions for the production of the enzyme through submerge fermentation technique were also optimized. The stability of the enzyme at various and temperature and pH was also examined.

2.0 Materials and Methods

2.1 Isolation of bacteria from the rhizosphere of maize plant

Bacteria were isolated from the rhizosphere of maize cultivars (LNTP-Y and ART98-SW6-W) for 12 weeks as the plants grew. Five test tubes were prepared for the isolation of bacteria. Nine millilitre (9 mL) distilled water was transferred into each of those test tubes. One gram of the soil sample was added to the first test tube to give a dilution of 10^{-1}. The content was mixed very well. One millilitre (1mL) of the mixture was added to the next test tube to make a 10^{-2} dilution. This process was repeated up to 10^{-5} dilution (Fawole and Oso, 2001).

Zero point one millilitre (0.1ml) of the 10^{-4} dilution was used to inoculate nutrient agar plates through the use of spread plate technique. Sterile spreader was used for spreading the inoculum over each plate. All the plates were incubated upside down at 37ºC for 24 hours. The plates were checked after 24 hours in order to isolate the bacteria (Fawole and Oso, 2001).

2.2 Phylogenetic Analysis of the Promising Isolate

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X following the procedure described by Tamura and Nei (1993) and Kumar *et al.* (2018). Sequence of the promising isolate and its closest relatives were used to construct a dendrogram tree.

2.3 Screening of isolated bacteria for enzymes production

Thirty nine bacterial isolates were screened for the production of *L-asparaginase*. The screening was carried out following the method described by Umayaparvathi *et al.* (2013).

Minimal agar media containing L-asparagine as carbon and nitrogen source was used. Chromogenic change from yellow to pink on the agar medium due to the growth of a particular microbial colony as used as indication of a change in pH (Sabu *et al.,* 2005). Minimal Asparagine Agar (MAA) medium was prepared and used for the screening of bacterial isolates for the production of *L-asparaginase*. Components of the medium was 4% (w/v) of L-asparagine, 0.2% KNO_3 (w/v), 0.2% KH_2PO4 (w/v), 0.005% $MgSO_4.7H_2O$ (w/v), 0.5% NaCl (w/v), 0.001% $FeSO_4.7H_2O$ (w/v), 0.002% $CaCO_3$ (w/v), and 15g/L agar with pH adjusted to 7.4. The medium was supplemented with 0.012 (g/L) of 2.5 % of phenol red as pH indicator. After inoculation, all the plates were incubated at 37°C for 24 hours. *L-asparaginase* activity was identified by the formation of a pink zone around colonies. The colony that formed highest pink zone was picked and maintained on the agar slants at 4°C (Hymavathi *et al.,* 2009; Abdallah *et al.,* 2012; Rahamat *et al.,* 2015).

2.4 Molecular characterisation of the bacterial isolate

Promising bacterial isolate was identified using polymerase chain reaction (PCR) technique. The 16S ribosomal RNA (rRNA) sequencing was used to identify bacterial isolate (B26). The DNA was isolated using AxyPrep Multisource Genomic DNA Miniprep Kit. The DNA was then subjected to cocktail mix containing forward and reverse primers. The amplification process and denaturation of the extracted genomic DNA was followed by the annealing of primers at 56°C for 30 second and extension at 72°C for 45 seconds. The amplicon from the reaction was loaded on 1.5% agarose gel. The ladder used was 50bp from NEB. The expected base pair of the amplicon was around 1500bp. The PCR product was then purified. The product from the purification process was loaded on 3130xl genetic analyzer from Applied Biosystems to produce the sequences. The 16S rRNA sequences were analysed and compared with sequences deposited in the National Center for Bioinformatics (NCBI) GenBank data base.

2.5 Production of enzyme

Enzyme was prepared using medium containing 0.2% KH_2PO4 (w/v), 4% (w/v) of L-asparagine, 0.5% NaCl (w/v), 0.2% KNO_3 (w/v), 0.005% $MgSO_4.7H2O$ (w/v), 0.001% $FeSO_4.7H2O$ (w/v) and 0.002% $CaCO_3$ (w/v) with pH adjusted to 7.4. Overnight culture was suspended in sterile deionised water and used as inoculum for pre culture to obtain an initial cell density and turbidity was adjusted to 0.5 McFarland standard (Veerapagu *et al.*, 2013). The submerged fermentation medium was maintained at 37°C for 72 hours at 200 rpm in a shaking incubator. At the end of the fermentation period, the broth was centrifuged at 10,000 rpm for 20 minutes at 4°C. The cell free supernatant was recovered as crude enzyme preparation and subjected to purification for further studies (Das and Prasad, 2010; Umayaparvathi *et al.*, 2013).

2.6 Purification and determination of molecular weight of the *L-asparaginase* produced

The supernatant containing crude enzyme was precipitated with ammonium sulfate. The precipitated enzyme was dissolved in 20 mM potassium phosphate buffer and dialyzed against same buffer. Protein extraction and dialysis was carried out using dialysis cassette. Extracted enzyme was centrifuged at 16,000 xg for 5 minutes to pellet and the pellet was repeatedly washed with 1x PBS until the supernatant becomes clear (Hasan *et al.*, 2013; Purwanto, 2016).

Molecular weight of the enzyme was determined by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gel was prepared with a 5% stacking and a 12% separating gel, 0.75 mm thickness. The (thawed) parasite protein extract was centrifuged at 16,000 xg for about 5

minutes and then the supernatant was transferred into a fresh tube. The required amount of 5x SDS sample buffer was added and incubated at 95°C for 5 min. The prestained protein ladder (4 µl and 8 µl) of each enzyme extract was loaded into the slots of the gel in the electrophoresis chamber filled with electrophoresis buffer and the gel was run at 200 V for 1 hour. Coomassie Brilliant Blue R-250 was used to stain the gel after the electrophoresis technique. The molecular weight of the enzyme was determined. This was done by comparing the weight with the migration distances of standard marker proteins on the ladder (Aley *et al.,* 1986; Ansorge *et al.,* 1996; Das and Prasad, 2010; Umayaparvathi *et al.,* 2013).

2.7 Enzyme assay

Activity of the enzyme was determined using 2.0% of L-asparagine. The quantity of enzyme solution used was 1 mL. This was mixed with 1 mL of L-asparagine in 50 mM Tris-HCl (pH 8.5) and this was incubated for 10 minutes at 30°C. Addition of 2 mL of 0.4 M trichloroacetic acid was used to stop the reaction after incubation. The precipitate was then separated by centrifugation at 10,000 rpm for 10 minutes. About 1 mL of each supernatant was neutralized with 5 mL of 0.4 M sodium carbonate and incubated with 1 mL of 1 N Folin Ciocalteu's reagent solution at 40°C for 20 min. Absorbance was then measured using spectrophotometer (Lenifield Model 752G, England). One unit of enzyme activity was defined as the amount of enzyme that caused absorbance at 340 nm to increase at a rate of 0.1 per min per ml of crude enzyme extract under the assay conditions (Das and Prasad, 2010; Geetha *et al.,* 2012; Umayaparvathi *et al.,* 2013; Reddy *et al.,* 2016).

2.8 Optimization of fermentation conditions

The isolate was examined under different fermentation conditions in order to derive the optimum conditions for enzyme production. Enzyme production was estimated at various incubation periods, substrate concentrations, temperatures, agitation rates, pH, carbon source and nitrogen sources, sodium chloride concentrations and inoculum sizes. Experiments were conducted in triplicate for each of the fermentation parameters (Das and Prasad, 2010; Umayaparvathi *et al.,* 2013; Wakil and Adelegan, 2015).

2.9 Effect of incubation period on enzyme production

The effect of incubation periods on enzyme production was studied. Each of the flasks were inoculated with equal quantity of standardized inoculum. The flasks were incubated at different incubation periods: 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 hours. The culture filtrates were collected and used for enzyme activity assay. The experiments were carried out in triplicate and the average values were recorded (Das and Prasad, 2010; Umayaparvathi *et al.,* 2013; Reddy *et al.,* 2016).

2.10 Effect of substrate concentration on enzyme production

The effect of each substrate concentration (L-arginine, L-asparagine and L-glutamine) was examined on enzyme activity. The substrate concentrations were ranged from 1.0 to 10% w/v and activity of each enzyme was determined at each concentration of the substrates (Das and Prasad, 2010; Umayaparvathi *et al.,* 2013; Reddy *et al.,* 2016).

2.11 Effect of temperature on enzyme production

Effect of temperature was examined on enzyme activity. The temperature range of 25^{o}C to 45^{o}C was used and the activity of each enzyme was determined at each temperature (Das and Prasad, 2010; Umayaparvathi *et al.,* 2013; Reddy *et al.,* 2016).

2.12 Effect of agitation rate on enzyme production

The fermentation medium was subjected to various agitation rates using shaker incubator. The agitation rates used were 50 rpm, 100 rpm, 150 rpm, 200 rpm and 250 rpm. Experiments were conducted in triplicate and recorded appropriately (Das and Prasad, 2010; Umayaparvathi *et al.,* 2013; Reddy *et al.,* 2016).

2.13 Effect of pH on enzyme production
Effect of pH was examined on enzyme activity. The pH values range of 4 to 10 were used and activity of each enzyme was determined at each pH value (Das and Prasad, 2010; Umayaparvathi *et al.,* 2013; Reddy *et al.,* 2016).

2.14 Effect of additional carbon source on enzyme production
Effect of additional carbon source was studied by changing the source of carbon in the broth and the flasks were incubated in triplicate and the average values were recorded. The carbon sources used were glucose, lactose, maltose, mannitol and sucrose at 4% w/v each (Das and Prasad, 2010; Umayaparvathi *et al.,* 2013; Reddy *et al.,* 2016).

2.15 Effect of additional nitrogen source on enzyme production
Effect of additional nitrogen source was studied by changing the source of nitrogen in the broth and the flasks were incubated in triplicate and the average values were recorded. The nitrogen sources used were KNO_3, meat extract, $NaNO_3$ and yeast extract at 4% w/v each (Das and Prasad, 2010; Umayaparvathi *et al.,* 2013; Reddy *et al.,* 2016).

2.16 Effect of various concentrations of sodium chloride on enzyme production
The effect of various concentrations of sodium chloride (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0%) on enzyme production was studied by varying the concentration of sodium chloride solution in the broth and the flasks were incubated in triplicate and the average values were recorded (Das and Prasad, 2010; Umayaparvathi *et al.,* 2013; Reddy *et al.,* 2016).

2.17 Effect of inoculum size on enzyme production
Effect of inoculum size was studied by varying the concentration of inoculum in the broth and the flasks were incubated in triplicate and the average values were recorded. The inoculum sizes used ranged from 0.1 to 1.0 mL after standardisation (Das and Prasad, 2010; Umayaparvathi *et al.,* 2013; Reddy *et al.,* 2016).

2.18 Study of enzyme production using optimum fermentation parameters
The enzymes *L-asparaginase* was produced using the optimum fermentation parameters. The production was done in triplicate and the average enzyme yield was recorded (Das and Prasad, 2010; Umayaparvathi *et al.,* 2013; Wakil and Adelegan, 2015; Reddy *et al.,* 2016).

2.19 In vitro analysis of the effect of extracted enzymes on the substrates
After production of the enzymes using optimum fermentation conditions, the broth was centrifuged at 10,000 rpm for 15 minutes. The supernatant was filtered using Whatman filter paper. The filtrate was then passed through a 0.22-µm filter. The filtrates obtained were then used to inoculate solidified agar containing respective substrates, and the agar plates were incubated for 24 hours at 37^{o}C (Kathiresan and Manivannan, 2006).

2.20 Effect of pH on the activity and stability of enzyme
L-asparaginase activity was evaluated at different pH values. Each partially purified enzyme was examined between pH 6.5 and 11, under assay conditions and the amount of ammonia and urea liberated was determined using spectrophotometer (Lenifield Model 752G, England). In case of pH stability experiment, each enzyme was incubated for 24 hours at 4±1^{o}C at different pH values (Dura *et al.,* 2002; Kumar and Chandrasekaran, 2003; Balagurunathan *et al.,* 2010).

2.21 Effect of Temperature on enzyme activity and stability
Optimum temperature for enzyme activity was determined by incubating the standard reaction mixture at temperatures ranging from 25^{o}C to 70^{o}C. Thermostability studies were carried out by pre-incubating the

enzyme at different temperatures for different time intervals (Dura *et al.*, 2002; Kumar and Chandrasekaran, 2003; Balagurunathan *et al.*, 2010).

3.0 Results

The chromogenic zones produced by the isolates were as shown in table 1. Isolate B38 produced the highest chromogenic zone of 35.00±0.09 mm and was selected for further studies.

The isolate was identified as *Alcaligenes faecalis* subsp. *phenolicus* and the nucleotide sequence is as shown in figure 1. The various conditions for the fermentation process were optimised in order to determine the best parameters for enzyme production. The optimum incubation period for the production of *L-asparaginase* was 84 hours (Figure 2). Effect of each substrate on enzyme production was examined. The optimum L-asparagine concentration that gave enzyme yield of 83U/mL was 7% (Figure 3). Temperature also had significant effect on enzyme yield. The optimum temperature for the production of *L-asparaginase* was 40^{o}C (Figure 4).

The optimum agitation rate that gave the highest *L-asparaginase* yield (95U/mL) was 200 rpm (Figure 5). The optimum pH for production of the enzyme was 7.5. However, enzyme yield dropped significantly between pH 8.0 and 10.0 and between 6.5 and 4.0. Lactose (4% w/v) had statistically significant effect on yield of the enzyme as shown in figure 7. Sucrose had the least effect on the production of *L-asparaginase*. Effect of additional nitrogen source was examined on enzyme production and yeast extract and KNO_3 (at 4% w/v each) had significant effect on the production of *L-asparaginase* as there was no significant effect between the effects showed by the two substrates. $NaNO_3$ had the least effect on the production of enzyme as shown in figures 8. Effect of NaCl was monitored between 1% and 10% and it was found that the optimum NaCl concentration for the production of *L-asparaginase* was 4% and the enzyme yield obtained was 92 U/mL as shown in figure 9.

The optimum inoculum size for the production of the enzymes ranged between 0.1mL and 0.3mL as shown in figure 10. The chromogenic zone obtained when the partially purified *L-asparaginase* was used against L-asparagine was as shown in table 3. The enzyme metabolised the substrate hydrolysing L-asparagine to L-aspartic acid and ammonia. The enzyme was produced using optimum parameters determined and the enzyme activity was 192±0.93 U/mL. The molecular weight of *L-asparaginase* was 39 kDa as shown in Table 5.

Table 1: Chromogenic zones for enzyme production

Bacterial Isolates	*L-asparaginase* (mm)	Bacterial Isolates	*L-asparaginase* (mm)
B1	10.00 ± 0.01[a]	B21	20.00 ± 0.01[f]
B2	0.00 ± 0.00	B22	20.00 ± 0.02 [f]
B3	0.00 ± 0.00	B23	0.00 ± 0.00
B4	0.00 ± 0.00	B24	0.00 ± 0.00
B5	27.00 ± 0.02 [i]	B25	0.00 ± 0.00
B6	15.00 ± 0.01[c]	B26	10 ± 0.02 [a]
B7	20.00 ± 0.01	B27	15.00 ± 0.01 [c]
B8	18.00 ± 0.02[e]	B28	10.00 ± 0.03 [a]
B9	0.00 ± 0.00	B29	0.00 ± 0.00
B10	0.00 ± 0.00	B30	0.00 ± 0.00
B11	21.00 ± 0.01[g]	B31	27.00 ± 0.01[i]
B12	0.00 ± 0.00	B32	11.31 ± 0.00[b]
B13	10.00 ± 0.02 [a]	B33	0.00 ± 0.00
B14	0.00 ± 0.00	B34	21.00 ± 0.02[g]
B15	0.00 ± 0.00	B35	0.00 ± 0.00
B16	0.00 ± 0.00	B36	15.00 ± 0.01 [c]
B17	0.00 ± 0.00	B37	23.00 ± 0.01[h]
B18	10.00 ± 0.02 [a]	B38	35.00 ± 0.09[j]
B19	17.00 ± 0.01[d]	B39	0.00 ± 0.00
B20	10.00 ± 0.00 [a]		

Means with the same letters are not significantly different ($p \leq 0.05$) at $\alpha = 5\%$

Nucleotide sequence of *Alcaligenes faecalis* subsp. *phenolicus* (Isolate B38)

TCAGATTGAACGCTAGCGGGATGCTTTACACATGCAAGTCGAACGGCAGCGCGAGAGAGCT
TGCTCTCTTGGCGGCGAGTGGCGGACGGGTGAGTAATATATCGGAACGTGCCCAGTAGCGG
GGGATAACTACTCGAAAGAGTGGCTAATACCGCATACGCCCTACGGGGGAAAGGGGGGGAT
CGCAAGACCTCTCACTATTGGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAAAGGCT
CACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGAT
CCAGCCATCCCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAA
AAGGTATCCCCTAATACGGGATACTGCTGACGGTATCTGCAGAATAAGCACCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA
GCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCA
TTTTTAACTGCCGAGCTAGAGTATGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAAT
GCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAATACTGACGCTC
AGACACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGA
TGTCAACTAGCTGTTGGGGCCGTTAGGCCTTAGTAGCGCAGCTAACGCGTGAAGTTGACCGC
CTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGT
GGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTCTGGAA
AGCCGAAGAGATTTGGCCGTGCTCGCAAGAGAACCGGAACACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTT
GCTACGCAAGAGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGGACAGAGG
GTCGCCAACCCGCGAGGGGGAGCCAATCTCAGAAACCCGATCGTAGTCCGGATCGCAGTCT
GCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGTCGCGGTGAAT
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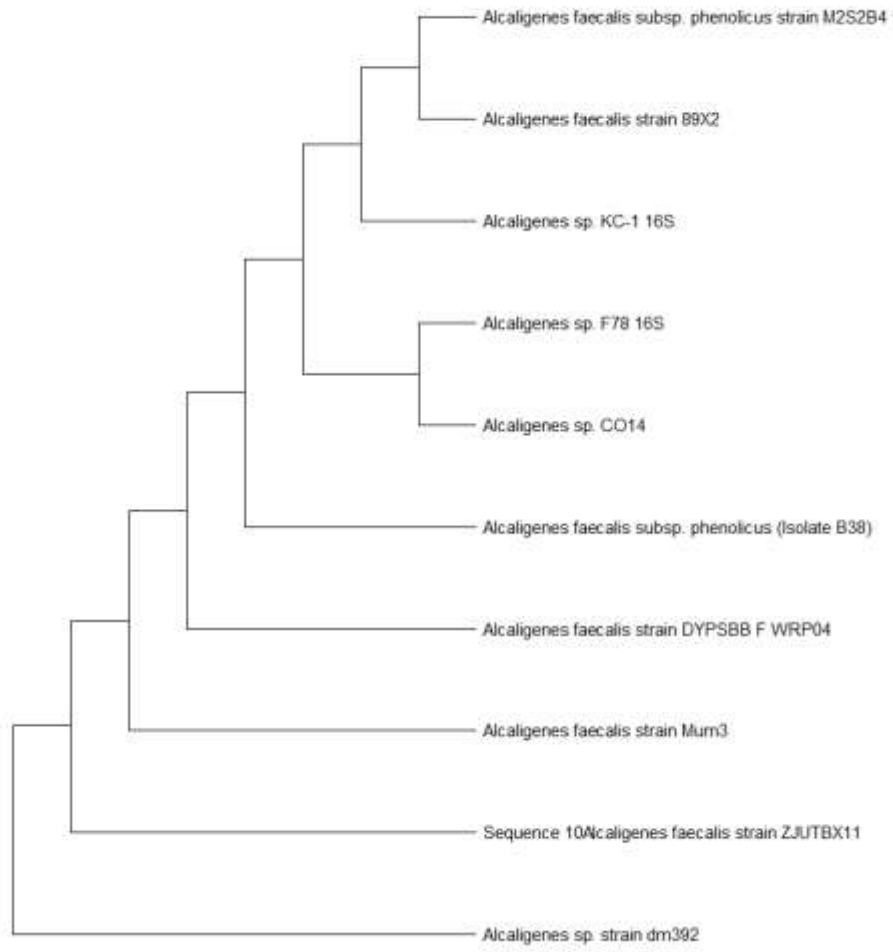

Figure 1: Maximum Likelihood (Molecular Phylogenetic Analysis) (Isolate B38)

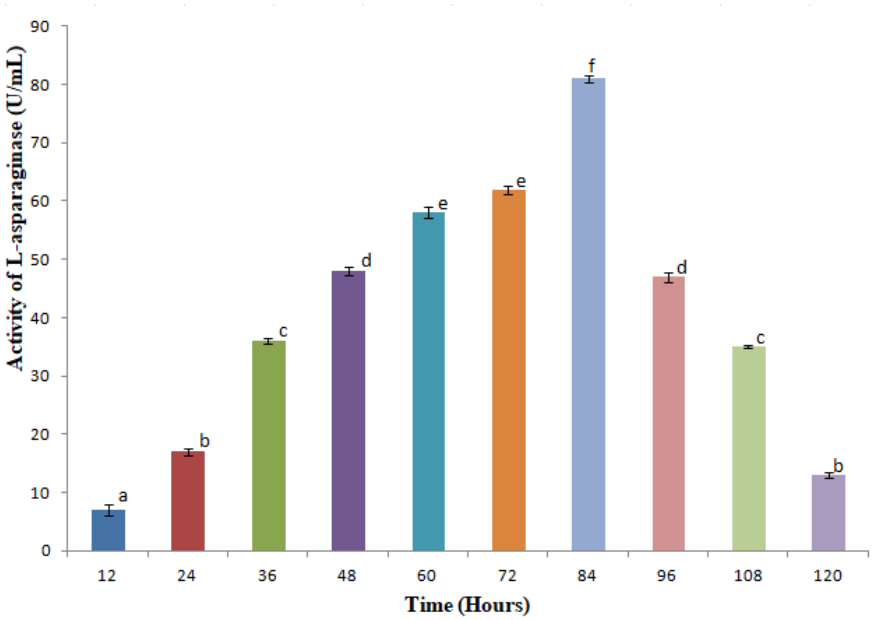

Figure 2: Effect of Incubation Period on *L-asparaginase* Production (produced by Isolate B38)
Means with the same letters are not significantly different ($p \leq 0.05$) based on Duncan's multiple range test
Error bars represent the standard error of mean (SEM)

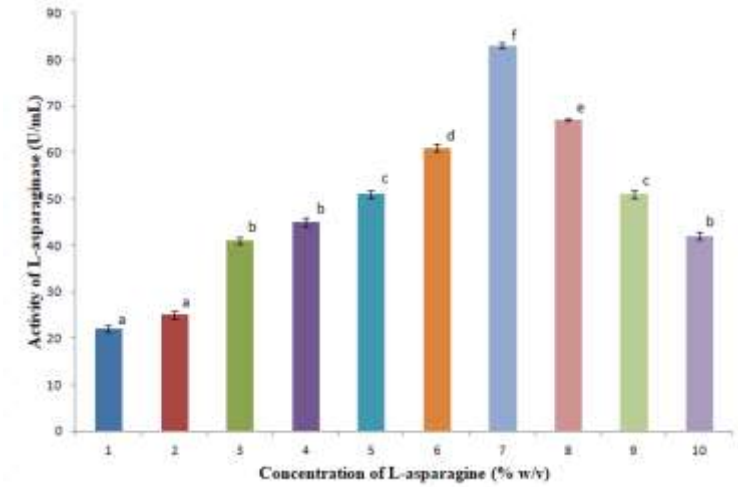

Figure 3: Effect of L-asparagine Concentration on the Production of *L-asparaginase* (Isolate B38)
Means with the same letters are not significantly different (p≤0.05) based on Duncan's multiple range test
Error bars represent the standard error of mean (SEM)

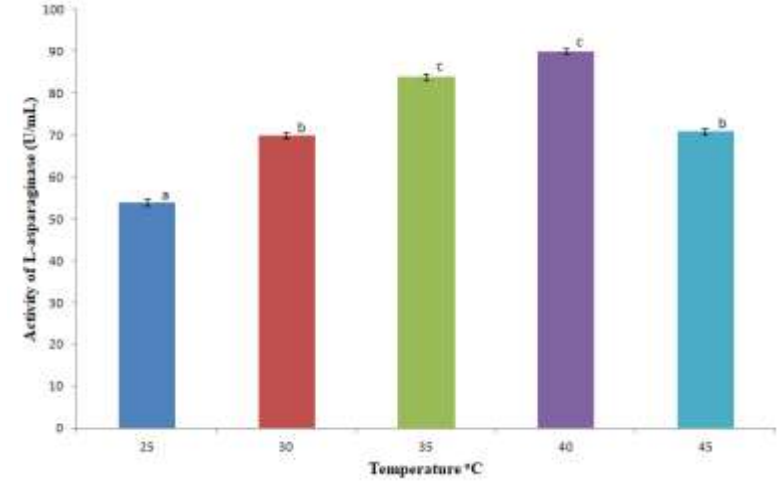

Figure 4: Effect of Temperature on the Production of *L-asparaginase* (Isolate B38)
Means with the same letters are not significantly different (p≤0.05) based on Duncan's multiple range test
Error bars represent the standard error of mean (SEM)

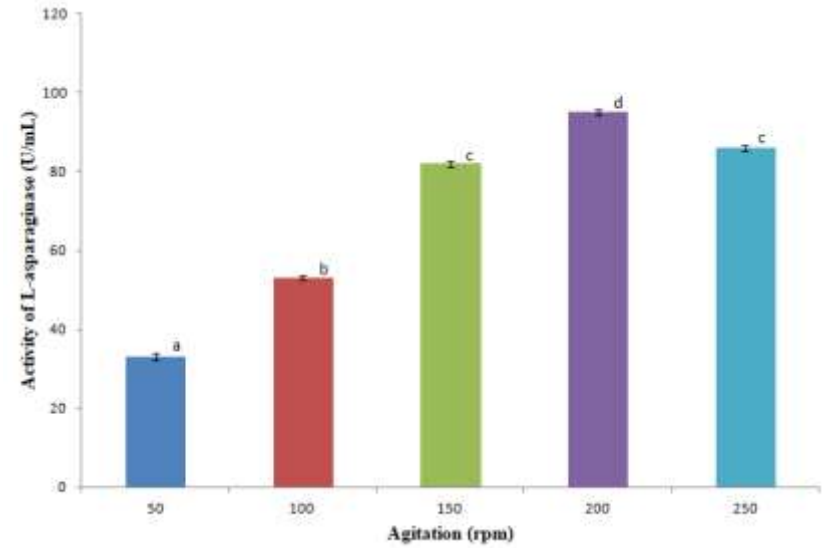

Figure 5: Effect of Agitation Rate on the Production of *L-asparaginase* (Isolate B38)
Means with the same letters are not significantly different (p≤0.05) based on Duncan's multiple range test
Error bars represent the standard error of mean (SEM)

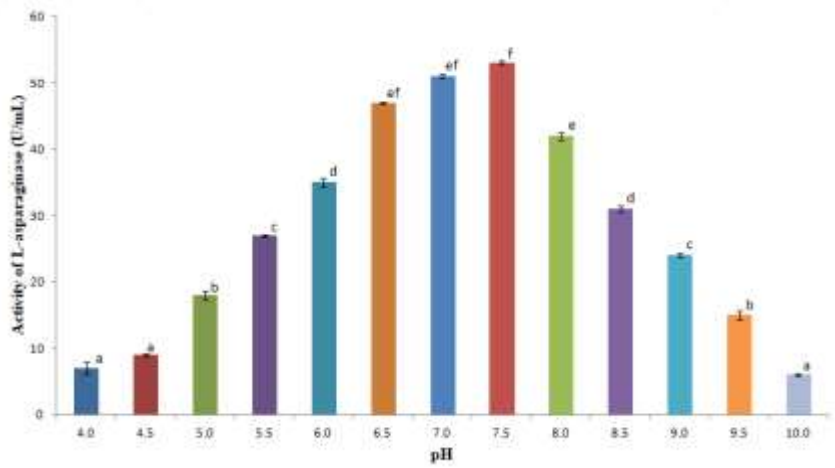

Figure 6: Effect of pH on the Production of *L-asparaginase* (Isolate B38)
Means with the same letters are not significantly different (p≤0.05) based on Duncan's multiple range test
Error bars represent the standard error of mean (SEM)

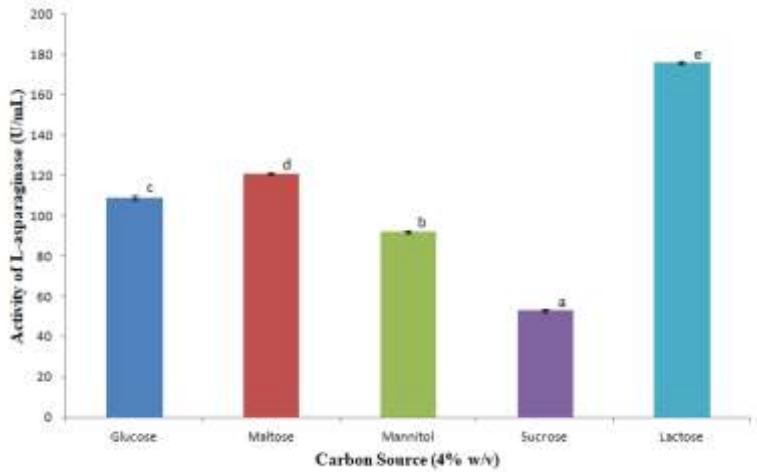

Figure 7: Effect of Additional Carbon Source on the Production of *L-asparaginase* (Isolate B38)
Means with the same letters are not significantly different (p≤0.05) based on Duncan's multiple range test
Error bars represent the standard error of mean (SEM)

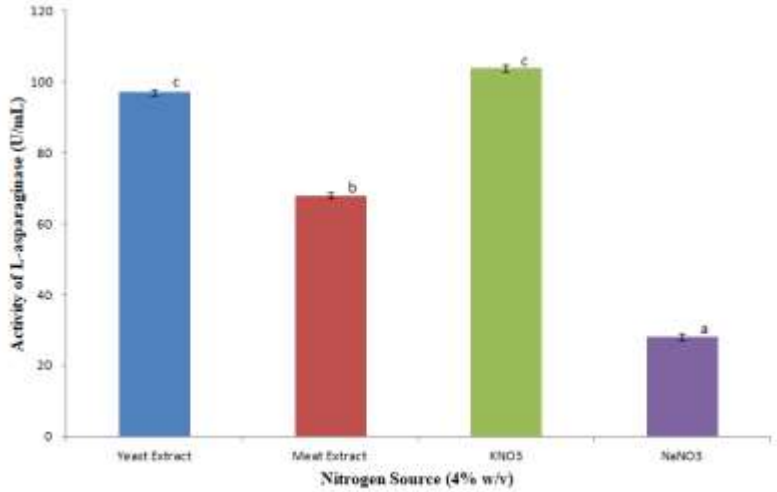

Figure 8: Effect of Additional Nitrogen Source on the Production of *L-asparaginase* (Isolate B38)
Means with the same letters are not significantly different (p≤0.05) based on Duncan's multiple range test
Error bars represent the standard error of mean (SEM)

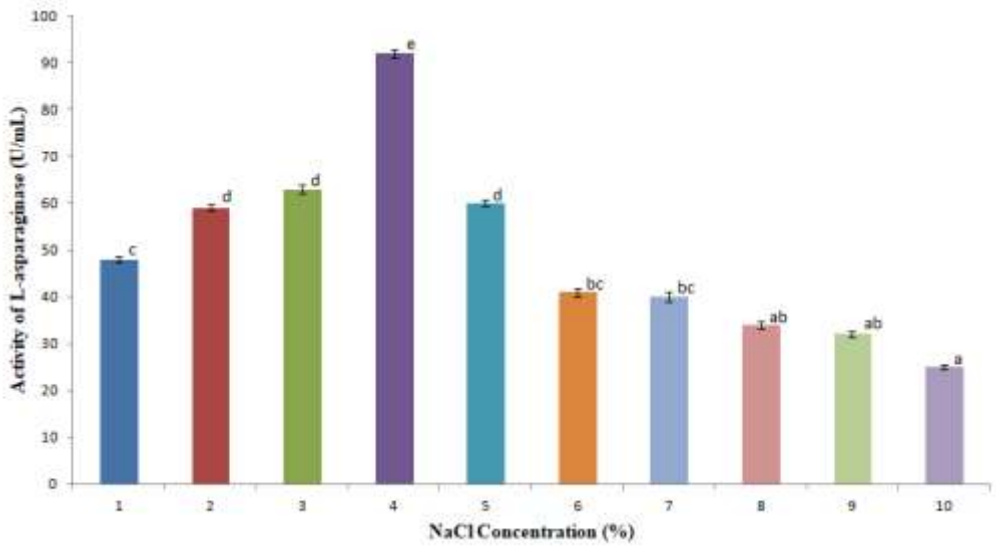

Figure 9: Effect of NaCl Concentration on the Production of *L-asparaginase* (Isolate B38)
Means with the same letters are not significantly different (p≤0.05) based on Duncan's multiple range test
Error bars represent the standard error of mean (SEM)

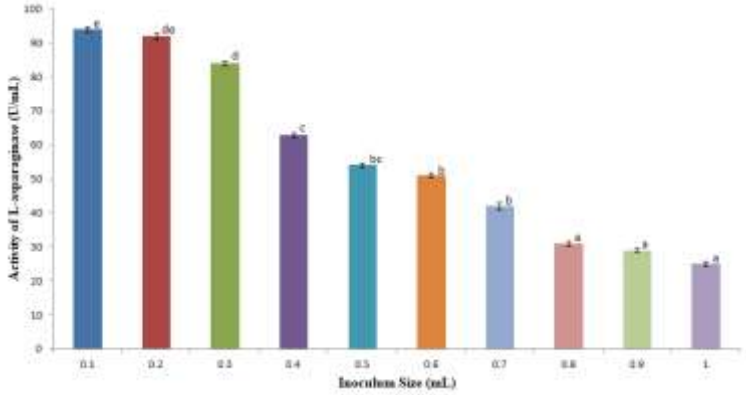

Figure 10: Effect of Inoculum Size on the Production of *L-asparaginase* (Isolate B38)
Means with the same letters are not significantly different (p≤0.05) based on Duncan's multiple range test
Error bars represent the standard error of mean (SEM)

Table 2: Optimum conditions for the production of enzymes

S/N	Fermentation Condition	*L-asparaginase* (Isolate B38)
1	Incubation period (Hours)	84
2	Substrate Concentration (%)	7 (L-asparagine)
3	Temperature (^oC)	40
4	Agitation rate (rpm)	200
5	pH	7.5
6	Additional carbon (4% w/v)	Lactose
7	Additional nitrogen (4% w/v)	KNO_3
8	NaCl Concentration (%)	4
9	Inoculum size (mL)	0.1

Table 3: In vitro analysis of the effect of enzymes on the substrates

S/N	Extracted enzyme	Bacterial isolate	Examined substrate	Chromogenic zone (mm)
1	*L-asparaginase*	*Alcaligenes faecalis* subsp. *phenolicus* (Isolate B38)	L-asparagine	57±2.08

Table 4: Enzyme yield using optimum fermentation parameters

S/N	Enzyme	Production Isolate	Enzyme activity (U/mL)
1	*L-asparaginase*	*Alcaligenes faecalis* subsp. *phenolicus* (Isolate B38)	192±0.93

Values are Mean±SEM

Table 5: Molecular weight of the enzyme

S/N	Enzyme	Production Isolate	Molecular weight (kDa)
1	*L-asparaginase*	*Alcaligenes faecalis* subsp. *phenolicus* (Isolate B38)	39

3.1 Discussion

Bacteria have been chosen from various microorganisms for large scale production due to their ability to produce high yield of various industrial products (Janette *et al.,* 2019). Morphological and biochemical characteristics of the bacterial isolates were used for their identification. The promising bacterial isolate was later identified using their 16S rRNA sequence through the GeneBank of NCBI. The promising isolate belong to the species of *Alcaligenes*.

The isolated bacteria were screened for the production of *L-asparaginase*. The isolate that produced highest chromogenic zone was selected for the production of *L-asparaginase*. The enzyme was then produced using the submerged fermentation process. The results obtained during the optimization process showed that fermentation parameters such as incubation period, pH, temperature, inoculum concentration, substrate concentration, and sodium chloride concentration significantly affected production of the enzyme.

Production of enzyme was monitored using optimum conditions. Enzymes yield was observed to be higher compared to the observed yield before optimization. The yield of *L-asparaginase* was 192±0.93 U/mL. The yield of *L-asparaginase* reported by Pejman *et al.* (2016) was 61.7 U/mL. This was lower compared to the value reported in this present study. The isolated bacteria used in the study were halophilic and halotolerant strains of *Bacillus* and *Salicola*. The existence of high salt content could be one of the reasons for low yield.

The molecular weight of *L-asparaginase* was 39 kDa. Varying molecular weights have been reported for enzymes (Snehal *et al.,* 2005). Microbial enzymes are preferred because of their high yields, ease of modification and optimization and economic friendliness. Microbial enzymes are not affected by seasonal fluctuations (Neelam *et al.,* 2013). It has been mentioned that some of the factors affecting the molecular weight of enzymes include the source of the enzyme and the molecular weights of enzymes can range between 12 000 to over 1 million Da (Saurabh, 2018).

When partially purified enzyme was used on the substrate, higher chromogenic zone was produced compared with the chromogenic zones obtained when viable microbial cells were used. This is possible because cell components that could affect enzyme quantity within the cells have been lowered during enzyme extraction.

The enzyme was stable at neutral to slightly alkaline pH. The pH values obtained ranged from 7.0 to 8.5. Reports have posited that these pH levels will allow enzymes to function effectively. High pH values have been known to have adverse effect on enzyme activity. One of the major challenges of using enzymes as biopharmaceutical is their vulnerability to extreme pH conditions (Fromm, 1975). For this reason

enzymes that are to be used as therapeutics are usually encapsulated using nanoparticles or other substances that will not affect delivery of the enzyme at target sites. Various enzymes have been PEGylated in order to immobilise them and prevent inactivation. *L-asparaginase* was stable between 25ºC and 55ºC. It has been demonstrated that enzymes can be denatured at higher temperature (Roy, 2008).

Maize plant rhizosphere environment has been shown as a source of biopharmaceutically important microorganisms. There is therefore a need to conduct further research in the rhizosphere of other plants in order to isolate other microbes that can be of medical and industrial importance.

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