American Journal of Microbiology and Biotechnology
2018; 5(1): 5-11
http://www.aascit.org/journal/ajmb
ISSN: 2375-3005

AASCIT American Association for Science and Technology

Keywords
Anti-cancerous Enzyme,
Optimization,
L-asparaginase,
E.coli,
Response Surface Methodology (RSM)

Received: March 22, 2017
Accepted: May 5, 2017
Published: February 27, 2018

Extraction and Optimization of L-asparaginase Produced by Local Isolates of *E.coli*

Hanan Moawia Ibrahim[1, *], Safeya Mohammed Shareef Mohammed Salih[1], Runda Eltayeb[1], Elrashied Elimam Elkhidir[2]

[1]Department of Biology, Central Lab., Ministry of Higher Education and Scientific Research, Khartoum, Sudan
[2]College of Agricultural Economic, Faculty of Agriculture, University of Khartoum, Khartoum, Sudan

Email address
hara111@yahoo.com (H. M. Ibrahim)
[*]Corresponding author

Citation
Hanan Moawia Ibrahim, Safeya Mohammed Shareef Mohammed Salih, Runda Eltayeb, Elrashied Elimam Elkhidir. Extraction and Optimization of L-asparaginase Produced by Local Isolates of *E.coli*. *American Journal of Microbiology and Biotechnology*. Vol. 5, No. 1, 2018, pp. 5-11.

Abstract
This study investigated the production of the anticancer enzyme L-asparaginase by the local isolate of *E.coli*. Sewage water samples were collected from different locations in Sudan. 96% out of The 180 samples collected were found to be positive *E.coli* while 4% were negative in EMB that showed pink color around the colony. 42% of the positive isolates were L-asparaginase producer with inhibition zone ranged from (15-40 mm) while 58% of The isolates not L-asparginase producer. These isolates were identified microscopically, biochemically and confirmed by molecular characterization. The optimization of the physical conditions in term of temperature and pH showed that the optimum pH is of 9.0 and optimum temperature is of 25°C using statistical experimental design, Response Surface Methodology (RSM) revealed in higher L-asparaginase yield of 2.2U/ml/min.

1. Introduction

L-asparaginase is known for its potent ability to be used as a therapeutic agent for the treatment of leukemia since many years, and is found to be responsible for the antitumor activity of guinea pig serum. Subsequently, it was identified as an effective antitumor agent in human clinical trials and today it is regarded as one of the useful components of the antitumor therapy. L-asparaginases have been found in many mammalian and bacterial species, but only the enzymes from *E. coli* and *Erwinia chrysanthemi* have been produced on an industrial scale. Although the drugs from both sources have identical mechanisms of action and toxicities, their pharmacokinetic properties are different, and patients allergic to one drug are frequently resistant to the other. Though important in cancer therapy, the clinical utility of L-asparaginase is often limited by three factors. First, the broad variety of side effects associated with L-asparaginase administration, including immune-suppression and pancreatitis. The toxic effects fall into two main categories, those related to immunologic sensitization to a foreign protein and those related to the inhibition of protein synthesis [1]. Microbial L-asparaginase has attracted considerable attention since the demonstration that L-asparaginase form *E.coli* has anti-

tumor activity [2]. Because of its long history of laboratory culture and ease of manipulation, *E. coli* plays an important role in modern biological engineering and industrial microbiology. The work of different authors became a foundation of biotechnology [3]. L-asparaginase IUPAC name is *E.coli* asparagines amido hydrolase, its formula is C_{1377}, H_{2208}, N_{382}, O_{44}, S_{17} and of molecular mass of 31731.9g/mol, it is approved biotech agent that converts asparagines to aspartic acid and ammonia. Some malignant cells lose the ability to produce asparagines and so the loss of exogenous sources of asparagines leads to cell death. L-asparaginase is on the World Health Organization (WHO) essential medicines list. L-asparaginase most common use is a processing food manufacturing for that purpose it is marketed under the brand names Acryl away and it reduces the formation of acryl amide, a suspected carcinogen in starch food product (Wikipedia). Attention has been given to isolate L-asparaginase enzymes by microorganisms. Different microorganisms were used for the screening, some of the *Pseudomonas* species were also proved for the production of L-asparaginaes [4]. Acute lymphoblastic leukemia is cancer of white blood cell (WBC) characterized by the excessive multiplication of malignant and immature WBC in bone marrow. Tumor cells have an unusually high requirement for the amino acid L-asparagine and cannot synthesize sufficient endogenous L-asparagines due to very low levels of L-asparagines synthesize and therefore are dependent on serum levels of L-asparagine for their proliferation and survival [5]. Treatment of acute leukemia includes chemotherapy steroids radiation therapy intensive combined treatments including bone marrow or stem cell transplants or of one more attributed reason is the inability of these cells to increase L-asparagine synthetase activity after L-asparaginase administration [6]. It demonstrated high potential against acute lymphoblastic leukemia [7]. So they use both L-asparagine from the diet (blood serum) as well as what they make themselves (which is limited) to satisfy their large L-asparagine demand. Thus, administration of L-asparaginase deprives dependent tumor cells of their extracellular source of L-asparagine and lead to apoptosis, among this chemotherapy is most preferred. However, healthy cells escape unaffected as they are capable of synthesizing asparagine de novo with the aid of the enzyme L-asparagine synthetase [8]. The requirement of the biotechnological process is high yielding organism to satisfy this requirement isolation of microorganism that could produce a higher amount of L- asparaginase is important.. Extensive clinical trials of this enzyme, however, were not possible because of inadequate production of this substance along with strain improvement of organisms which can enhance the production of enzymes, for this purpose, strain development and optimization of environmental factors using statistical experimental design is preferred. So the general objective of this study is to extract L-asaraginase from a local isolate and to optimize the physical conditions in term of pH and temperature using.

2. Materials and Methods

2.1. Sampling

One hundred eighty sewage water samples were collected from different locations in Sudan using 50 ml pre-sterile tubes, samples has been taken every one meter from the collection area. This study has been conducted from September 2014 to May 2016.

2.2. Isolation of Micro-organism

Water samples were serially diluted using soil dilution plate technique [9]. In this technique: 1g of each soil sample was taken in 9 ml of sterilized normal saline (0.85%) in pre-sterilized test tube. Serial aqueous dilutions were prepared by transferring 1 ml of the soil suspension into 9 ml of sterilized normal saline still water in sterilized test tubes. Different aqueous dilutions (10^{-4} - 10^{-10}) of the soil suspension were applied separately into sterilized petri-dishes containing sterilized plate of Eosin Methylene Blue (EMB) agar and incubated at 37°C for 24 hours. Pure cultures were obtained by streak plates using wire loop.

2.3. Primary Screening for L-asparaginase

Plates of sterilized modified ISP5 medium were inoculated with the collected water samples and isolated by a loop-full of isolate in the centre of each plate. All plates were incubated at 37°C including the control plates without carbon source (Lactose).

2.4. Identification of the Isolates

2.4.1. Biochemical Characterization

Microscopic and biochemical examinations were performed [10], [11]. Motility test, Gram stain, Oxidase test, Urease test, Voges-Proskauer test, Indole test, Catalase Test, Nitrate Reduction Test, Citrate Utilization Test and Hydrogen sulphide production test.

2.4.2. Molecular Identification

DNA extraction

DNA was extracted by taking I ml of overnight grown brain heart media culture, centrifuged at 6000 rpm for 5 min, discard the supernatant, the pellet was washed with distilled water then put in water bath at 95°C for 30 min and immediately cooled on ice for 10 min, and centrifuged at 12000 rpm for 10 min, the supernatant was the DNA extraction. [12].

Polymerase Chain Reaction (PCR)

PCR was used to confirm *E.coli* isolates using universal primer. For (pal gene) the primer sequence are:

Forword Primer: 5'GGCAATTGCGGCATGTTCTTCC-3'

Reverse primer: 5-CCGCGTGACCTTCTACGGAC-3'

PCR Mixture

The PCR mixtures were prepared as follows: 2 µl of 10 mL of both forward and reverse primers, 2µl of DNA sample,

16µl de-ionized water were added to adjust the final reaction volume to 20µl in PCR premix tube. PCR conditions were carried out as follows: 3 min at 94°C followed by 35 cycles of 94°C for 30 sec. 60°C for 30 sec, and 72°C for 90 sec. finally by 72°C for a minute [13].

Agarose Gel Electrophoresis

10 µl PCR Products were analyzed with 1.5% agarose gel which prepared by dissolving 1.125 g of molecular grade agarose in 75 ml of 1X TBE buffer, boiled for 5 min at microwave, then cooled, 2 µl of ethidium bromide was added, the gel poured in tank and cooled. 10 µl of PCR product were run out on the gel for 30 min., then examined under gel documentation system [13].

2.5. Extraction of L-asparaginase

The secondary screening of bacterial isolates for L-saparaginase production was done using modified broth medium (pH 7.0) supplemented with L-asparagine which was inoculated with *E.coli* cultures and incubated for 42 hours at 37°C. After completion of the incubation period culture broth was centrifuged at 6,000 rpm for 15 min. at 4°C to assay extracellular L-asparaginase activity, the supernatant was identified as crude enzyme.

2.6. Enzyme Assay

Activity was measured by direct Nesslerization of ammonia. The activity catalysis L-aspargine to L-aspartic acid and ammonia and the latter react with the Nesslers reagant to produce an orange colored product. The enzyme assay mixture consisted of 100ul of freshly prepared L-asparaginase (189mmol/L) in Tris-HCL buffer (pH8.6) and 100uL of crude extract of the enzyme The reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by adding 100uL of 15% trichloroacetic acid (TCA) the reaction mixture was centrifuged at 6000 xg for 5 min at 4°C to remove the precipitates. The ammonia released in the supernatant was determined using colorimetric technique by adding 500 ml Nesslerization sample containing 200 uL supernatant and 4.3 mL distilled water. The contents in the sample were vortexed and incubated at room temperature for 10 min scanned for measured at 396nm against the blanks that received TCA before the addition of crude enzyme the ammonia produced in the reaction was determined based on the standard curve obtained with ammonium sulfate. One Interaction Unit (IU) of L-asparaginase activity was defined as the amount of the enzyme that liberates 1umol/L of ammonia /min at 37°C [14].

2.7. Time Course of Enzyme Production

The colonies with large zone on ISP5 medium were used for inoculum preparation. inoculate the colonies in brain heart infusion broth and incubated at 37°C for 42 hours in rotary shaker incubator at 140 rpm, then a volume of 5 ml was taken from this culture and added to 45 ml of GTA medium. Samples were removed periodically every 6 hr. and the enzyme activity and cell growth were determined Spectro- photometeracally. [15].

2.8. Optimization of the Physical Conditions

Response surface methodology [16] was used for the optimization of the variables, the variables were temperature and pH. The variables (coded and un-coded) of each constituent at level -1, 0 and +1 are given in Table 1. The surface response for enzyme production as a function of selected key variable was determined. A two-level full fractional factorial design with two variables consisting of two blocks and with 14 runs (8 combinations with 6 replications of the center points) were used. The MINITAB package version 16.0 was used to describe the response surface method.

Table 1. *Experimental design.*

	C1	C2	C3	C4	C5	C6
	stdor	Runor	ptType	Blocks	Temp	pH
1	1	1	1	1	25	6.0000
2	7	2	0	1	35	7.2500
3	2	3	1	1	45	6.0000
4	4	4	1	1	45	8.0000
5	5	5	0	1	35	7.2500
6	3	6	1	1	25	8.5000
7	6	7	0	1	35	7.2500
8	11	8	1	2	35	9.0177
9	13	9	0	2	35	7.2500
10	9	10	1	2	49	7.2500
11	8	11	1	2	20	7.2500
12	10	12	1	2	35	5.4822
13	14	13	0	2	35	7.2500
14	12	14	0	2	35	7.27500

3. Result

One hundred eighty water samples collected from 4 different locations in Sudan, serial diluted, then 10^{-1} were cultured in EMB media. 173 water samples (96%) showed metallic green color indicating the presence of *E.coli.* while seven water samples (4%) were negative as shown on Figure 1. that may be because *E.coli* is commonly known as colon bacteria so it is easily available in sewage water with fecal contamination, Those negative results may be due to the depth of the water from where the sample obtained or may be genetically different from those positive ones. *E.coli* was confirmed to be an active producer of L-asparaginase which in agreement with published research [17]. Also this study agreed with others results [18] in that ISP5 modified media is used to identify coliform for L-asparaginase production. The importance of micro-organism as L-asparaginase sources has been focused since the time was obtained from *E.coli* and its anti-neoplastic activity demonstrated in guinea pig serum [8]. It was occur naturally and is distributed among living organisms including animals, plant and microbes [19].

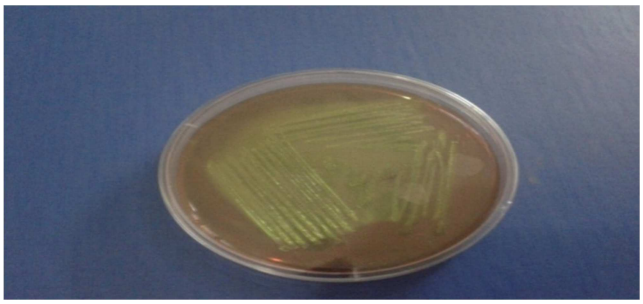

Figure 1. *Illustrate the finding of the metallic green in EMB media.*

3.1. Primary screening of L-asparaginase Production

42% out of 96% of the positive isolates were found to be of L-asparaginse producer, where the other 58% could not produce L-asparaginase. All samples gave high inhibition zones ranged from 15-40 mm, when cultured in ISP-5 media. Samples No (10), (15) and (18) gave highest inhibition zone of 40 mm where samples No (16) and (17) showed lowest Inhibition zones of 15mm. The above finding showed that Sudan habitant were an excellent source of L-aspararaninase producer. Biochemical identifications of the isolates illustrated in Table 2. All the tested isolates were positive in oxidase, catalase, urease and indole tests. except sample No (1) and (2) are negative while in H_2S production twenty nine of the samples were negative except sample No. (2) was positive. Tested isolates showed negative result in Voges Proskauer test, most of the isolates were able to reduce nitrate only samples No. (3), (5) and (9) not reduce nitrate. All of the isolates were strong in utilization of citrate except samples No. (1), (8) and (11)

Table 2. *Biochemical identification of the isolates.*

Sample No.	Motility	Gram	Indole	Oxidase	(VP)	HS	Urease	Lactose	Catalase	Citrate	Nitrate
1	+	-	+	+	-	-	-	+	+	-	+
2	+	-	+	+	-	+	-	+	+	+	+
3	+	-	+	+	-	-	+	+	+	+	-
4	+	-	+	+	-	-	+	+	+	+	+
5	+	-	+	+	-	-	+	+	+	+	-
6	+	-	+	+	-	-	+	+	+	+	+
7	+	-	+	+	-	-	+	+	+	+	+
8	+	-	+	+	-	-	+	+	+	-	-
9	+	-	+	+	-	-	+	+	+	+	+
10	+	-	+	+	-	-	+	+	+	+	+
11	+	-	+	+	-	-	+	+	+	-	+
12	+	-	+	+	-	-	+	+	+	+	+
13	+	-	+	+	-	-	+	+	+	+	+
14	+	-	+	+	-	-	+	+	+	+	+
15	+	-	+	+	-	-	+	-	+	+	+
16	+	-	+	+	-	-	+	+	+	+	+
17	+	-	+	+	-	-	+	+	+	+	+
18	+	-	+	+	-	-	+	+	+	+	+
19	+	-	+	+	-	-	+	-	+	+	+
20	+	-	+	+	-	-	+	+	+	+	+
21	+	-	+	+	-	-	+	+	+	+	+
22	+	-	+	+	-	-	+	+	+	+	+
23	+	-	+	+	-	-	+	+	+	+	+
24	+	-	+	+	-	-	+	+	+	+	+
25	+	-	+	+	-	-	+	-	+	+	+
26	+	-	+	+	-	-	+	+	+	+	+
27	+	-	+	+	-	-	+	+	+	+	+
28	+	-	+	+	-	-	+	+	+	+	+
29	+	-	+	+	-	-	+	+	+	+	+
30	+	-	+	+	-	-	+	+	+	+	+

(+) Positive samples. (-) Negative samples

3.2. Molecular Identification

The extracted DNA of three samples was collected for amplification in PCR. PCR results revealed that the isolates were *E.coli* compared to the standard. The specific primers used produced a 270 bp band as shown in electrophoresis gel and measured using a 100 bp DNA ladder. The identification of this strain showed that all the isolates were positive to the presence of *E.coli* Figure 2.

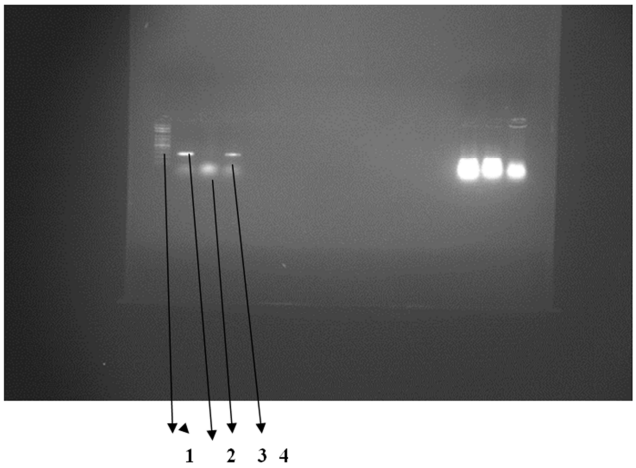

Figure 2. *Agarose gel electrophoresis of PCR amplification product*

Lane 1: DNA ladder of 100bp
Lane 2: Sample 15
Lane 3: Sample 10
Lane 4: Sample 1

3.3. The Time Course of L-asparaginase Activity

The enzyme activity and the bacterial growth kinetics are depicted on Figure 3. Enzyme production from the local isolated *E.coli*in different time periods (0-42) hr. Maximum growth of the bacterium was obtained with 18hr of cultivation based on turbidity of the culture (at 650 nm). The activity of the enzyme reached a maximum within 18 hr after inoculation (2.4 U/ml), beyond 18hr of growth no increase in enzyme activity or bacteria growth was recorded. The two profiles were similar and show that the fermentation kinetics of L-asparaginase production by *E.coli* might be classified as growth associated enzyme, production was found to be concomitant with growth. This is a significant improvement over other study [20] whom reported that the highest (0.06 U/ml) enzyme activity was at 24 hr, and the enzyme activity decreased at 48 hr throughout till 72 hr while this results showed that higher activity of 2.2 U/ml at 18 hour and the enzyme activity decreased throughout till 42 hour. This result also agree with published research [21]. They found that enzyme was consistently produced during log phase and continued to increase until the highest yield was achieved following decline phase of the microbes.

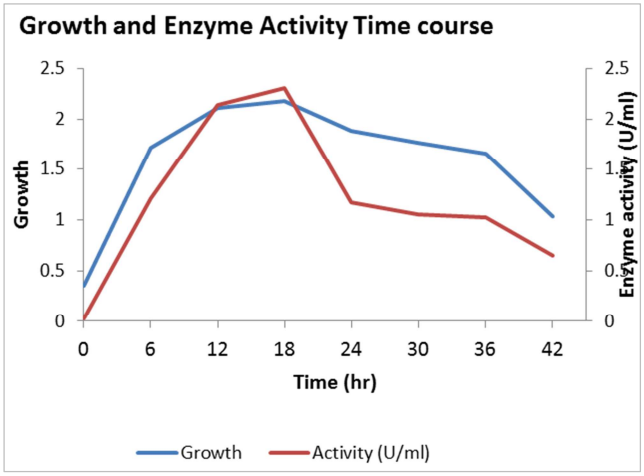

Figure 3. *The profile of the L-asparaginase activity and bacterial growth.*

3.4. Response Surface Methodology

The requirements of the biotechnological processes is high yielding organisms to satisfy this requirement, the knowledge of various fermentation parameters are necessary, for this purpose optimization of environmental factors is a must. The experimental design constitutes an efficient tool and is well adopted for treating problems with large number of variables and allows simultaneous, systematic and efficient variables of all components [16], [22]. The extracellular pH has a strong influence on the pathways of metabolism and product generation by micro-organism, the temperature is also important as it affects the conversion efficiency of substrate into cell mass which affect the product particularly when product is growth associated. So in this study we optimized pH and temperature as environmental factors for increasing the L-asparaginase yield. RSM allowed calculation of maximum production based on a set of experiments in which all the factors were varied within chosen ranges. Enzyme activity achieved for each variables concentration and the experimental combination for the two factors two level 2^2 resopnse surface analysis are shown in Table 3. The overall effects of experimental operation factors, temperature and pH on L-aparaginase activity are shown in Table 3.

Table 3. *Variables in experimental plan.*

Trails	stdor	ptType	Blocks	pH	Temp	Enzyme Activity U/ml
1	1	1	1	6	25	1.7573
2	7	0	1	7.2	35	2.5563
3	2	1	1	6	45	1.4586
4	4	1	1	8.5	45	0.5473
5	5	0	1	7.2	35	3.0238
6	3	1	1	8.5	25	3.1478
7	6	0	1	7.2	35	1.9715
8	11	1	2	9	35	2.9215
9	13	0	2	7.2	35	2.3747

Trails	stdor	ptType	Blocks	pH	Temp	Enzyme Activity U/ml
10	9	1	2	7.2	49	0.2792
11	8	1	2	7.2	20	1.2447
12	10	1	2	5.4	35	0.7091
13	14	0	2	7.2	35	2.3622
14	12	0	2	7.2	35	2.3426

Table 4. *Regression result of a polynomial model for L-asparaginase.*

Term	Coef	SECoef	T	P
Constant	-9.03796	6.16562	-1.466	0.181
temp	0.35755	0.14210	2.516	0.036
pH	1.14407	1.34841	0.848	0.421
temp*temp	-0.00327	0.00132	-2.472	0.039
pH*pH	-0.00860	0.08565	-0.100	0.922
temp*pH	-0.02341	0.01494	-1.567	0.156
S = 0.373651	PRESS = 6.03595			
R-Sq = 72.25%	R-Sq(pred) = 0.00%	R-Sq(pred) = 0.00%		

It was found that those factors all exerted certain effect on response and the percent variability explained (R^2) was (72.25). (R^2) value also indicates that only (27.75) percent of the total variation is not explained by the model. This indicated that the variation in those selected factors could explain the variation in L-asparaginase activity up to 72.25 percent. Table 4. The analysis was done using un-coded units. Estimated Regression Coefficients for Activity.

Regression coefficients obtained from MINITAB package (Relaese 16) analysis are shown in Table 4, coefficients in the second-order polynomial in terms of linearity and quadratic were significant at 5%.

By applying regression analysis on the experimental data, the following second-order polynomial equations 1 and 2 were found to explain L-asparaginase activity

$$Y = \beta^{o} + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \ldots\ldots. \quad (1)$$

$$\text{Enzyme activity} = -9.03769 + 1.4407 + 0.003 - 0.008 - 0.02341\ldots\ldots\ldots \quad (2)$$

Figure 4 and 5 represent the isoresponse contour and surface plots for the optimization of temperature and pH on L-asparaginase activity. The derived optimum levels of the physical environment were temperature of 25°C and pH of 9 at which maximum yield was 2.2U/ml/min. However, the pH optimum of L-asparaginases from several plants ranged from 8.0-8.5 [23]. Most of L-asparaginases from bacteria showed alkaline pH optima (8.0–10) [24], [25]. Temperature optimum of asparaginase from *V. unguiculata* (40°C) was reported before [26]. This temperature was also similar to that reported for *Pseudomonas aeruginosa* and *Pectobacterium carotovorum* [27], [25]. The optimum activity of *Streptobacillus sp.* asparaginase was recorded at 35°C [24].

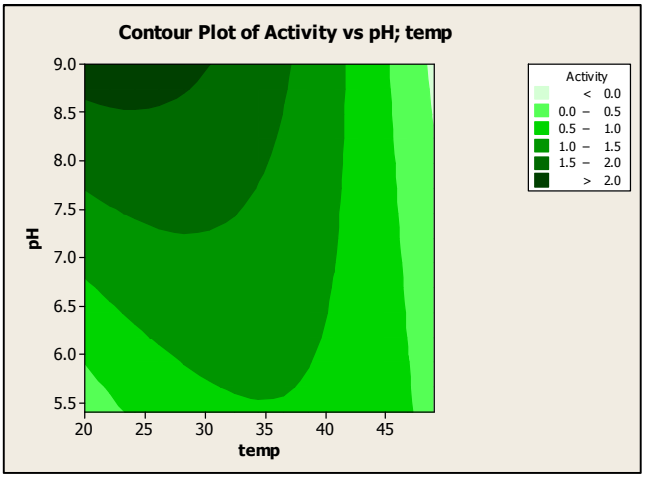

Figure 4. *Contour plot of enzyme activity, pH and temp.*

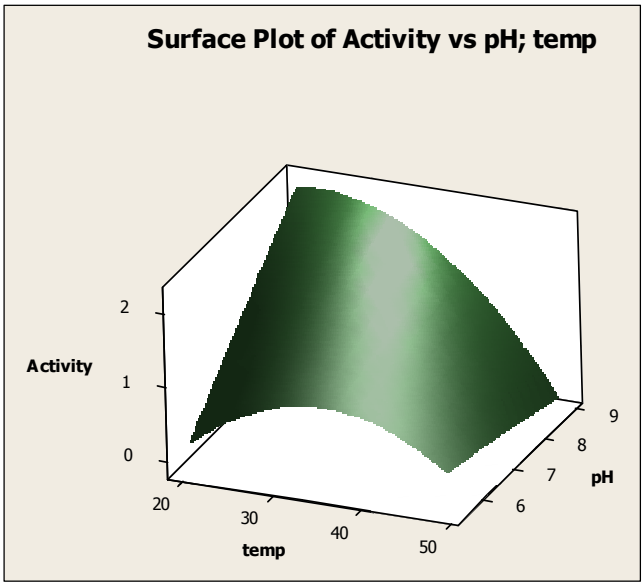

Figure 5. *Surface plot of enzyme activity, pH and temp.*

4. Conclusions

96% out of 180 samples of sewage water were found to be *E.coli,* positive while 4% were negative. 42% out 96% of the positive isolates were found to be of L-asparaginse producer, where the other 58% could not produce L-asparaginase. The optimization of the physical conditions in term of temperature and pH showed that the optimum pH is 9 and optimum temperature is 25°C using statistical experimental design, Response Surface Methodology (RSM), revealed in higher L-asparaginase yield of 2.2U/ml/min

References

[1] Wang B, Relling MV, and Storm MC (2003). Evaluation of immunologic crossreaction of antiasparaginase antibodies in acute lymphoblastic leukemia (ALL) and lymphoma patients. Leukemia, 17: 1583-1588.

[2] Broome JD (1961). Evidence that L-asparaginase activity of guinea pig serum is responsible for its anti lymphoma effects. Nature, 191: 1114-1115.

[3] Kamada N, Inoue N, Hisamatsu T, Okamoto S, Matsuoka K, Sato T, Chinen H, Hong KS, Yamada T, Suzuki Y, Suzuki T, Watanabe N, Tsuchimoto K, Hibi T (2005). Nonpathogenic *Escherichia coli* strain Nissle 1917 prevents murine acute and chronic colitis. Inflammatory Bowel Diseases, 11 (5): 455–63.

[4] Subha M, Amaresh S, Ramakrishna S, and Chakrabrty SL (1995). Purifiction characterization and antitumor activity of L-asparaginase isolated from *pseudomonas stutzeri* MB-405. Curr. Microbiol., 30: 291-298.

[5] Amena S, Vishalakshi N, Prabhakar M, Dayanand, A, Linappa K (2010). Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis.* Brazilian Journal of Microbiology, 41 (1): 137-178.

[6] Basha N (2009). Production of extracellular antileukemic enzyme L- a sparaginase from marine actinomycetes by solid state and submerged fermentation: purification and characterization, Tropical Journal of Pharmaceutical Research, 8 (4): 353-360.

[7] Oettgen HF, Old LJ, Boyse EA, Campbel HA, Philips FS, Clarkson BD, Tallol L, Leeper RD, Schiwartz MK, Kim JH (1967). Inhibition of leukemias in man by L- asparaginase. Caner Research, (12): 2619-2631.

[8] Boyes EA, Old LJ, Campbell HA, Mashburn LT (1967). Suppression of murine leukemias of various types: Comparative inhibitory activites of guinea pig serum L-asparaginase and *Escherichia coli* L-asparaginase. Journal of Experimental Medicine, 125: 17–31.

[9] You KM, Prak, YK (2004). A new method for the selective isolation of Actinomycetes from soil. Biotechnol. Technol., 10: 541 546.

[10] Barrow G L, and R KA FeLtham (2003). Cowan and steels manual for the Identification of medica Bacteria 3rd Edn. Cambridge unive. pres, Combrridge, pp: 352.

[11] Murray P R, EJ Baron, JH Jorgensen, ML Landry and MA Pealler (2007). Mannual of clinical microbiology. 9th Edn., ASM Prees USA.

[12] Atlas RM, Handbook of microbiological media. 2nd Edn, RcR. press, Boca Florida. 1997.

[13] Maniatis T, J Sambrook, and EF Fritsch (1989). Molecular cloning laboratory manual, 2nd Edn., Cold Spring Harbor Laboratory Press, USA.

[14] Ramaiah N, Chandramohan D (1992). Production of L-asparaginase by the marine luminous bacteria Indian J. of Mar Sci., 21: 212-214.

[15] Ibrahim Hanan Moawia, wan Mohtar wan yuoff, Aidil Abdul Itamid, Rosli Md Illis, Othman Hassan and Othman omar (2004). Optimization of medium for the production of B. cyclodextrin glucanotrans ferase using central composite-Design (CCD). Process Biochemistry, 40 (2): 753-758.

[16] Box GP, WG Hunter, and JS Hunter (1978). Statistics for experiments: An introduction to design, data analysis and model building. John Wiley and Sons Inc., New York.

[17] IrnadaA, and J Gen (1972). Asparaginase and glutaminase activities of microo rganisms. J. Gen Microbiol, 76: 85-99.

[18] Peterson RE, and ACiegler (1969). L-asparaginase production by various bacteria. Applied Microbiol., 17: 929-230.

[19] MukherjeeJ, Majumdar S, Scheper T (2000). Studies on nutrition and oxygen requirements for production of L-asparaginase by *Enterobacter aeorgenes*. Appl. Microbiol Biotechnol. 53 (2): 180-184.

[20] Ramakrishan (1994). Characterization ofan extracellular asparaginase of *Rhodosporidium torulo ides* CBS 14 exhibiting unique physicochernical properties. Can. J. Microliol., 42: 316-325.

[21] Varavinit S, Sanguanpong V, and shobsngob S (1998). Utilization of brew yeast waste and Thai glutinous rice starch in the Production of cyclodextrin glycosyltransferase. Food and Ingredients Journal of Japan, 176: 88-95.

[22] Davis L (1993). Efficiency in research development and production: the statistical design and analysis of chemical experiment. Royal Society of Chemistry, Cambridge.

[23] Khalaf ZA, N K Al-Ani, and H M Jasim (2012). Optimum conditions for asparaginase extraction from *Pisum sativum subspp*. Iran Journal of Plant Physiology, 2: 517–521.

[24] Makky EA, YC Loh, and M R Karim (2014). Purification and partial characterization of a low molecular weight L-asparaginase produced from corn cob waste. Biocatalysis and Agricultural Biotechnology, 3:(4), 265–270.

[25] ShanmugaprakashM, C Jayashree, V Vinothkumar (2015). Biochemical characterization and antitumor activity of three phase partitioned L-asparaginase from Capsicum annuum L. Separation and Purification Technology, 142: 258–267.

[26] Ali EMM (2009). Purification and characterization of Vigna unguiculata cultivar asparaginase," Journal of Biological Research, 11: 29–36.

[27] Kumar S, V Venkata Dasu, and K Pakshirajan (2011). Purification and characterization of glutaminase-free l-asparaginase from *Pectobacterium carotovorum* MTCC 1428, Bioresource Technology, 102 (2): 2077–2082.