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# Bioprocessing Of Ar Isolates For Economical Production Of L – Glutaminase By Solid State Fermentation

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**Abstract:** L-glutaminase (L- glutamine amidohydrolase EC, 3.5.1.2) is a significant enzyme found to possess antileukaemic properties and also having many other applications. The main objective of the present work was to produce potential strains in producing the enzyme with agro-industrial wastes such as Decaffeinated Tea Dust Waste (DTDW) and Coconut Fiber Waste (CFW) which were fortified with whey. Rainforest soil samples from different places were collected for potential strains and were introduced into selective medium where L-glutamine was the only carbon and nitrogen source. Such grown organisms were screened by rapid plate assay. From the total 16 strains 3 prominent strains (AR-glut 5, AR-glut 6, AR-glut 7) were selected from its zone of colour formation. Further they were identified through different biochemical tests of Bergey's manual of bacterial classification. Organisms were optimized to yield more enzyme for their medium pH, incubation temperature and inoculum volume. The effect of additional phosphate and metal ion sources were also studied. Enzyme assay was carried out by nesslerization and analyzed using spectrophotometer at 450 nm. Enzyme activity was calculated from the standard graph of ammonium sulphate. The optimized temperature, pH, inoculum volume, and incubation were found to get a maximum yield of enzyme at $37^0$C, 7.4 pH, 15 mL and 48 hours respectively. Addition of Phosphate and metal ions were found to improve the yield of enzyme slightly. At the optimum pH, temperature, inoculum volume and incubation time the maximum yield of the enzyme was 150 IU/gds with AR-glut 7 on DTDW medium. DTDW and CFW prove to be wonderful materials in producing the enzyme L-glutaminase economically. Under optimized conditions the DTDW was found to be superior to that of CFW in enzyme production.
**Keywords:** Solid state fermentation, L- glutaminase, Decaffeinated Tea Dust Waste medium, Coconut Fiber Waste medium, AR isolates.

## Introduction and Experimental

**Introduction:** L-Glutaminase (L- glutamine amidohydrolase EC, 3.5.1.2) is an enzyme which catalyses deamidation of L- glutamine to L-glutamic acid and ammonia. It plays a significant role in cellular nitrogen metabolism. It is found to be a potent antileukaemic agent by reducing the glutamine pool, which is being avidly consumed by the tumor than the normal cells and there by destroying tumor cells selectively[2-5]. Some studies suggest it has a promising effect against retro viruses[1, 5]. It is also being used as analytical agent[11] and a bio-sensing agent in the determination of glutamine of biological products[10]. With all these potential properties

of this enzyme, it is essential to find out an economical method in producing this enzyme. Solid state fermentation proves to be efficient and results in enzyme of high purity[7] and of higher in concentration[8,12]. For the economical production of L Glutaminase Decaffeinated Tea Dust Waste (DTDW) and Coconut Fiber Waste (CFW) were tried. The reason for the selection of DTDW as a substrate was for the fact that it possess high nitrogen source which is a prerequisite for the enzyme synthesis[14] and the CFW was a waste left by coir industries and found to have organic carbon[13]. They function as a good support in SSF by their micro fibers which increase the surface area. It has a high fibrin content which is derived from cellulose. Another reason behind using DTDW and CFW waste was that they were abundantly available and they were used for nothing more than in the production of fertilizer and as they were economically feasible in the commercial manufacture of this enzyme. Whey is a byproduct of dairy Industries which consists of nitrogenic bases and good supply of minerals. Thus fulfills the nutritive requirement needed for the growth of microorganisms.

**Objective:** Soil samples were collected in sterile screw capped tubes from different places of rain forests. From that L-glutaminase producing microorganisms were separated by using selective minimal agar media (L-glutamine as a sole carbon and nitrogen sources). Potential colonies were sub cultured and used for further studies. DTDW, CFW with whey were used as media for the culture of potential microorganisms and the production of the enzyme. The main objective was to generate L- Glutaminase economically by using the above said waste products. Commercial production of L-Glutaminase has been carried out using submerged fermentation (SmF) technique. But nowadays, solid state fermentation (SSF) has been emerging as a promising technology for the development of several bioprocesses and products including the production of therapeutic enzymes on a large scale. The primary advantage of SSF was the fact that many metabolites were produced at higher concentration.

## Experimental methods:

### Materials and methods:

Minimal agar media, Phenol red, Buffer tablets(pH 5.4-9), DTDW, CFW, Whey, Nesslers reagent, TCA, L-Glutamine, Ammonium sulphate, Erlenmayer flasks, Temperature controlled Orbital shaker, Refrigerated centrifuge and Spectrophotometer were the important chemical and equipments used. Rapid plate assay [6], and Glutaminase assay based on calorimetry method[9] were the assay methods followed.

### Collection of soil samples & isolation of potential strains:

Soil samples were collected from different places of rain forests of Keeriparai, Tamilnadu. About 1g of each of above samples was taken into 50 ml of sterile water. The Suspension was kept on rotary shaker for 30min and kept aside to settle the soil matter. One ml of the suspension was serially diluted five times with sterile water and 0.5 ml of each of these dilutions was added to 50 ml of sterile isolation medium which consist of L-glutamine as a sole agent for both carbon and nitrogen sources. After sufficient mixing the medium were plated in 6-inch diameter sterile petridishes and incubated at $37^0$C. Cycloheximide 10µg/ml is used as antifungal agent[15]. Subsequent subcultures were done every three days using the minimal agar media until potential strains develop. Such obtained potential strains were isolated based on the semi qualitative rapid plate assay done by *Gulati .et al. 1997.*

### Initial Screening for the production of the enzyme:

From the total 16 strains 3 prominent strains viz. AR-glut 5, AR-glut 6, and AR-glut 7 were selected dependant on its zone of colour formation. Further they were identified through different biochemical tests of Bergey's manual of bacterial classification.

Culture flasks were autoclaved and inoculated with 5 mL of inoculum volume and incubated at $37^0$C temperature using temperature controlled orbital shaker at 150 RPM and samples were collected at regular intervals of every 24 hours up to 72 hours of incubation. The collected samples were estimated for growth profile and were centrifuged at 12000 rpm at $4^0$C in a refrigerated centrifuge. The supernatant was estimated for the crude enzyme production. Enzyme assay was carried out as explained by *IMADA et. al* and analyzed using a spectrophotometer at 450 nm. Enzyme activity is calculated from the standard graph of ammonium sulphate. (1 IU is equal to 1µ mol of ammonia/min/mL.)

### Optimiztion of Fermentation parameters

With the DTDW and CFW as media pH, Incubation temperature, inoculum volume, Incubation time and additional supplements of Phosphates and metal ions influencing L-glutaminase production in these bacterial

strains were considered for designing the experiments. To achive optimized activity each parameter is varied one at a time. Different concentration of 1% - 4% potassium di hydrogen phosphate was added as a phosphate source to study its effect on the production of L-glutaminase. To determine the effect of various metal ions such as $Mg^{2+}$, $Mg^{2+}$, $Zn^{2+}$, and $Fe^{2+}$ were incorporated to the substrate at concentration of 0.03% w/w. Phosphates are found in nucleic acid, phospholipids, lipopolysaccharides, and in cytoplasm. Bacteria are found to accumulate more inorganic phosphates and made them to poly phosphates which are used up in scarcities[16]. Such all these physical parameters and additional phosphate and Metal ions were optimized. All these experiments were done in triplicates and average value is taken. Finally DTDW 5 gm with 10 mL of whey mixture as one medium and coconut fiber waste coarsely grounded 5 gm with 10 mL whey mixture as another medium for the growth of micro organisms in optimized parameters were used to yield maximum production of the enzyme.

**Assay of the enzyme:**

Enzyme assay was carried out as explained by *IMADA et. al* with slight modification. 0.5 mL of 0.2m glutamine with 1 mL of 0.2m acetate buffer of pH 5.4 is added with 0.5 mL of enzyme preparation. This reaction mixture is incubated at about 30 min to allow the enzymatic reaction. The action is stopped by adding 1 mL of 10% trichloro acetic acid. 0.1 ml of the reaction mixture with 3.7 dil. water and 0.2 mL nessler's reagent is analysed using a spectrophotometer at 450 nm. Enzyme activity is calculated from the standard graph of ammonium sulphate. 1 IU is equal to 1µ mol of ammonia/min/mL.

**Substrate media:**

**DTDW medium:** Fine tea dust were subjected to hot water and the residue was filtered out. Then the residue was stored with 2 mL of Whey per gram of DTDW at $4^0$C until it is being used.

**CFW Medium:** Coconut fibre wastes were collected from coir industries and were boiled at high pressure. Then they were dried in a hot air oven. It was then stored with 2 mL of Whey per gram of CFW at $4^0$C until it is being used.

**International Unit:**

One IU of L- glutaminase activity was the amount of enzyme which catalyses the formation of 1 µmole of $NH_3$ per minute under optimal assay conditions.

**Results and discussion:**

**Substrate Selection**

The solid substrates used here play a dual role in supply of nutrients to the microbial culture growing and also serve as anchorage for the growing cells. In SSF selection of a suitable substrate for a fermentation process is a critical factor and thus involves of a number of residual wastes for the microbial growth and product formation. In the present study, two substrates, viz. DTDW, CFW were screened as the substrates.

**Potential strain isolation:**

From the 16 colonies of microorganisms 3 were found potential strains in producing L-glutaminase by forming the zone of colour which is the end point of rapid plate assay. The isolates AR-glut 5, AR-glut 6, and AR-glut 7 zones were found to be 20 mm, 15 mm, and 22 mm respectively.

**Biochemical Screening of AR Isolates:**

| Biochemical test | AR glut 5 | AR glut 6 | AR glut 7 |
|---|---|---|---|
| TSI | ac/ak | ac/ak | ac/ac |
| Citrate | - | - | - |
| Urease | +/- | + | + |
| Nitrate | + | + | - |
| MR | + | + | - |
| VP | + | - | - |
| Indole | + | - | - |

TSI-triple sugar iron, MR-methyl red, VP-voges proskauer, ac-acid, ak-alkaline

**Gram staining result:** AR glut 5-gram negative rod,
AR glut 6-gram negative rod,
AR glut 7-gram negative rod.

**Morphological characteristics of AR isolates:**

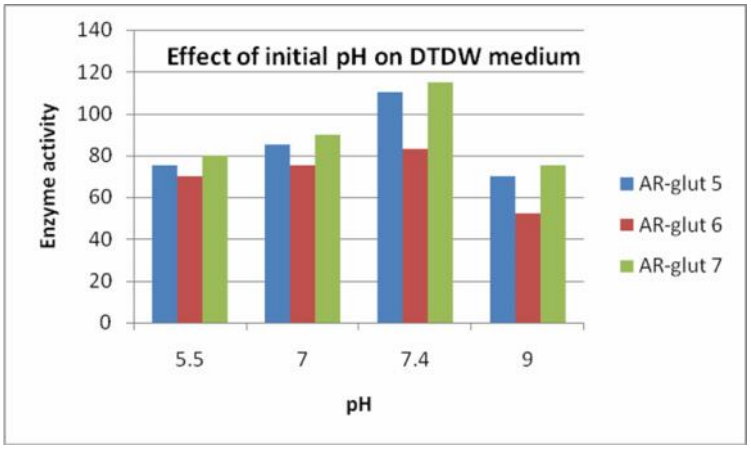
| Colony morphology | AR Glut 5 | AR glut 7 | AR glut 6 |
|---|---|---|---|
| Shape : | Circular | Circular | Circular |
| Margin : | Entire | Entire | Entire |
| Elevation: | Flat | Convex | Convex |
| Size : | Moderate | Moderate | Moderate |
| Texture : | Smooth or rough | Smooth | Smooth or rough |
| Appearance : | Glistening | Shiny | Glistening |
| Pigmentation : | Non pigment | Pigmented | Non pigment |
| Optical: | Translucent | Translucent | Translucent |

**Optimization of Physical parameters:**

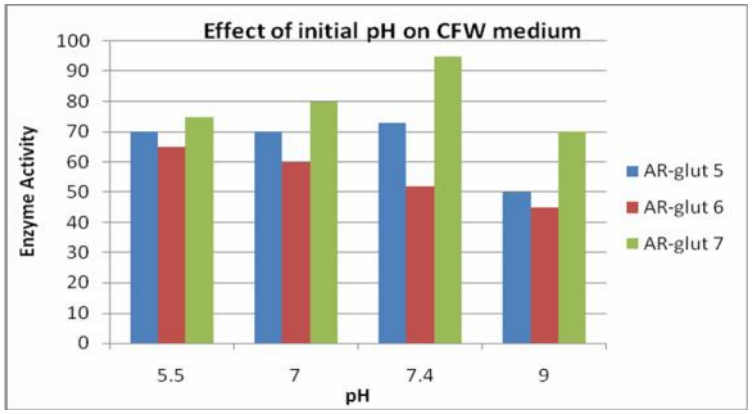
**Optimization of pH:**

In order to maintain the favorable conditions for increased L-glutaminase production initial pH was optimized. This was established by carrying out the fermentation by varying the pH from 5.5 to 9(adjusted with 1N HCl or 1N NaOH). The significance of initial pH of the fermentation on the production of L-glutaminase production was observed. The maximum L-glutaminase production of 70 to 115 IU/gds was obtained at 7.4pH for the three isolates when using DTDW waste media and CFW as substrates**.** The isolate AR-glut 7shows the maximum yield of 115 (IU/ gds) with DTDW which is 15 to 20 IU/gds more than the CFW. The significance of pH may be attributed to the balance of ionic strength of plasma membrane.

**DTDW media substrate:**



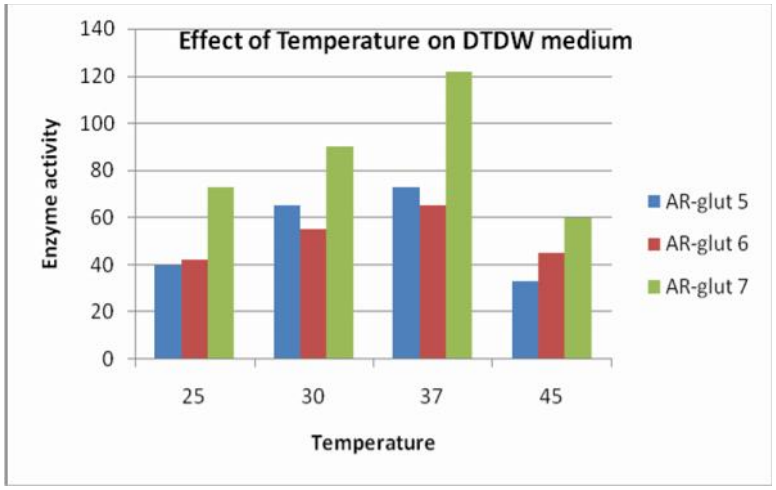
**CFW media Substrate:**



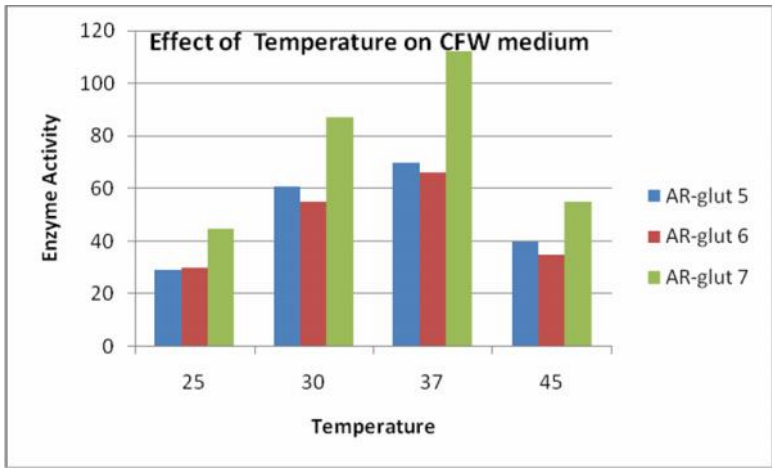

**Optimization of Temperature:**

Fermentation was carried out at various temperatures such as $25^0$C, $30^0$C, $37^0$C and 45°C to study their effect on enzyme production. Incubation temperature had a profound effect on L-glutaminase production by AR- GLUT isolates under solid cultural conditions. The yield of enzyme varies widely from 40 to 122 IU/gds. The maximum yield 122 $IUgds^{-1}$ was obtained with AR glut 7 on DTDW. when SSf was carried out at $37^0$ C for the three isolates. From the temperature parameter tested $37^0$C was found to be the optimum temperature in producing maximum L-glutaminase concentration.

**DTDW media substrate:**



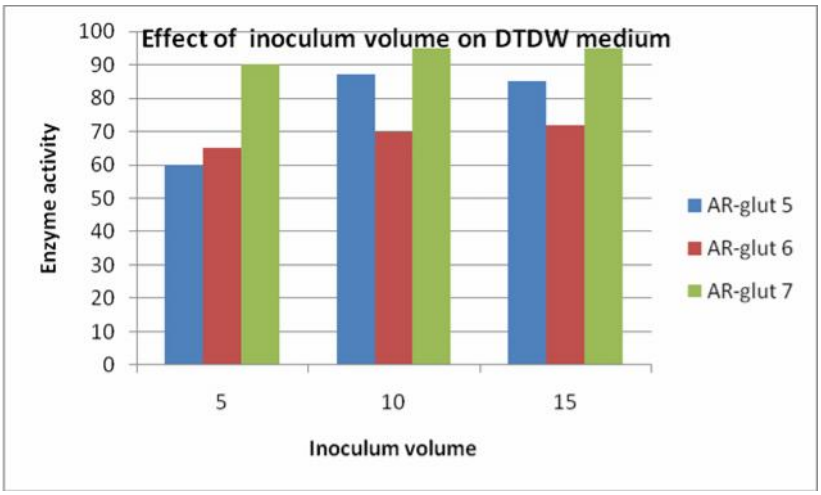

**CFW media Substrate:**



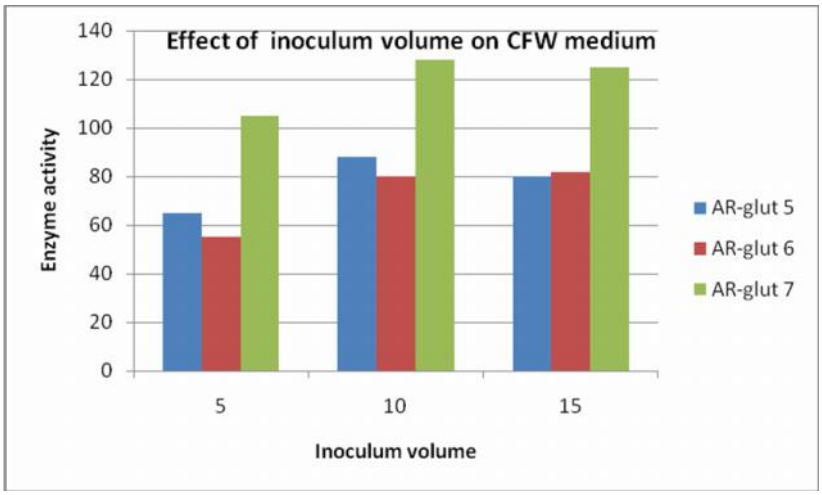
**Optimization of Inoculum volume:**

Fermentation was carried out with different inoculum volumes varying from5-15 ml for a period of 72h to study its effect on the production of L-glutaminase. Maximum L-glutaminase production ranges from 9 0 to 128 I U/gds. T h e h i g h e s t y i e l d was obtained with 10 ml of 4 day old culture of isolates. There is no significant change by increasing the inoculums volume from 10 to 15 mL. This may be attributed to nutrient depletion and the formation of other metabolites that affects the growth of these microorganisms.

**DTDW media substrate:**



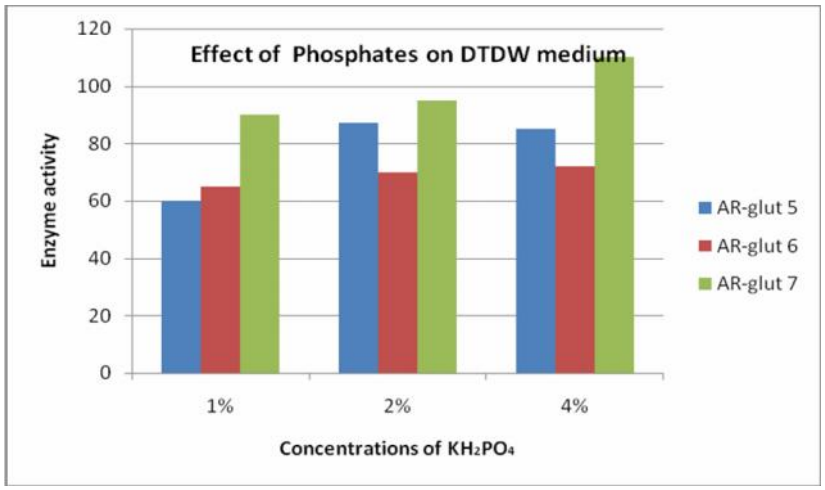

**CFW media Substrate:**



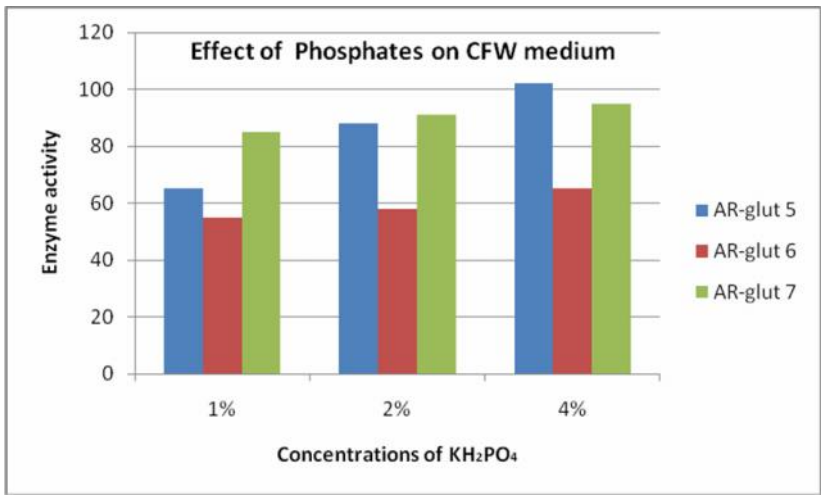

**Effect of Phosphate Source:**

Apart from the structural component phosphates function as regulators in enzyme metabolism. Fermentation was carried out with different concentration of 1% to 4% of potassium di hydrogen phosphate to study its effect on the production of L-glutaminase. Optimal L-glutaminase production of 85-110 I U/gds was obtained with 2 - 4% of potassium di hydrogen phosphate. Overall a proportional increment of enzyme production is seen with the increase in phosphates with all the strains and in both substrates. The least production of enzyme 60 and 55 IU/gds were found with DTDW of AR glut 5 and CFW of AR glut 6 respectively. AR glut 7 strain shows to be superior in the DTDW medium whereas ARglut 5 shows to be good at CFW medium.

**DTDW media substrate:**



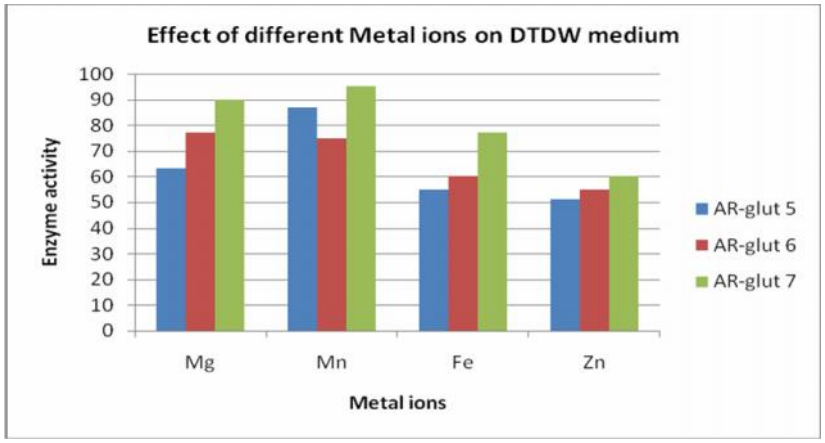

**CFW media Substrate:**



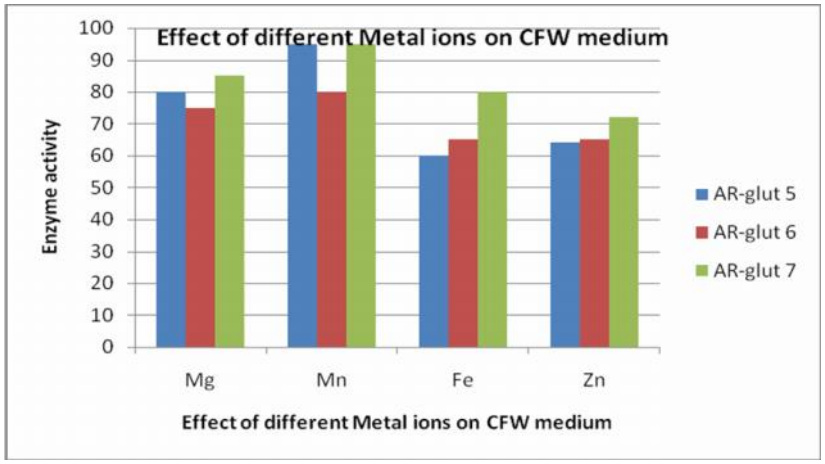

**Effect of Metal Ions**

Fermentation was carried out with different metal ions like $Mg^{2+}$$Mn^{2+}$,$Zn^{2+}$ & $Fe^{2+}$ incorporated to the substrate at concentration of 0.03% to study its effect on the production of L-glutaminase. Maximum L-glutaminase production was obtained with the introduction of the metal ions $Mg^{2+}$ and $Mn^{2+}$ slightly improves the yield of the enzyme. It may be due to the fact they act as cofactors in the biosynthetic pathway of enzymes where these are essential but are needed in minute quanties.

**DTDW media substrate:**

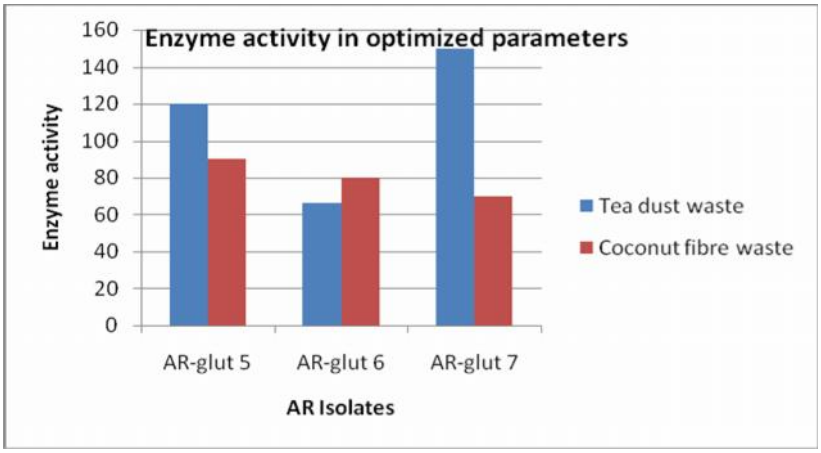


**CFW media Substrate:**




**Optimized parameters with DTDW and CFW as Media:**

The optimized parameters of initial pH 7.4, Temperature $37^0$C, incubation period of 48 hours, the inoculum volume of 10 ml, 0.03% w/v of metal ions and 4% w/v of potassium dihydrogen phosphate were fixed. AR glut7 was found to yield maximum enzyme of 150 IU/gds in DTWD medium. 120 IU /gds were obtained with AR glut 5 isolate in DTDW medium. CFW medium yielded a maximum of 90 IU/ gds with AR glut 5 isolate. The SSF was carried out and the results were shown below.




## Conclusion:

Out of the 16 isolates selected three of the AR isolates showed good zone of colour formation which reveals the production of L- glutaminase. In the present study it was found that the CFW and DTDW prove to be wonderful materials in producing the enzyme L-glutaminase economically. Under optimum pH, temperature and inoculum volume the AR isolates produced more of the enzyme with DTDW medium. Solid state fermentation of such medium proves to be efficient and economically feasible to produce this enzyme in a large scale. Critical factors such as pH, incubation Temperature, inoculums volume and additional nutrients play a vital role in the production of this enzyme. DTDW medium found to be superior when compared with CFW, most probably due to the fact it possess all the essential nutrients along with whey in adequate quantity over the time of incubation. Micronized tea dust was succulent which were vital as it supplies a steady release of nutrients for the growth of microorganisms. AR glut 7 with the DTDW medium produced a significant amount of 150 IU/ gds L-glutaminase enzyme. Further bioprocessing of this isolates are being carried out for the economical production of L-glutaminase from the AR isolates.

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