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Study On Effect Of Different Parameters On Production Of Anticancer Enzyme L-Asparaginase By Halotolerant Bacteria.

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Abstract : The enzymes L-asparaginase, L-glutaminase, and L-arginase were chosen according to amino acids starvation in cancer cells and screened in halophilic and halotolerant bacteria.L–Asparaginase is a potential anti-cancer enzyme. We report production of L-Asparaginase from halotolerant bacterial isolates. Maximum L-Asparaginase production by submerged fermentation showed by R8 i.e 30 IU/min.After purification enzyme was characterized with respect to pH, temperature, different carbon source and substrate concentration. The enzyme, L-Asparaginase, shows maximum activity at an optimal temperature of 37^0C. L-Asparaginase from G7 showed greater stability compared to R8 & R6.The production of enzymes increased as substrate concentration increased.The optimum temperature and pH for L-asparaginase and L-glutaminase activities in selected strains were similar to the physiological conditions of human body and the enzymes could tolerate NaCl up to 5% concentration.

Key words:- L-Asparaginase, Halotolerant enzymes, Anticancer enzyme, Submerged fermentation.

INTRODUCTION:

Among therapeutic proteins, enzymes represent a tiny low and after all profitable market. they will be wont to treat vital, rare, and deadly diseases. Catalyst medical aid is the only obtainable treatment for shooting disorders. Therapeutic enzymes have a broad style of specific uses: as oncolytics, as anticoagulants or thrombolytics, and as replacements for metabolic deficiencies. Typical samples of oncolytic enzymes are unit L-asparaginase, L glutaminase, etc. The oncolytic enzymes fall under 2 major classes: one. Those who degrade little molecules that grow tissues have a demand for a pair of. those who degrade macromolecules like membrane polysaccharides, structural and useful proteins or nucleic acids. Use of catalysts as therapeutic agents entails their administration to neoplasm bearing subjects in conjunction with a pro-drug thirty conjugated to a useful cluster that's vulnerable to attack by Associate in Nursing enzymes. to attain the requisite property, the subsequent 2 options are a unit of sizeable importance. 1. The acidic living thing surrounds the many neoplasms as compared to traditional tissues. 2. Associate in Nursing catalyst with Associate in Nursing acidic pH-activity optimum: Therapeutic enzymes area unit cosmopolitan in plant and animal tissues and microorganisms together with bacterium, yeast and fungi. though microorganisms area unit potential sources of therapeutic enzymes, utilization of such enzymes for therapeutic functions is restricted attributable to their incompatibility with the physique, however there's Associate in Nursing accrued specialize in utilization of microorganism enzymes attributable to economic practicableness. A serious potential application of therapeutic enzymes is within the treatment of cancer. Antineoplastic has proven to be promising for the treatment of acute leukemia. Its action depends upon the actual fact that neoplasm cells are a unit deficient in aspartate-ammonia ligase activity, that restricts their ability to synthesize the ordinarily non-essential aminoalkanoic acid L amino alkanoic acid. Attributable to that they're forced to rely on body fluids. The action of the antineoplastic doesn't have an effect on the functioning of traditional cells that are a unit ready to synthesize enough for his or her own necessities, however reducing the free exogenous concentration induces a fatal starvation within the prone neoplasm cells. Since their biological action hinges on contact action, a property that enhances thirty one efficiency therapeutic enzymes can cause a good variety of diseases and conditions. Therapeutically helpful enzymes are unit needed in comparatively little amounts however at a really high degree of purity and specificity.

L-Asparaginase:-

L-asparaginase ((L-asparagine amino hydrolase EC 9.5.1.1)) could be a potent anticancer protein that catalyzes the chemical reaction of L-asparagine to L-aspartic acid and ammonia. Antineoplastic drug is Associate in Nursing protein that's loosely distributed among the plants, animals and microorganisms. The foremost usually used organisms to provide antineoplastic drugs are Escherichia coli. enterobacteria carotovora, Thermus thermophilus, Proteus vulgaris, bacteria species, mycobacterium bovis, Streptomyces griseus, seventeen animal organs like liver of guinea pig, placenta, urinary organ and viscus of beef and horse and in plant part as common pea and rice. amino acid is Associate in Nursing organic compound needed by cells for the assembly of macromolecule, amino acid isn't a vital organic compound in traditional cells and that they synthesize this organic compound by the chemical change activity of amino acid synthetase from amino acid and amino alkanoic acid but, growth cells cannot

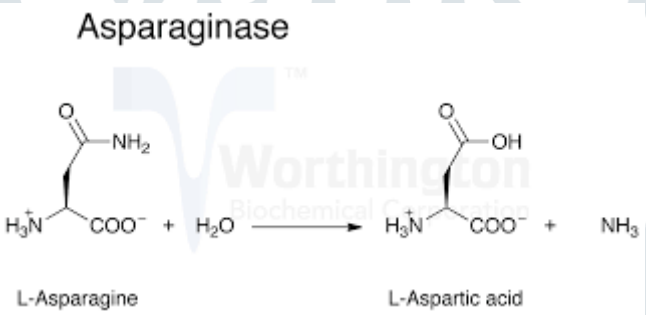
manufacture L-asparagine because of the absence of L-asparagine synthetase and that they rely on cellular pools of L-asparagine for his or her growth. The principle behind the utilization of antineoplastic drug as Associate in Nursing anti-tumor agent is that it takes advantage of the very fact that each one leukemic cells are unable to synthesize the non-essential organic compound asparagines their own, that is extremely essential for the expansion of the growth cells, whereas traditional cells will synthesize their own amino acid, so leukemic cells need high quantity of amino acid. These leukemic cells rely on current asparagines for his or her ample nourishment and diet.

antineoplastic drug, however, catalyzes the conversion of L-asparagine to amino acid and ammonia. This deprives the leukemic cell of current amino acid and prevents them from the fast malignant growth. microorganism L-asparaginases are enzymes of high efficiency utilized in treating numerous kinds: of cancers, chiefly acute lymphocytic leukemia. microorganism L-asparaginases are either high affinity periplasmic enzymes or low affinity cytoplasmic enzymes. This protein has been widely exploited within the treatment of bound types of cancer, particularly acute lymphocytic leukemia since the time it had been obtained from escherichia and its antineoplastic activity incontestable in guinea pig body fluid. growth cells, a lot of specifically, humor growth cells need vast amounts of asparagines for his or her fast and malignant growth. L-asparaginase exploits the remarkably high demand growth cells have for the organic compound amino acid. This protein has been isolated. refined Associate in Nursing by experimentation studied very well as an opposing cancer agent in human patients and ascertained its high potential against childhood acute lymphocytic leukemia throughout the induction of remission or the intensification phases of treatment.

Asparaginases are cosmopolitan in nature from bacteria to mammals and play a central role within the organic compound metabolism and utilization. In soma, L-aspartate plays a very important role as a precursor of amino alkanoic acid within the organic compound cycle and in trans -amination reactions forming salt within the gluconeogenic pathway resulting in aldohexose formation L-asparaginase could be a distinctive cancer therapy agent.

Asparaginase Hydrolysis:-

The simple hydrolysis reaction of the side chain amide bond of L-asparagine is catalyzed by a group of amidohydrolases known as asparaginases 151 This enzymatic hydrolysis of L-asparagine was first observed by Lang (1904) who detected asparaginase activity in several beef tissues.



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Application Of Asparaginase:-

The simple reaction of the aspect chain organic compound bond of L-asparagine is catalyzed of amidohydrolases referred to as cancer drugs 151.This protein reaction of L-asparagine was 1st ascertained by Lang (1904) World Health Organization detected asparaginase activity in many beef tissues.

Microbial enzymes, like cancer drugs, were performed to plant or animal sources thanks to their economic production, consistency and simple method modification. In recent years, cancer drugs have attracted a lot of attention in pharmaceutical and food industrial applications. within the food trade, it had been wont to confirm and eliminate. acrylamide, from bread victimization sequence technology by degrading L-asparagine, the precursor of amide, before backing fifteen. Another vital application of cancer drugs is in biosensors once the Indian team of Neelam Verma use cancer drugs for development of a completely unique diagnostic biosensor for the detection of L-asparagine in cancer of the blood cells. Recombinant, immobilized and changed cancer drugs have been created from microorganism sources with exaggerated activity than wild sort sources. cancer drug may be a therapeutically vital macromolecule utilized in combination with different medication within the treatment of acute leukemia (mainly in children), Hodgkin's disease, acute myelomonocytic cancer of the blood, chronic leukemia, lymphosarcoma, reticulosarcoma and melanosarcoma.

L-Asparaginase & it's importance:-

L-asparaginase is the initial therapeutic protein with antineoplastic properties that has been studied loosely by researchers and scientists way and wide. L-asparaginase was initially discovered by Lang in 1904 . Analysis on physiological capabilities of L-asparaginase was below manners for over 0.5 a century, and a true step forward was accomplished in 1922 once Clementi] unconcealed the presence of L-asparaginase within the body fluid of guinea pigs. master in 1953 did a progression of experiments to prove the power of guinea pig humor as tumor matter. He applied various sets of research wherever he concocted the findings that 2 forms of subcutaneously dead lymphosarcoma in mice didn't proliferate once animals got injections of guinea pig humor, whereas untreated controlled mice died attributable to carcinomas. Within the second set of experiments, 2 differing kinds of lymphomas, specifically exocrine gland cancer and fibrosarcoma of mice, didn't degenerate once treated with guinea pig humor. In 1961, ANother|yet one more|one more} breakthrough was achieved once with Broome incontestable L-asparaginase as a growth agent in guinea pig humor with substrate specificity. Later on, another substrate specific L-asparaginases were found to inhibit tumors . McCoy through his in vitro experiments well-tried the position of amino alkanoic acid amino alkanoic acid for Walker cancer 256. Atenbern in 1954 and Broome in 1965 rumored the growth activity of L-asparaginase in microorganism and yeast, respectively. L-asparaginase is found within the humor of guinea pigs and rodents however is absent in humans . Tumors in mice, dogs, and rats may be regretted by the Elspar protein refined from guinea pig humor, E. coli, and to a lesser degree by the liver [15–17]. 2 isozymes of L-asparaginase, specifically kind I and sort II, are known by Ohnuma in 1967.. 2

Studied Elspar, that changes amino alkanoic acid to yield aspartate and ammonium ions extensively. The impetus for this work for the most part has been the invention that Elspar from some organisms exhibit marked antimitotic activity. however these responses ar led to is unknown. The yeast yeast is capable of synthesizing 2 clearly totally different Elspar. L-Asparaginase is particular for L-Asparagine, is organic in synthesis, is unaffected in activity by the product of its activity, is active solely upon intracellular L-Asparagine, and is maybe a chemical compound protein consisting of identical monomers coded for by one gene referred to as aspl. Elspar II, that was discovered solely recently, is inhibited once cells ar full-grown in media wealthy in simply obtainable chemical element, however once cells are incubated in chemical element free medium containing associate energy supply, the protein is derepressed to a high level, a method that needs First State novo macromolecule synthesis.. 3

Joseph Robert et al.,rumored that the L-Asparaginase is understood to be a potent opposing growth agent in animals and has given complete remission in some human cancer. In depth clinical trials of this protein, the very best yields of protein were obtained once cells were full-grown aerobically in an exceedingly com steep medium. sensible protein production was related to media containing L-glutamic acid. L-methionine, and carboxylic acid. The addition of aldohexose to the medium, however, resulted in depressed production of L-Asparaginase. atomic number 11 ions seemed to suppress L-Asparaginase production. With the procedure delineated for isolation of biologically active L-Asparaginase from E. coli, stable L-Asparaginase preparations with a particular activity of 620 IU per mg of protein(1.240-fold purification with four-hundredth total recovery) were obtained. 4

Moriji Miura et alStudying the increasing proof of the numerous roles competed by immunity within the management of cancer, it becomes terribly fascinating for the antineoplastic agent to satisfy the necessity that it ought to be void of immunological disorder and however effective against cancer. All told, it's been thought that L-Asparaginase may belong to a replacement category of therapeutic agents for cancer simply compatible with the higher than necessity. However, many investigators have rumored that this protein pent-up DNA and RNA synthesis in rat liver and PHA' -induced white blood cell blastogenesis which the patient treated with the protein attended suffer from such toxicities as liver harm. symptom, coagulopathy, symptom, etc. These report merely that the protein itself might need associate action directed against the metabolism of bound traditional cells through the deprivation of exogenous L-Asparagine.5

Riccardo Riccardi et al., expressed that L-Asparaginase has been widely used for the treatment of acute lymphocytic leukemia. Therapeutic and harmful effects within the central systema nervosum are noted with general treatment. so as to raised outline the link between L-Asparaginase administration and humor (CSF) amino alkanoic acid levels, L-Asparaginase and amino alkanoic acid were measured within the CSF of Macaca mulatta monkeys following intrathecal and v. administration. Following intrathecal injection, the protein activity of E Coli L-Asparaginase within the CSF incontestable a a lot of fast terminal half-life than did that of "In-labeled diethylene triamine penta carboxylic acid, a marker of CSF bulk flow. Intrathecal injection of E. coli Elspar resulted in complete depletion of Communications Security Establishment amino alkanoic acid for a minimum of five days. An identical amount of CSF amino alkanoic acid depletion was discovered following the administration of L-Asparaginase Similar results were found in seven patients undergoing general L. Elspar medical aid The negligible plasma level of L-Asparaginase necessary to eat CSF asparagines in each species was zero.1 III/ml. 2 alternative enzymes. enteric bacteria L-Asparaginase and succinylated Acinetobacter glutaminase-asparaginase, were cleared from the CSF at an equivalent rate as bulk flow. These information indicate that general L-Asparaginase medical aid could also be a possible means that of treating central systema nervosum involvement in patients with acute lymphocytic leukemia which there's therapeutic advantage to mistreatment intrathecal L-Asparaginase. 6

Isolation:-

Collection of samples:-

Sea water samples were collected from several seaside areas including Goa, Ratnagiri, Alibag and Mumbai and stored at 3°C until use.

Streak plate method:-

Streak plate method used for isolation of microorganisms.

- Sterile nutrient agar plates with different salt concentration like 5%, 7% and 10% were used.
- A loopful of sea samples were streaked by quadrant methods on plates with different salt concentrations.
- After that plates were incubated at 25°C for 24 hours.

Maintenance of Pure cultures:-

The Following procedure is used for maintenance of pure cultures,

- The molten nutrient agar was gently poured into the sterile test tube and was allowed to solidify as slants.
- After agar was solidified, the inoculation loop was sterilized and a loopful of the culture was taken from the pour plates and streaked in the solidified agar slants.
- The tubes were incubated for 24 hours at 25°C.

Figure:-Halotolerant bacterial isolates

Figure:-Halotolerant bacterial isolates

Screening:-

The following procedure used for Rapid plate assay

- Screening for L-Asparaginase production was performed by the method of rapid plate assay.
- M9 medium was prepared, phenol red solution 2.5% stock solution in ethanol was added to the media as a pH indicator.
- Samples were incubated at 25°C for 48 hours.
- The indicator's colour changes yellow to pink appeared around bacterial colonies after 24 and 48 hours were used as a sign for L-Asparaginase production.

Identification :-

Gram Staining:-

The Following procedure used for Gram Staining.

- A smear of organisms was made on a glass slide. It was allowed to dry and heat fixed.
- Crystal violet was added and allowed stain for 30 sec. The slide was washed with distilled water.
- Gram's iodine was added and allowed to stain for 60 sec.
- The iodine was removed by drop wise addition of 95% ethanol. The slide was washed with distilled water.
- Safranin dye was added to slide and allowed to stain for 30 sec for counter staining. The slide was washed with distilled water and dried with blotting paper.
- The slide was examined microscopically.

Monochrome staining :-

The following Procedure used for Monochrome staining

- A clean grease free slide is taken. Slide is made grease free by washing the slide with detergent wiping the excess water and the slide is passed through flame.
- On these grease free slide smears are made by using a sterile wire loop and cell suspension.
- These slides are allowed to air dry.
- After air drying these slides are rapidly passed through a flame for three to four times for heat fixation.
- After heat fixation the slide is placed on the staining rack and flooded with a particular stain and these stains are allowed to react for three minutes.
- Further the slide is washed under running water. The slide is air dried and watched under oil immersion.

Spore staining :-

The following Procedure used for spore staining

- Cover the smears with a piece of absorbent paper.
- Place the slide over a staining rack that has a beaker/water bath of steaming water.
- Flood the absorbent malachite green for 3-5 minutes. paper with and let it steam
- Remove the stained paper carefully and discard and allow it to cool for 1-2 minutes.
- Gently rinse the slide with tap water by tilting the slide to allow the water to flow over the smeared stain. This is to remove the extra dye present on the slide on both sides and to also remove extra dye staining any vegetative forms in heat-fixed smear.
- Add the counter stain, safranin for 1 minute.
- Rinse the slide with water, on both sides to remove the safranin reagent.

● Ensure the bottom of the slide is dry before placing it on the stage of the microscope to view with the oil immersion lens, at 100x for maximum magnification.

Observations :-

Different strains selected on the basis of colony characteristics such as color, size, shape, elevation, and margin were maintained and preserved on NA slants at 4°C for further use.

Screening :-

Different bacterial isolates were obtained from sea water samples, among these isolates some showed the production of pink zones around the colonies on M9 agar containing phenol red indicator, indicating the production of L-asparaginase.

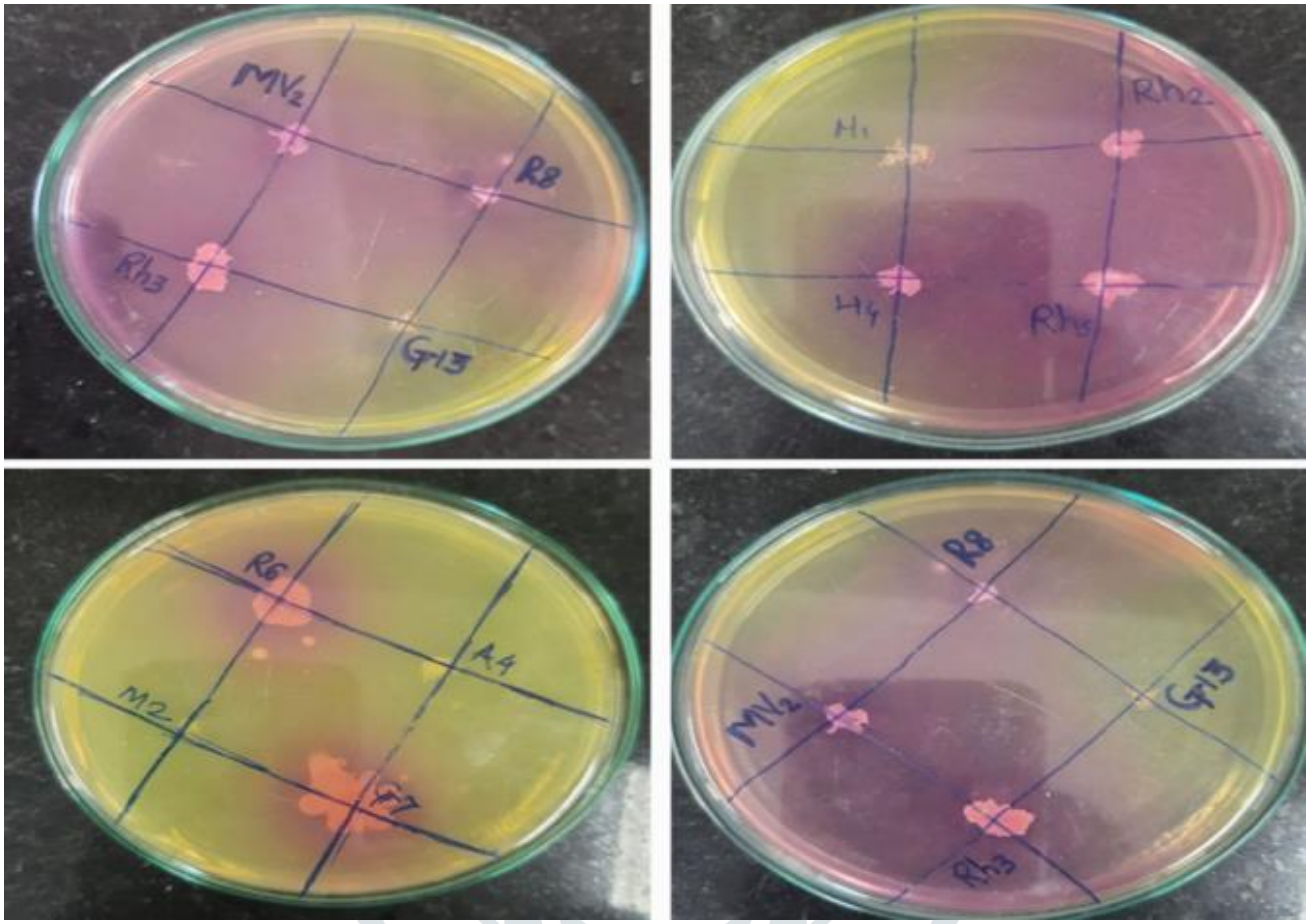


Fig:-Screening of halotolerant bacterial isolate

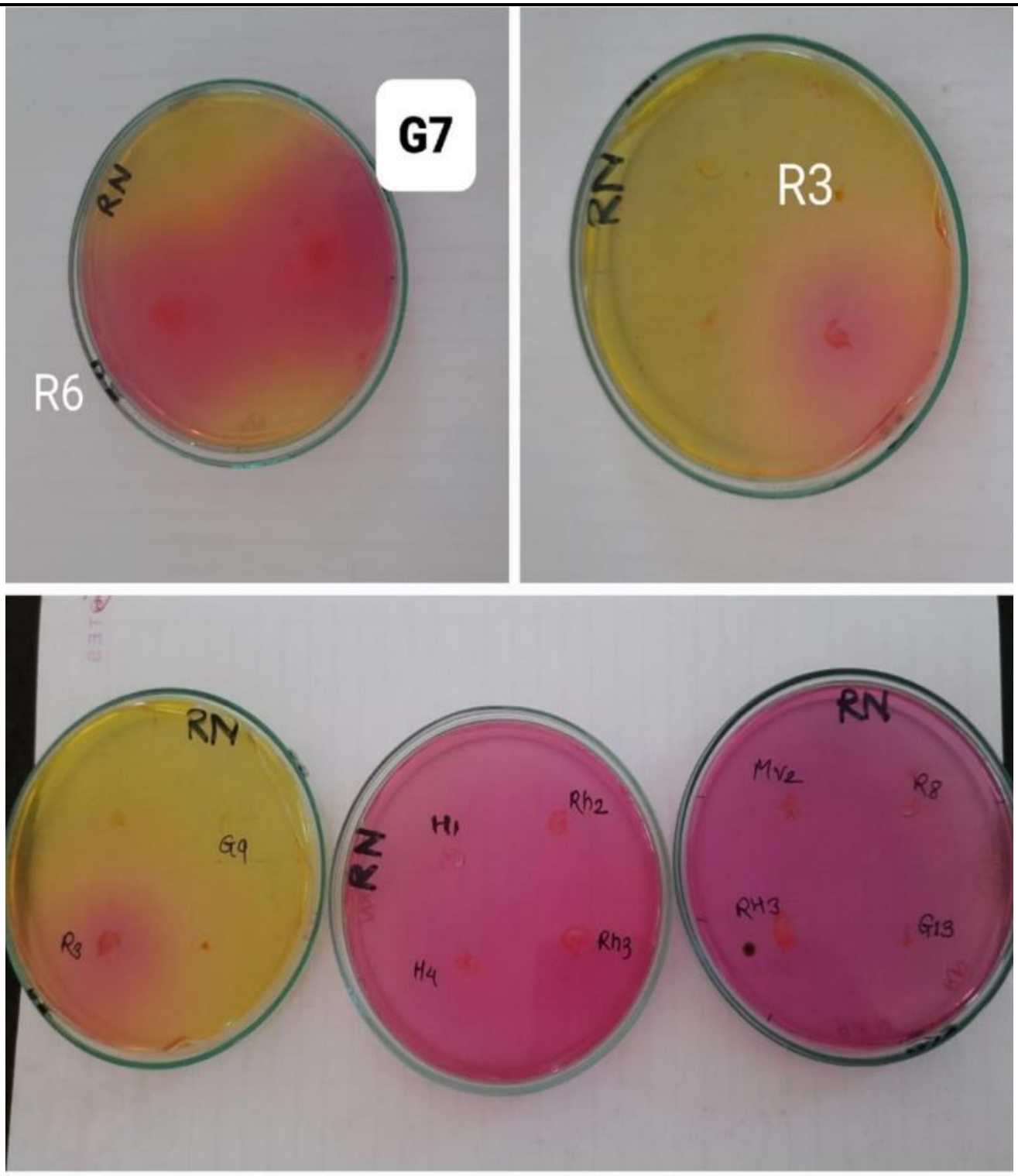


Fig:-Screening of halotolerant bacterial isolates

Observation Table:-

Table :-Zone size produced on M9 medium by isolates for L-Asparaginase

Microorganisms	Size of zone (Cm)	
	After 24 hours	After 72 hours
G7	0.5	1.2
MV2	0.1	0.5
G13	-	0.7
R8	0.3	1.3
R3	0.2	0.5
R6	0.4	1.2
RH1	-	0.3
RH2	0.3	0.6
RH3	0.4	1
RH4	0.2	0.8

Identification:-

Microscopic Identification:-

Table:-Colony Characteristics of Isolates

Bacterial isolates	Size	Shape	Colour	Margin	Elevation	Consistency	Opacity	Gram character	Motility
G7	1mm	circular	white	Irregular	flat	smooth	opaque	Gram positive rod	Motile
R8	1mm	circular	white	Entire	convex	smooth	opaque		
R6	1mm	circular	Creamish white	Entire	convex	smooth	opaque	Gram positive rod	Motile
R3	pinpoint	circular	white	Entire	convex	smooth	opaque	Gram positive cocci	-

Results:-

Isolation:-
Halotolerant bacteria were isolated from sea water samples and characterization was done.

Screening :-
Bacterial isolates G7, R6 and R8 showed a broad zone of pink color around the colony.

Identification:-
Microbial characterization and identification was done based on morphological and biochemical tests and the results were compared with Bergey's manual of Determinative Bacteriology.

Production And Quantitative Estimation Of L-Asparaginase

Production and quantitative estimation of L-Asparaginase

Material:-

Production of L-Asparaginase:-

Cultivation of L-Asparaginase producing organism

The composition of M9 medium is as follows : (in gm/liter)

- KH_2PO4 - 2.0
- NaCl - 5.0
- Asparagine - 5.0
- Glucose - 2.0
- pH - 7
- Phenol red solution (2.5%) - 3.6

Cultivation of L-Asparagine producing microorganisms Ammonium Sulphate Precipitation:-

The following material was used for ammonium sulfate assay.

Centrifuge tubes

Ammonium Sulfate

Graduated cylinder

Balance Pipettes

Chemicals:-

- 0.05M Sodium borate buffer (pH-8.5)
- 0.1N TCA (trichloroacetic acid)
- 1N NaOH
- 0.1 M EDTA
- Nesseler's Reagent

Preparation Of Stock:-

Stock solution of 10mM It was prepared by dissolving 132 mg of ammonium sulphate in 100ml distilled water in a volumetric flask.

Working solution of 1mM:-

It was prepared by taking 5ml of stock solution and made up to 50ml with distilled water.

Enzyme assay**:-**

The following materials used for enzyme assay

Enzyme sample

0.05M Sodium borate buffer (pH-8.5)

0.IN TCA IN NaOH

0.1 M EDTA 0.04 M

L-Asparagine Nessler's Reagent

Method:-

Production of l-Asparaginase

Cultivation of L-Asparagine producing microorganisms-

The following procedure used for cultivation of L-Asparaginase producing organisms, 100 ml of production media was prepared and autoclaved at 121°C for 20 min, after cooling a loopful of organisms were inoculated. The inoculated media was kept in a shaker incubator at 250 rpm at 25°C for 120 hours. After incubation of culture broth was centrifuged at 5000rpm for 5 min. The supernatant was stored at 4°C.

Quantitative estimation:-

Ammonium Sulphate method for Standard graph:-

Into a series of test tubes, 0.5ml of (0.05M) borate buffer pH 8.6 was added. To this 1 ml of each 0.1 mM, to 1 mM of working solutions were added. From this 1 ml sample was withdrawn and delivered into 2.5 ml of 0.1N trichloroacetic acid. To this 5 ml of distilled water were added (for dilution), to this 1 ml of IN NaOH was added. 0.2 ml of EDTA (0.1M) was added to each sample to overcome the encountered turbidity. After 10 min, 0.5 ml of Nessler's reagent was added. The OD was measured after 5 min at 440 nm. Blank was prepared by adding 1 ml of water instead of ammonium sulfate solutions. The data is shown in Table 4.1. A standard curve was constructed by taking ammonium sulfate.

Enzyme assay:-

A 0.5 ml sample of cell suspension, 1.0 ml of 0.IM sodium borate buffer (pH 8.5) and 0.5 ml of0.04M L-asparagine solution were mixed and incubated at 37°C for 10 min. The reaction was then stopped by the addition of 0.5 ml of 0.IN trichloroacetic acid, Then and 5 ml of D/W water.

The liberated ammonia was determined by direct Nesslerization. Each sample was individually mixed with I ml of IN NaOH and 0.2 ml of 0. IM EDTA was added. After 2 min 0.5 ml of Nessler's reagent was added and mixed. Suitable blanks of substrate and enzyme containing sample were included in all assays. After 5 min from the addition of Nessler's reagent to the sample, the Absorbance of the sample was read at 440 nm.

Observation:-

Production of L-asparaginase:-

Fig:(a) Production of L- asparaginase

Fig:(b) Production of L- asparaginase

Quantitative estimation:-

Ammonium sulfate precipitation method

Table: Construction of standard graph for ammonium sulfate

Concentration of Ammonia (mM)	Absorbance (440nm)
0.1	0.10
0.2	0.17
0.3	0.23
0.4	0.31
0.5	0.39
0.6	0.46
0.7	0.53
0.8	0.63
0.9	0.68
1	0.75

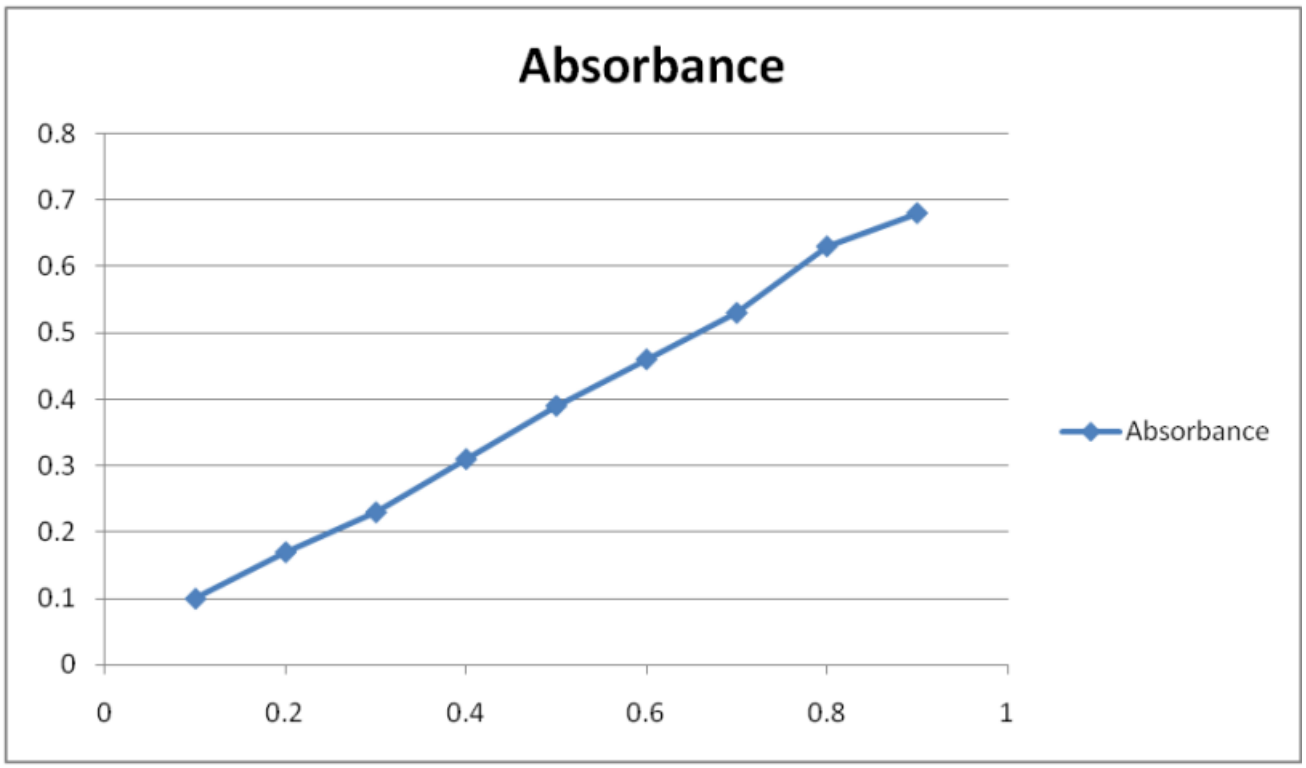


Fig.Enzyme assay standard graph for ammonia

Enzyme assay:

The result are presented in given table

Units/ml = Micromoles ammonia release/ Incubation time × ml enzyme in reaction

Table: L-asparaginase activity of selected isolates

Microorganisms	Enzyme Activity IU/ml							
	24hrs		48hrs		72hrs		96hrs	
G7	0.31	14.16	0.51	19.1	0.80	22.9	0.85	23.1
R6	0.24	12	0.50	20	0.69	20.8	0.60	19.8
R8	0.50	16.6	0.80	24.1	1.56	29.1	1.21	30.4
RH1	0.14	11.6	0.35	19.1	0.39	17.7	0.50	16.1
RH3	0.28	12.9	0.52	18.2	0.70	21.6	0.81	20.5
R3	0.32	9.1	0.34	12.5	0.43	18.1	0.53	17.0
RH4	0.17	10	0.34	15.0	0.57	19.3	0.61	19.1
RH2	0.9	10.8	0.12	12.5	0.5	15.6	0.10	14.9

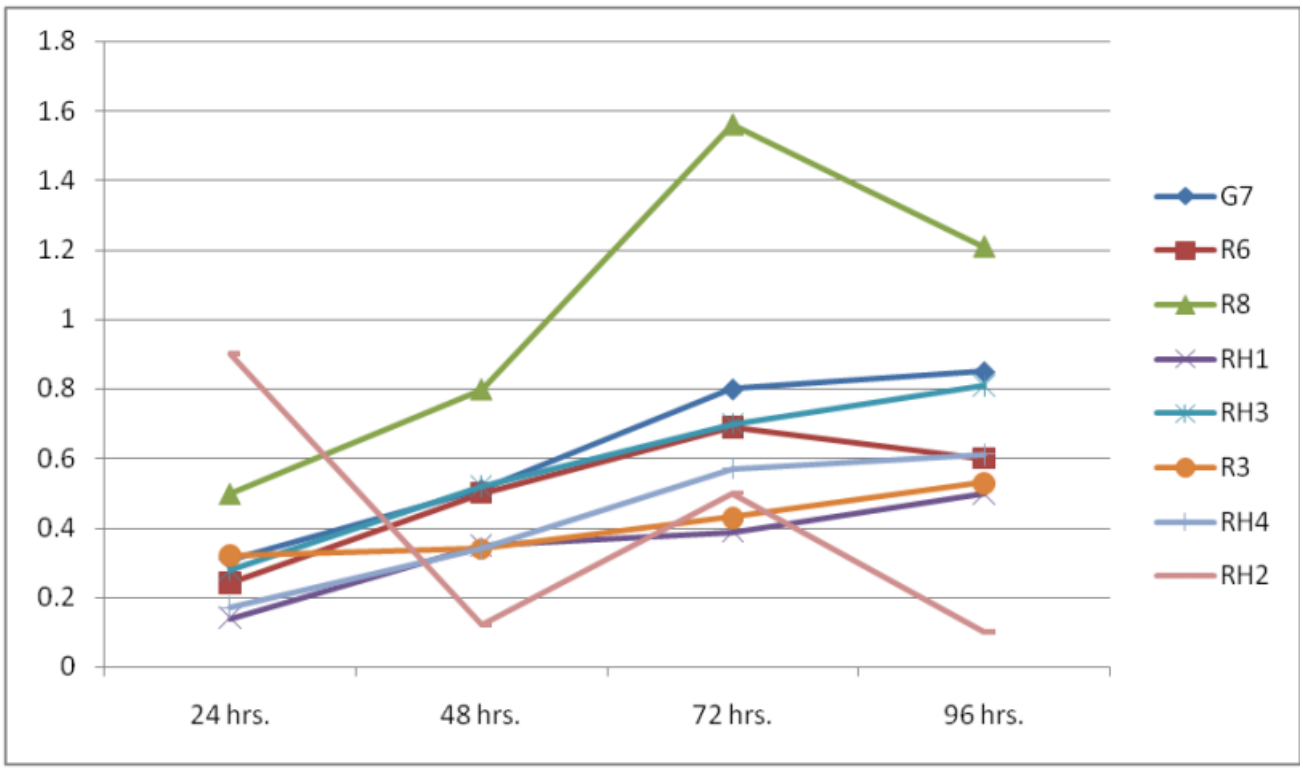


Fig: Graph of bacterial growth

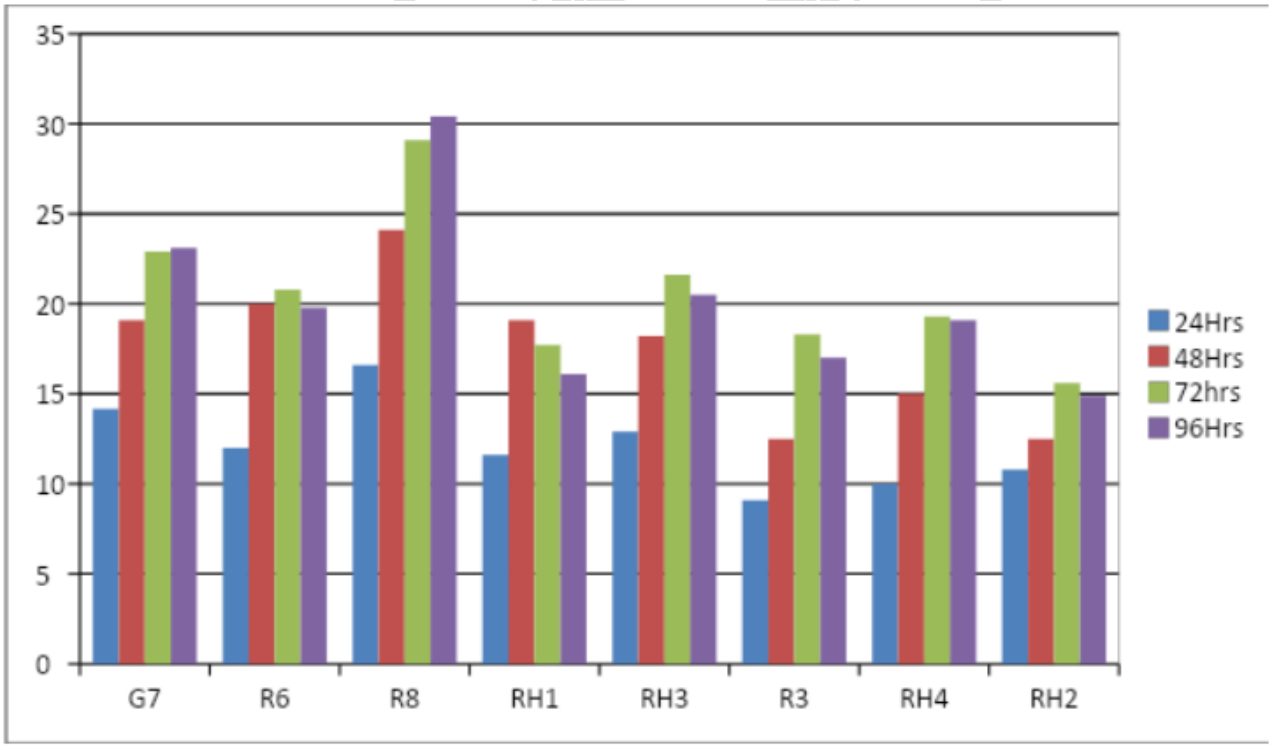


Fig: Enzyme activity IU/min

Result :-

Ammonium sulphate method for standard graph Powdered ammonium sulfate was added to a 80% saturation. The mixture was left for 12hrs at 4°C, followed by centrifugation at 8,000 rpm for 20 min at 4°C to separate the precipitate. The precipitate was used for further tests.

Enzyme assay (quantitative): The maximum L-asparaginase activity of 30 IU/ml was achieved by R8 by submerged fermentation. R8 was found to be a cultivable microorganism for L-asparaginase.

Effects of different physicochemical parameter on L-asparaginase activity

Effects of physicochemical parameters on L-Asparaginase activity:-

Materials

Effects of pH-

The following materials are used for assay,

- M9 medium with different pH (5,7,9)
- 0.05M Sodium borate buffer (pH-8.5)
- L-Asparagine 0.IN TCA
- D/W ● IN NaOH
- 0.1 M EDTA
- Nesseler's Reagent

Effect of Temperature-

The following materials are used for assay,

- M 9 medium with different temperature (20°C, 37°C, 55°C)
- Enzyme sample
- 0.05M Sodium borate buffer (pH-8.5)
- L-Asparagine
- 0.1 N TCA
- D/W
- 1N NaOH
- 0.1 M EDTA
- Nesseler's Reagent

Effect of substrate concentrations-

The following materials are used for assay,

- M 9 medium with different substrate concentration (0.2,0.4,0,0.6,0.8)
- Enzyme sample 0.05M Sodium borate buffer (pH-8.5)
- L-Asparagine
- DIN TCA
- D/W
- IN NaOH
- 0.1 M EDTA
- Nesseler's Reagent

Effect of carbon sources-

The following materials are used for assay:

● M9 medium with different Carbon sources (Glucose, Fructose and Maltose)

● Enzyme sample ● 0.05M Sodium borate buffer (pH-8.5)

● L-Asparagine

● 0.IN ICA

● D/W

● IN NaOH

● 0.1 M EDTA

● Nessler's Reagent

Methods:-

Enzyme assay is followed by Nesslerization method which is given below;

Nesslerization method:

● All the production flasks were incubated on a rotary shaker for 24 h. At the end of incubation period, 10 ml of the cell suspension was taken and centrifuged.

● Quantitative detection was carried out by a Nesslerization method.

● A 0.5 ml sample of cell suspension, 1.0 ml of 0.1M sodium borate buffer (pH 8.5) and 0.5 ml of 0.5M L-asparagine solution were mixed and incubated at 37°C for 10 min.

● The reaction was then stopped by the addition of 0.5 ml of 0. 1N trichloroacetic acid, and 5 ml of D/W water.

● The liberated ammonia was determined by direct Nesslerization. Each sample was individually mixed with I ml of 1N NaOH and 0.2 ml of 0.1M EDTA was added.

● After 10 min. 0.5 ml of Nessler's reagent was added and mixed. Suitable blanks of substrate and enzyme containing sample were included in all assays.

● After 5 min. from the addition of Nessler's reagent to the sample, the optical density of the sample was read at 440 nm.

Effects of pH:

● Inoculate isolates in M9 broth at different pH (5,7,9).

● The enzyme solution of three isolates was taken.

● Enzyme assay was performed using Nesslerization method.

Effect of Temperature-

● Inoculate the organism in M9 broth and incubate the flasks at various temperatures (20°C, 37°C ,55°C). ● The enzyme solution of 3 isolates was taken. Enzyme assay was followed using Nesslerization method.

Effect of substrate concentration-

● Inoculate the isolates in M9 broth which have different substrate L-Asparagine concentration.

(0.25, 0.50, 0.75).

- The enzyme solution of three isolates was taken.
- Enzyme assay was followed using Nesslerization method.
- Inoculate the organism in M9 broth which having different carbon sources (Galactose, Fructose and Maltose)
- The enzyme solution of three organisms was taken.
- Enzyme assay was followed using Nesslerization method.

Observations:-

Effect of pH: Observation Table

Table: Enzyme Activity of L-Asparaginase at different pH

pH	Enzyme activity IU/min.		
	R8	R6	G7
5	17	07	12
7	35	19	30
9	10	13	21

Fig: Effect of pH

Effect of Temperature:

Table-Enzyme Activity of L-Asparaginase at different temperature

Temperature	Enzyme activity IU/min.		
	R8	R6	G7
20	19	20	13
37	23	13	21
55	10	07	19

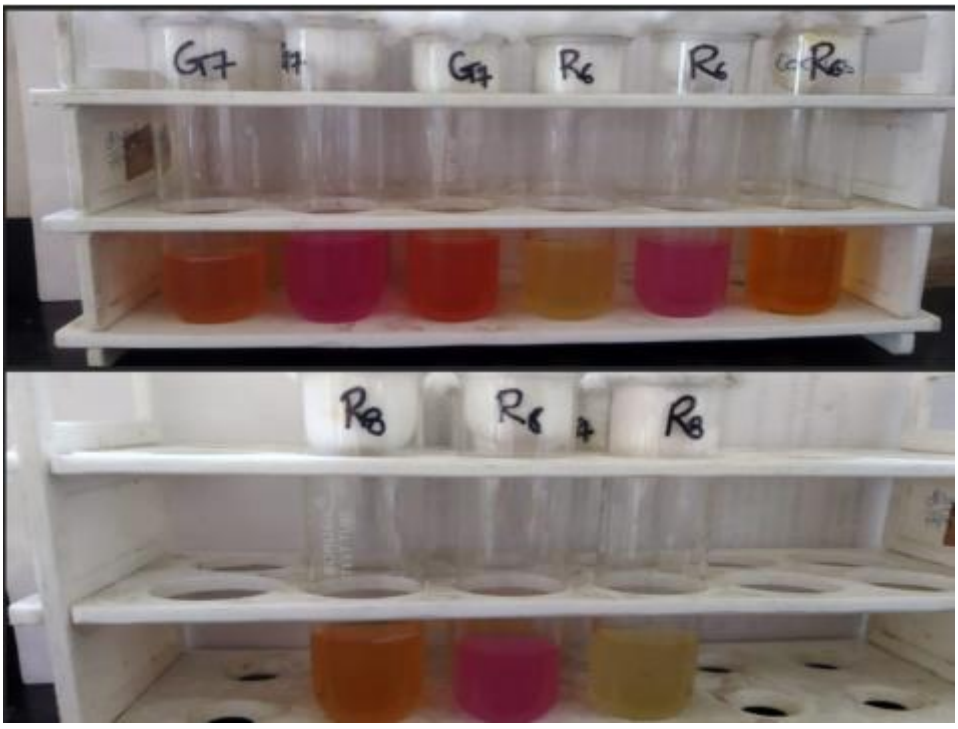


Fig. Effect of temperature

Effect of substrate concentration-

Table:Enzyme Activity of L-Asparaginase at different substrate concentration

Substrate concentration of L-Asparagine	Enzyme Activity IU/min		
	R8	R6	G7
0.2	2	2.4	3
0.4	5	4.4	6
0.6	4.5	1.8	3.5
0.8	3	2.8	4.5

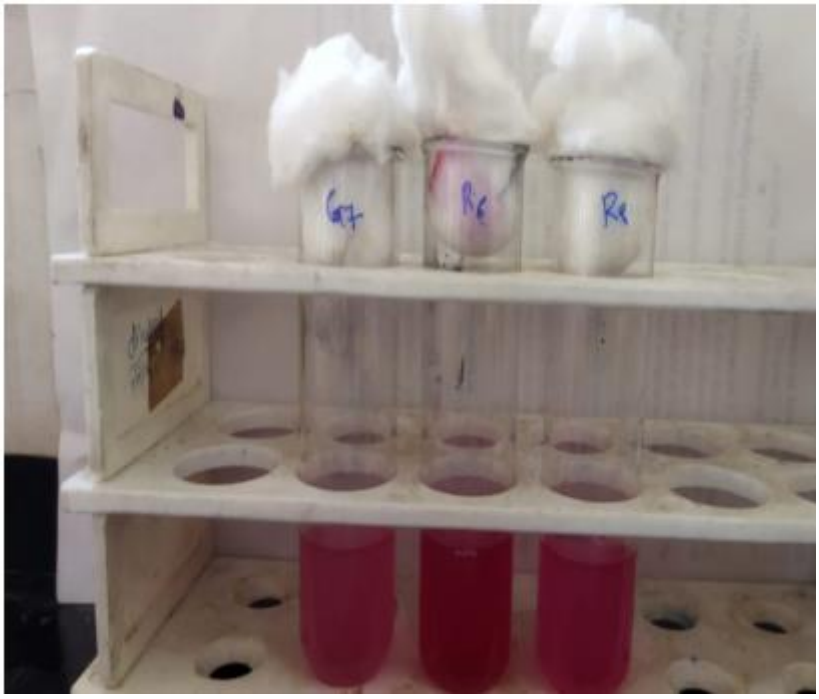


Fig-Effect of substrate concentration

Effect of carbon sources-

Table:Enzyme Activity of L-Asparaginase at different carbon sources

Carbon sources	Enzyme Activity IU/min.		
	R8	R6	G7
Glucose	14	10	20
Maltose	23	17	11
Sucrose	15	24	29

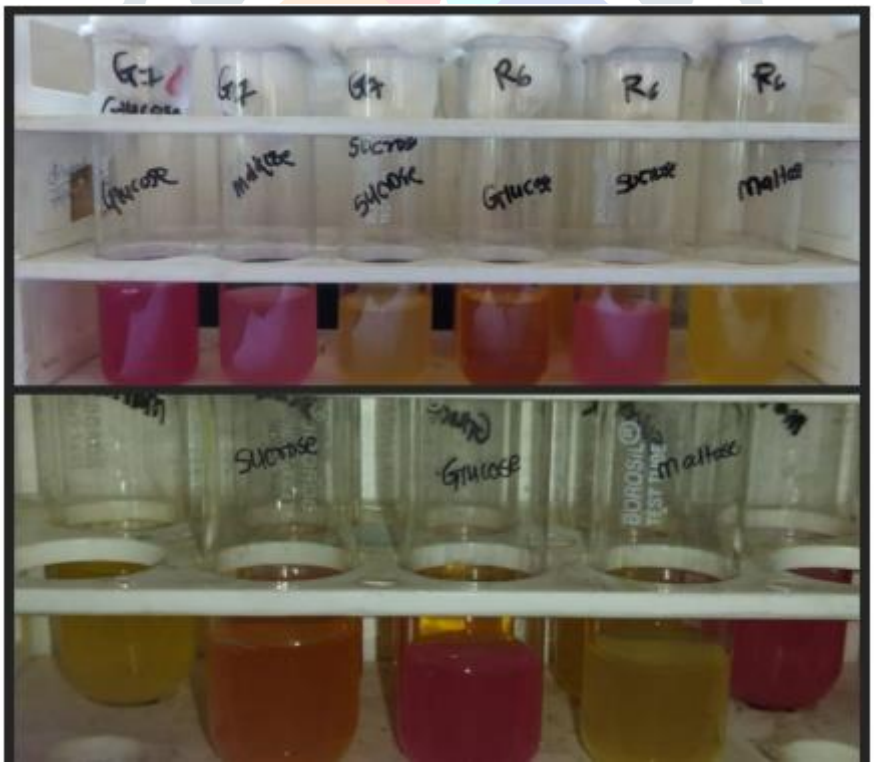
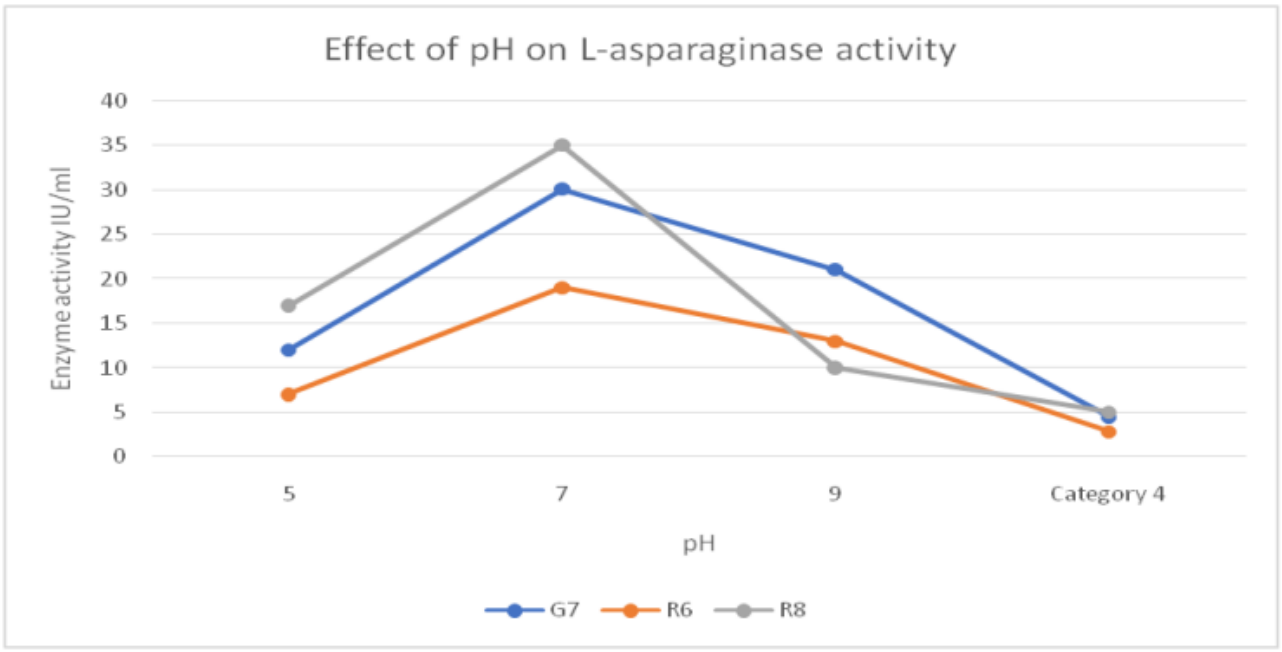


Fig -Effect of carbon sources

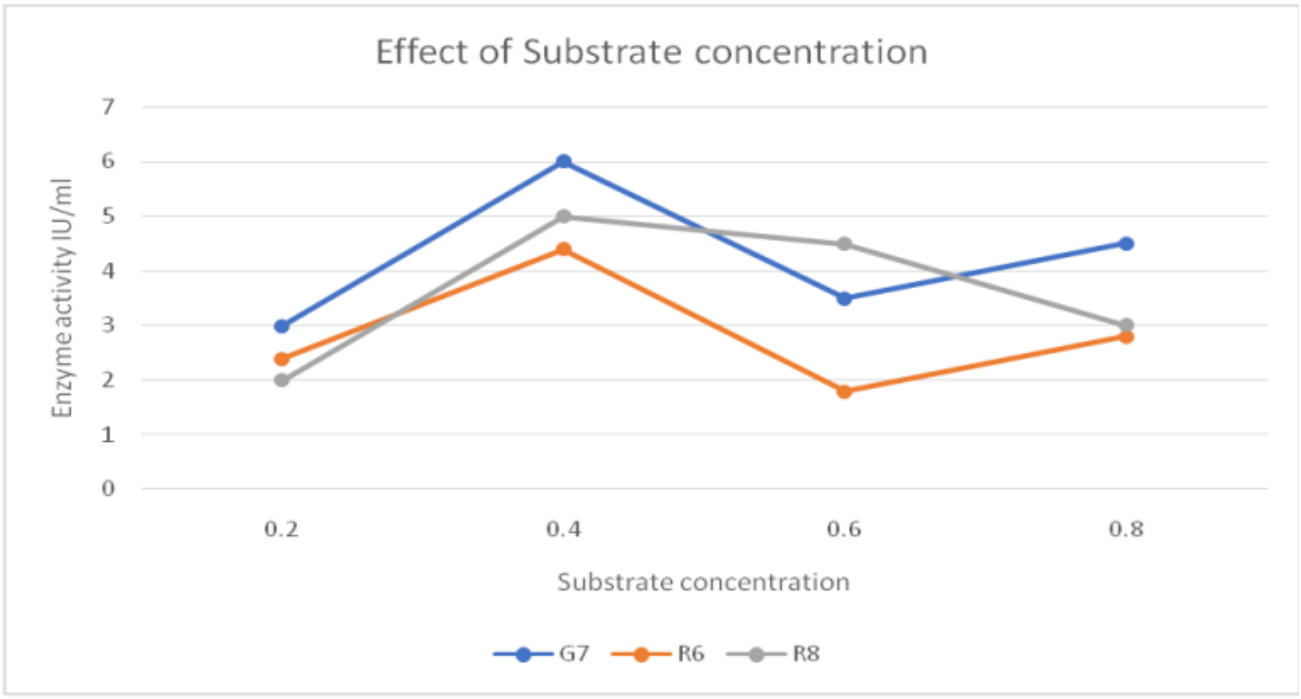
Results:-

Effect of pH-



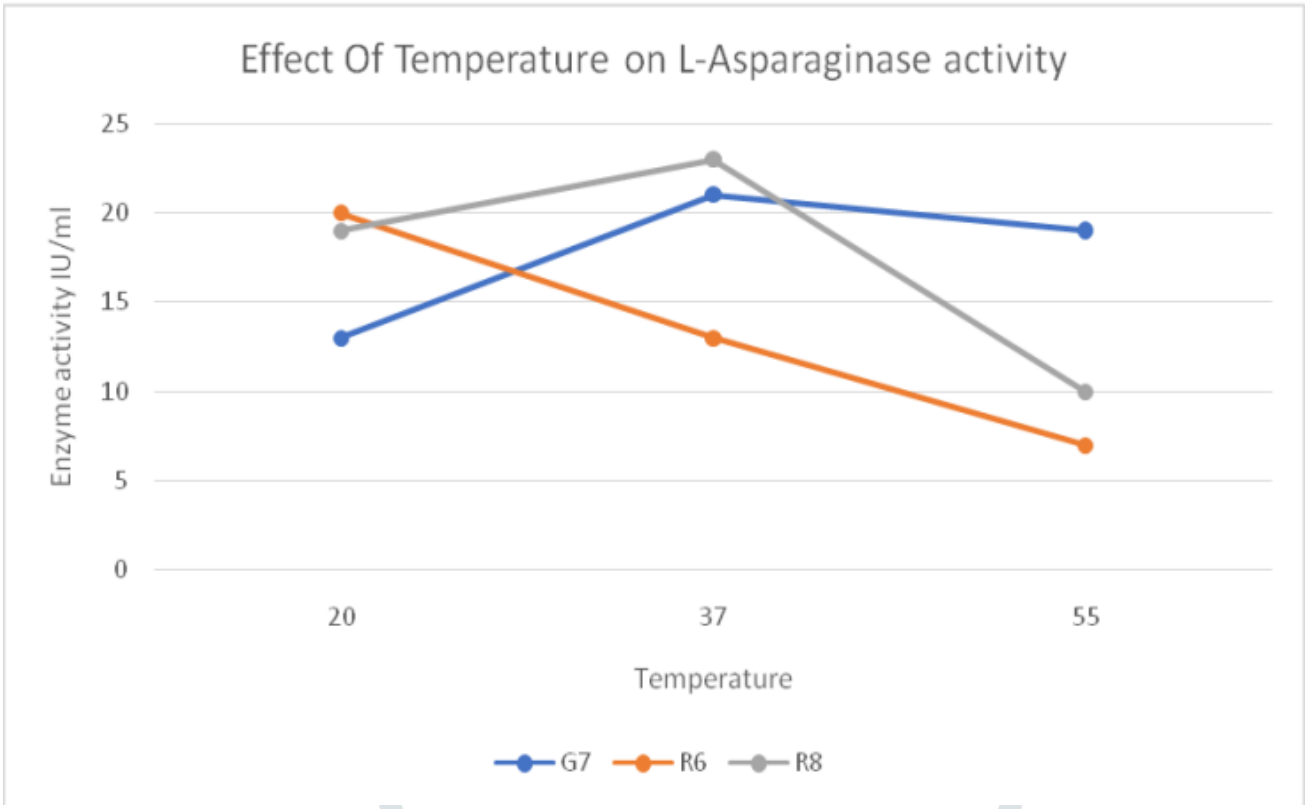
The optimum pH was found to be 7 for all three isolates

Effect of substrate concentration-



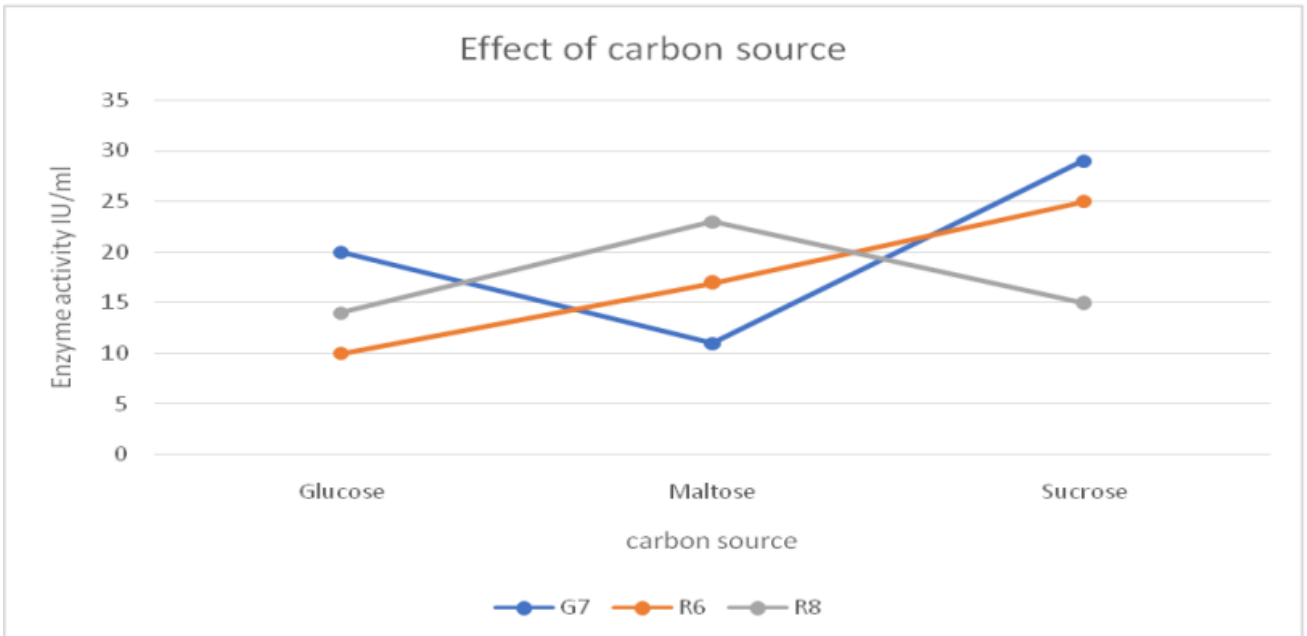
The optimum substrate concentration was found to be 0.4 these isolates.

Effect of temperature-



The optimum temperature for G7 and R8 found to be 37°C

Effect of carbon sources-



The optimum carbon source is sucrose for G7 and R6 and for R6 its maltose.

Conclusion:-

Effect of pH-

L-asparaginase activity was studied as a function of pH in the range between 5-9. L-asparaginase was active over broad pH ranges (5-9). The enzyme activity increases gradually till pH 7. After that Activity was decreased. All three isolates show the maximum activity at pH 7,which were similar to the physiological conditions of the human body.

Effect of Temperature-

The effect of temperature was studied by the incubation of the selected strains at different temperatures in the range of 20°C to 47°C with 55°C intervals. The optimum temperature for G7 and R8 was found to be 37°C. Which were similar to the physiological conditions of the human body and the enzymes could tolerate NaCl up to 7.5% concentration.

Effect of Substrate concentration-

The influence of substrate concentration on L-asparaginase activity was examined by using different concentrations of substrate ranging from 0.2 to 0.8 Molar to determine the optimum concentration of substrate required to give the highest L-asparaginase activity.gradual increase in the enzyme activity with the increase in substrate concentration from 0.2 to 0.4 Molar. However, further increase in substrate concentration(0.5–0.8 Molar) lead to decrease in enzyme activity.The optimum substrate concentration for all three isolates is 0.4 M.

Effect of Carbon sources-

The effect of carbon source on the enzyme activity. Optimum carbon source for G7 & R6 is sucrose and for R6 is Maltose.

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