Optimized Solid State Production of L – Glutaminase and Its Pharmacological Applications

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Abstract:

Production of L - glutaminase was carried out by *Bacillus halotalerance PHN 1* under solid state fermentation. Fermentation was done in presence of high salt concentration and using wheat bran as agro-industrial substrate. The processes of L-glutaminase production was optimized by using Plackett Burman, CCRD and RSM methods. Results showed better yield of L - glutaminase in presence of mannitol, $MgSO_4$ and NaCl at pH 7 and 30^oC. Purification of enzyme was done by using 60 % ammonium sulfate precipitation followed by ion exchange chromatography. Overall yield obtained after purification is 4.68 fold with 1.56 U/mg. Pharmacological activities like Anti-inflammation, antioxidant and anti-angiogenesis of crude and purified enzymes were checked. It has been observed that crude enzyme sample showing good pharmacological activities than purified enzyme. Cytotoxicity of both enzyme sample were checked by MTT assay on HepG2 (Liver Cancer Cell Line) and MCF7 (Breast Cancer Cell Line). These extract showed good cytotoxic activities on both cancer cells cell. lines.

Key words: Anti-inflammatory, Cytotoxicity, L – glutaminase, Medium optimization

1 Introduction:

Microbial products, especially like enzymes have numerous applications in industrial, agriculture, and ecological sectors. L- glutaminase (EC 3.5.1.2) is one of such enzymes that catalyses the amido-hydrolase reaction that converts L -glutamine to L -glutamate and ammonia. Glutaminase is one of the crucial enzymes for nitrogen metabolism and nearly found among all types of living cells and animals (Bülbül & Karakuş, 2013). Bacterial species like *Pseudomonas, Bacillus*, *E. coli* and *Actinobacterium* are known for their ability to produce glutaminase enzyme either extracellularly or intracellularly (Amobonye et al. 2019) whereas, in moulds *Aspergillus sp*. and *Trichoderma sp*. were commonly known for glutaminase production (Singh and Banik, 2013).

Recently, explored various pharmacological properties of glutaminase has attracted huge attention of medical as well as pharmaceutical fields (Roberts et al. 2001). It can also be used in production of food flavouring agent and can be utilized in food like soy sauce and other fermented foods (Yokatsuke 1985). Glutaminase biosensors also served as an analytical tool to detect residual concentration of glutamine and theanine (Sathish & Prakasham, 2010). Due to such and all other additional applications, this enzyme can be treated as one of the industrially important enzymes. A major a ttempt was made to isolate this enzyme from halophilic microorganisms from the marine ecosystem (Iyer and Singhal 2010; Chasanah et al. 2013). Because, glutaminase obtained from normal terrestrial microorganism showing incompatibility with human physiology basically with blood that can induce various side effects in humans (Sabu 2003). Therefore, there is a need of finding efficient, sustainable, eco-friendly cost-effective processes for glutaminase production from isolated halophilic microorganism (Amobonye et al. 2019; Janda & Abbott, 2010).

Organisms are cultured on moistened solid substrates during solid-state fermentation (SSF), which occurs when there is little to no free water available. SSF has drawn the attention in fermentation technology for development of different valuable enzymes and

metabolites on higher scale (Dutta et al., 2015). To carry out different bioprocesses in SSF industrial waste material or agricultural raw materials were utilized as ideal substrate. However, in case of enzymes production selection a good substrate for the SSF is a highly important factor because that significantly influences enzymes productivity (Nathiya et al., 2011). There are some reports available for the production of extracellular L-glutaminase using wheat bran and SSF technology (Sathish et al., 2016) but there is a need to optimize this processes by using various parameters.

Hence, in this study the attempt was taken to obtained optimized yield of L-glutaminase by using wheat bran as agro-industrial substrate and exploring the pharmacological activities of obtained crude and purified enzyme of SSF.

2. Materials and methods

2.1 Chemicals and media components:

In 500 ml Erlenmeyer flasks 25g of wheat bran is mixed with the 50 mL of other medium as described in further section. The pH of medium adjusted to 7. After sterilization by autoclaving the medium, 5 mL of inoculum with cell density 8 $x10^5$ /mL was inoculated. Inoculum was prepared by growing *Bacillus halotolerance* PHN1 for 18 - 24h. Medium was incubated at 37° C. The production of L-glutaminase was monitored by calculating glutaminase activity after interval of 24h for next few days. For calculation of enzyme activity enzyme was extracted from each flask by diluting fermented content of flask in 500 ml with vigorous shaking. The content was centrifuged and the supernatant was used as the enzyme source.

2.2 Optimization of media:

The preliminary optimization of medium was carried out by using OFAT design, in this optimization the effect of different sugars like glucose, sucrose, maltose, starch, cellulose, and mannitol were studied at (0.01 g/gds). Similarly, the as N_2 sources, yeast extract, malt extract beef extract, peptone, and $NaNO_3$, at (0.03 g/gds) concentration were

taken. Other medium components like KH_2PO_4, $MgSO_47H_2O$, and KCL were also studied by supplementing them in production medium at 0.05%. These all experiments were performed in triplicates.

2.3 Media optimization using statistical tools:

2.3.1. Plackett–Burman design:

Variables (components of the medium) of production medium were further optimized by using Plackett–Burman design using (Design Expert STAT Ease software 9.0 version, Minneapolis, USA) software. To recognise the influential media components amongst tested medium for highest L -glutaminase production as per design every individual variable was tested as shown in (Table 1).

Based on experimental design given in (Table 2) the experiments were run and results were obtained. This design of statistical optimization is based on the first order model having following equation.

$$R1 = \beta_\circ + \sum \beta i \, Xi \quad \text{..... (1)}$$

Where,

R_1: response for productivity of Glutaminase,

$\beta_\circ$: intercept, and

β_i : linear coefficient.

The evaluation of influencing component was done by using regression analysis of overall data at significant p value (<0.05) for the optimized production of L -glutaminase.

Due to insufficient interaction amongst identified variable this model cannot be further used for the medium optimization. For next step of optimization interaction of selected influential variable were studied. These variables were identified from PB design. selected variables were further optimized by Central Composite Rotatable Design (CCRD) and Response Surface Method (RSM).

2.3.2. Central Composite Rotatable Design for media optimization

In this step of optimization four influencing variable from PB design which are wheat bran, NaCl, $MgSO_4$ and mannitol were selected for CCRD as shown in (Table 3). The (3D) response surface contour plots were developed by using obtained data which is based on quadratic equation as follows.

$$R_2 = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_1 \beta_2 AB + \beta_1 \beta_3 AC + \beta_1 \beta_4 AD + \beta_2 \beta_3 BC + \beta_2 \beta_4 BD + \beta_3 \beta_4 CD + \beta_1 \beta_1 A^2 + \beta_2 \beta_2 B^2 + \beta_3 \beta_3 C^2 + \beta_4 \beta_4 D^2 \quad \text{..... (2)}$$

Where,

R_2: response enzyme activity in units;

A, B, C and D: coded independent variables;

β_1, β_2, β_3 and β_4: linear coefficients;

β_0: intercept term;

$\beta_1\beta_1$, $\beta_2\beta_2$, $\beta_3\beta_3$and $\beta_4\beta_4$: *quadratic* coefficients

$\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_1\beta_4$, $\beta_2\beta_3$, $\beta_2\beta_4$ and $\beta_3\beta_4$: interactive coefficients.

The CCRD design was run using Design Expert STAT Ease experimental set up as shown in (Table 4).

2.4 Enzyme extraction, purification:

L- glutaminase was purified by preparing 1L cell free broth (as stated in earlier section) by cold centrifugation (10000 rpm at 4°C) after 144h. This cell free broth then precipitated by 60 % saturation of ammonium sulfate. After precipitation the pellet obtained was dissolved in 50 ml of sodium phosphate buffer pH=7, 50 mM and overnight dialysis carried out against same buffer 20 ml of this enzyme was used in further studies as crude

enzyme. After dialysis, 30 ml of remaining enzyme was monitored for the on exchange chromatography using of DEAE – Cellulose resin and increasing concentration of NaCl gradient (0.025 M, 0.05 M, and 0.2 M NaCl). Followed by completion of column elution, the protein contents of each eluted sample (fractions) was recorded by taking its absorbance at 280 nm and enzyme activity of each fraction was also determined. Fractions showing glutaminase activities were then selected and mixed together and this enzyme was labeled as purified enzyme and used for the pharmacological applications.

2.5 Enzyme activity:

The enzyme activity is measured by calculating number of ammonia molecules released. Ammonia reacts with Nessler's reagent to from brown colour complex. Intensity of brown color is measured at 450 nm.

Reaction-

The activity of glutaminase was calculated using the Nesslerization principle as stated by Jesuraj et al. (2013) with some modification. The reaction cocktail contains 0.5mL enzyme, 0.5mL of 0.2M glutamine. The enzyme and substrate were prepared in phosphate buffer 50mM and 7 pH. The enzyme reaction is carried out for the ½ h then activity stopped by adding (10% TCA) 100 µl in reaction mixture. After stopping the reaction, the activity was measured by diluting 0.1 ml of reaction cocktail with 3.7ml distilled water and 0.2 ml of Nessler's reagent. The activity was calculated by taking absorbance at 450 nm.

2.6 Pharmacological activities of enzyme extracts:

2.6.1 *In vitro* anti-inflammatory activity by Protein denaturation method

The reaction mixture containing 4.6 ml of phosphate-buffered saline (PBS) pH 6.4, and 0.2 mL of egg albumin (from a fresh hen's egg) and 0.1 ml of the crude and purified enzyme samples respectively. As a control, the same volume of double-distilled water was

used and allowed to incubate this reaction mixture for 37°C ± 2 for 15 minutes then heated for 5 minutes at 70°C. After cooling of samples the absorbance was measured at 660 nm using distilled water as a blank. Diclofenac sodium at the same concentration served as a reference drug and tested as above. Using the following formula, the % inhibition of protein denaturation was determined.

% Inhibition = C –T / C x 100

Where, T is the measured absorbance test and

C is the absorbance of control sample

2.6.2 Cytotoxicity on cancer cell lines

The cytotoxic impact of the crude and purified enzyme was checked by MTT assays on 3 cell lines.

a) HepG2 (Liver Cancer Cell Line)

b) MCF7 (Breast Cancer Cell Line)

c) Normal cell line: NPK52E cell line

Assay – MTT assay

Cell culture medium containing 1 x 10^4 cells/ml were grown at 5% CO_2, 37°C for 24h. 100 µl of culture medium containing cells were poured in tissue culture grade, and 96 wells at 10^4 cells / well concentration. Stepwise increase concentration of crude as well as purified enzymes was added in each well from 50 µl, 100 µl, 150 µl, 200 µl, and 250 µl. As a control instead of sample 0.2% DMSO in PBS was mixed with cells at similar concentration. All samples were incubated in triplicates in CO_2 incubator for 24 h at 37°C and 5% CO_2. Following incubation, the medium was entirely taken out. 20 µl of MTT reagent (5 mg/ml PBS) was added in each well. The reacted cells allowed to incubate at 37°C for 4h. Cell cultures were kept in wells and examined under the microscope for production of formazan crystals.

Followed by the removal of medium DMSO (200 µl) was added in the wells and allowed for the incubation at 37°C for 10 minutes. The % of living cell of cell lines after reaction with samples and control was determined by taking absorbance of micro plate in ELISA reader at 550 nm.

2.6.3 Anti-Angiogenesis Activity:

A sterile microbe free embryonated 72 h age egg was obtained from hatchery. With the help of Egg Candler, the presence and location of embryo was determined. With anchor the egg kept horizontal in incubator equipped with indicator of humidity and temperature. At 37°C and 50% humidity incubation of egg was carried out for an hour in steady situation. After incubation eggs were taken from incubation chamber to sterile laminar hood. The horizontally positioned eggs then open gently with scalpel. In same position of egg and perpendicular to the egg's axis circular cutting disc cups of filter paper having 1mm thickness and 6 to 7mm in diameter was prepared and sterilized by autoclave. The embryos were taken in these cups and kept for incubation for 120 h in presence of samples to be tested (crude and pure enzyme- filter sterilized). After incubation these embryos were placed under microscope and their microscopic and morphological characteristics of generating blood vessels were visualized.

2.6.4 Antioxidant Activity:

The antioxidant activities of the extract were determined by the improved ABTS+ radical cation assay with the slight modification (Re et al. 1999; Sarma et al., 2016). ABTS+ radical cation was synthesized by mixing 7 mM 2, 2′- azino-bis (3- ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS) and 2.45 mM potassium persulfate ($K_2S_2O_8$), incubated at room temperature in the dark. To determine the ABTS radical scavenging activity, 3 mL of ABTS+ solution was mixed thoroughly with 0.2 mL of different concentrations (25, 50, 100µL/mL) of crude enzyme sample. Ascorbic acid was taken as a

standard. The reaction mixture was allowed to stand at room temperature for 6 min. The percentage inhibition was calculated by the following formula:

$$\%\,\textbf{Inhibition} = \text{OD of control} - \text{OD of sample} / \text{OD of control} \times 100$$

2.7 Statistical analysis:

Graph Pad software (Graph Pad Instat version 3.00, Graph Pad software, San Diego, CA, USA) was used to obtained results where, obtained result for each experiment is the mean of three or more determinants. ANOVA was done for all data at $P< 0.05$ using

3 Result and discussion:

3.1 solid state fermentation of L -glutaminase:

It was observed that the *Bacillus halotolerance* PHN 1 was able to grow on wheat bran. The maximum enzyme activity 0.956 U was observed after 144 h and after this point of incubation the activity remains nearly constant.

Ahmed et al., (2016) reported that wheat bran itself can induce glutaminase enzyme in solid state fermentation. However, in this study the wheat bran is used as a substrate which can fulfil both requirements but using mannitol at 0.01 g/gds concentration have positive impact on enzyme production. According to Jesuraj et al., 2017, using supplementary C and N_2 sources gives prominent production of enzyme as compared to the medium with limited growth supplements. Some other reports are available for other enzymes used same model with various supplementary component (Bhagwat et al., 2015; Jhample et al., 2015).

3.2 Classical media optimization studies by OFAT strategy

Within all tested sugar sources mannitol shows positive effect on production of L – glutaminase. In presence of mannitol enzyme shows higher activity (Figure. 1). In case of N_2 sources it was observed that nearly all N_2 source impact similarly for the production of enzyme with very small effect (Figure. 2).

3.3 Medium optimization by using Plackett Burman design

Amongst 11 studied variables, wheat bran, NaCl, mannitol and $MgSO_4$ were selected as influential medium component from Plackett–Burman experimental runs acquiring significant p values. The overall response of selected variables with their effects on enzyme production was shown in (Table 5). The "Prob > F" values for model is less than 0.005 which indicates the significance of used variables in experimental design. Remaining other components like yeast extract, malt extract, $NaNO_3$, K_2HPO_4 and Sucrose, were showing greater p values so treated as insignificant. The model shows 91.40 F-value that suggested optimization design is significant and there is only 0.20% chance of large differences due to noise. It was also observed that the P-values of model are less than 0.0001 indicates A, B, D, G are influential model variables.

PB experimental model only displayed the impacts of variable on production of L-glutaminase and unable to inspect interactions among variables so, any interactive impacts among selected medium components were studied using CCRD and RSM. The optimization of L- glutaminase enzyme production was studied previously by traditional methods however there are few reports available for the medium optimization by using Plackett Burman design and CCD designs for solid state fermentation Mao et al., (2013).

3.4 Central composite rotatable design:

Further interactive analysis of screened components was carried out with CCRD of RSM. The experimental runs using variables for the CCRD design are according to bi factorial levels. The CCRD results validated by using equation - 4 (second order polynomial equation based on quadratic regression analysis).

R1 Glutaminase activity U = +13.66 + 1.14 A – 0.6225 B + 0.3175 C + 0.3350 D - 0.4400 AB + 0.1625 AC + 0.0.2175 AD + 0.1513 BC - 0.1562 BD - 0.4262 CD – 2.13 A^2 - 1.63 B^2 - 1.22 C^2 - 0.8321 D^2 ..... (4)

The effectiveness of selected variables was detected by obtained response after different statistical analysis (Table 6).

The obtained F-value is 33.43 which signify the design of model, having only 0.01% chance of large values that might found because of noise. Results obtained for "prob > F" below 0.0500 for (A, B, D, AD, AB, BC, CD AC, A^2, B^2, C^2, D^2) interacting variable are significant. Values greater than 0.1000 indicated model terms are nonsignificant.

"Lack of Fit F-value" 4.43 implicated that Lack of Fit is nonsignificant when compared with pure error. Insignificant values of F established this model's equation and it will efficiently use to determine L - glutaminase production. CV% (value for coefficient) is seen to have 5.71 which authenticate accuracy and consistency for given model.

Pre-determined R^2 values is 0.8347 in rational impact on adjusted R^2 0.9400, the difference is less than 0.2 indicated appropriateness for expected response for this model. Satisfactory accuracy shows precision ratio above 4, which is necessary and this model has 21.090, so can be navigated for the design space.

3.5 Interaction of variables:

The interactions among selected variables were checked to obtain optimized production of L - glutaminase activity in SSF. This can be achieved by adjusting one component for its optimum concentrations while keeping other component at their lowest (near about zero) or constant. Obtained results were expressed graphically on response surface and 3D contour plots. The 3D response surface and contour plot obtained for the wheat bran and mannitol shown (Figure. 3 A, B) spherical contour lines in graph which indicates a slight positive interaction in mannitol and wheat bran.

The impact of mannitol in submerged fermentation of L- glutaminase production medium with glutamine was seen more precisely as compared to solid state medium. However, in SSF medium containing wheat bran mannitol concentration was seems to increased slight high activity. Whereas, previously obtained reports indicates positive impact

of wheat bran alone that is without any supplemented sugar (Iyer and Singhal, 2008). On the other hand other reports also indicates glucose associated increased enzyme activity in *sp.* like *Pseudomonas, Streptomyces rimosus, Providencia and T. koningii* (ElSayed, 2009; Sivakumar et al., 2006; Kumar and Chandrasekaran, 2003; Iyer and Singhal, 2008).

(Figure 3. C, D) shows elliptical contour lines of surface response and indicating a positive and supportive interaction between wheat bran and NaCl for the L - glutaminase optimized activity. The graph signifies effect of NaCl concentration on increased enzyme activity and enzymes stability in SSF. It was observed that the L – glutaminase activity is not hampered up to 14% of NaCl concentration.

Conventionally used fungus in SSF (koji mould) *Aspergillus oryzae* was use in production of soy sauce can tolerate 17–18 % NaCl concentration and shows optimum glutaminases production (Yano et al., 1988). Mao et al., (2013) reported 68% retained residual activity of extracellular L-glutaminase at 20% NaCl concentration from isolated *Bacillus amyloliquefaciens* Y-9. The salt concentration recognised as one of the important criteria during soya sauce fermentation along with L - glutaminase and hence there is a large demand of salt tolerating glutaminase producing microorganism in such food industries. L – glutaminase producing halophilic bacteria can obtain from saline habitat especially marine water since this is an ideal ecosphere for halophilic microbial communities. L – glutaminase producing *Bacillus halotalerance* PHN1 was isolated from saline agriculture soil having substantial enzyme activity at elevated salt concentration. However, the maximum activity of enzyme can obtained at lower to moderate concentration of NaCl. Earlier reports of SSF production L- glutaminase by using wheat bran was observed at higher salt concentration. Since, addition of salt and brine is a necessary condition in some fermented food products the indicated positive interaction in NaCl and solid state substrate like wheat bran and other sources seems to be very useful for production of enzymes used in food fermentation (Nandakumar et al., 2003).

Response surface and Contour and plots (Figure. 3. E, F) of wheat bran and $MgSO_4$. Interaction shows elliptical forms of contour lines for L -glutaminase production and signifies importance of $MgSO_4$ for enzyme production. Interaction among $MgSO_4$ and wheat bran can have increased enzyme productions up to certain limit but at higher concentrations of both variables the enzyme activity remains constant.

The subsidiary variables interaction between NaCl, $MgSO_4$ and Mannitol were also checked. This is essential to determines models effectiveness for the higher production of L -glutaminase. In this interaction wheat bran was kept constant and other varying concentrations of testing components were used. This interaction also underlined the cost effectiveness of SSF designed for the production of glutaminase which can allow skipping of non-influential component from the designed medium. Contour and RSM plot for all interaction like NaCl with Mannitol, NaCl with $MgSO_4$ and $MgSO_4$ with Mannitol presented by (Figure 4. A, B; C, D and E, F). Elliptical form of all counter plots confirms their influence on enzyme activity along with wheat bran. All interaction sows positive influence for the enzyme production and signifies their importance in model.

There are several reports available for SSF production of glutaminase using different type of raw material as a substrate. Mostly used and preferred solid-state substrate is wheat bran (Prabhu and Chandrasekaran, 1995). Renu and Chandrasekaran (1992) reported production of glutaminase from *Vibrio costicola* using wheat bran having uniform particle (1.4 to 2.0 mm). Renu et al., (2003 and 2004) reported comparative studies on glutaminase produced by SSF and submerged fermentation in which solid state fermentation was increased impact (25 to 30 fold) than submerged fermentation protocol. The wheat bran was chosen by Dutta et al., (2015) used wheat bran containing SSF medium in order to synthesize the higher concentration of extracellular glutaminase by *Pseudomonas aeruginosa* PAO1. The production was increased when 90% moisture was maintained in medium.

3.6. Checking efficiency of optimized medium composition:

Based on results obtained of PBD and CCRD of RSM for SSF production of glutaminase using wheat bran an actual medium was prepared having only identified key variables like wheat bran, NaCl, $MgSO_4$, Mannitol medium was design having following composition, 21.25 g Wheat bran, 13.75 g NaCl, 0.9000 g $MgSO_4$, 4.50.g mannitol concentration. This modified media composed by screened variable at their lower concentration. After completion of fermentation, it is observed that the production medium shows 13.96 U glutaminase enzyme activity.

To obtained high concentration of glutaminase from *Aspergillus flavus* MTCC 9972 with SSF using Bengal gramme husk & wheat bran, Sathish et al., (2016) used a sequential optimisation technique made up of a modern fermentation application like ANN, GA etc. They got maximum activities by using this method and microorganism under derived optimum conditions. In additional work, *Fusarium nelsonii* KPJ-2, which is identified from coastline sediment, used for production of glutaminase by SSF which is optimised by using RSM approach (Soren et al., 2020).

3.7 Enzyme extraction and purification:

The overall 80 mg of protein was obtained after ammonium sulfate precipitation of 850 ml cell free medium having 44.20 U/mL enzyme activities. Ion exchange chromatography shows peaks having protein content as well as enzyme activities were confirmed the presence of enzyme in these fractions shows 17.00 U/mg of protein.

Amongst all studied bacterial sp. production yield obtained of *Bacillus halotolerance* PHN1 shown to have slightly less than another reported *bacillus sp.* Like *Bacillus cereus; which have* 19.64 U/ml individually (Ye et al., 2013).

3.8.1 Anti-inflammatory Activity:

The anti-inflammatory activity of crude and purified glutaminase enzymes was examined, and it is observed that the enzyme shows good anti-inflammatory activity. The

percent inhibitions of protein for crude and purified enzymes were 62.23 and 56.64 respectively, (Table 7).

Anti-inflammation is one of the significant biological properties that have been demonstrated by many metabolites obtained from microorganism, algae, photosynthetic bacteria like cyanobacteria etc. These metabolites, chemical makeup is divided into three categories: carotenoids, polysaccharides, and polyunsaturated fatty acid (Costa et al., 2015; Natarajan et al., 2016; Ianora et al. 2016; Calleja et al., 2003). Generally, aerobic glycolysis provides sufficient intermediates from a rapid tri-carboxylic acid cycle for de novo synthesis of nucleotides, non-essential amino acids (NEAAs), and fatty acids (Porporato et al., 2014). However, the "Warburg effect" is nothing but increased glucose absorption which is mostly observed in proliferating cells with altered glucose catabolism patterns i.e., in an anaerobic condition that prefers the conversion pyruvate to lactate rather than sending it for a tricarboxylic acid cycle for high energy output (Cairns et al., 2011).

3.8.2. Cell toxicity of L-glutaminase on cell lines

Cytotoxic activity of glutaminase against HepG2 Liver cancer cell line and MCF7 breast cancer cell line was determined. To Investigations of cyto-protective as well as cytotoxic potential glutaminase, MTT assay was performed. In this study, the enzyme samples were compared with standard drug 5 Fluorouracil (5FU). It is observed that crude enzyme efficiently induces cytotoxicity in HepG2 (liver cancerous cell line) and MCF7 (breast cancer cell line) at their increasing concentration than purified enzyme sample. The cytotoxic impact of crude and purified L – glutaminase sample on HepG2 and MCF7 cell lines was seen in (Figures 5,6 and 7,8) The IC_{50} value of the crude enzyme for HepG2 cell was found to have 30.29 ± 2 and 16.59 ± 0.65 µl/mL and for MCF7 is 53.26 ± 4 and 26.89 ± 0.32 µl/mL respectively. The obtained results underline the cytotoxic activity mediated by glutaminase against HepG2 and MCF7 cells. A determined IC_{50} concentration of this

enzyme will make this enzyme compatible for further activities related to anticancer investigations.

Lunt et al., (2011) stated the glutamine is the most abundant free amino acids found in the blood and is required at higher concentration in cancer cells. The uptake of glutamine is seen from extracellular fluid to intracellular cytoplasm and maintenance of intracellular glutamine concentration was achieved through glutamine transport pathways (Cheng et al., 2012). Although the state of art of most cancer cells usage of high concentration of glutamine as fuel, which is one of the crucial mechanisms of cancer metabolism but remain poorly studied (Anastasiou and Cantley, 2012). Reitzer et al., (1979) observed that the HeLa cells exhibit a higher glutamine dependence compared to other cancers and this property can be used for the deprivation of glutamine in blood stream by using any either mechanism and may leads to cancer cells to undergo programmed cell death. Angiogenesis phenomena are recognized as a key target in malignancy (Weis and Thompson, 2010). Anti-angiogenic medicines, that control formation of unusual blood vessels in transformed tissue and shown to increase the effectiveness of immunotherapy and chemotherapy and even improve drug delivery into tumors (Petrillo et al., 2018).

3.8.3. Anti-angiogenesis activity

The anti-angiogenesis effect of glutaminase was studied by using the Chick Ex-Ova CAM assay protocol. The glutaminase acts as a good anti-angiogenesis compound when compared to control results depicted in Figure 9.

The Anti-angiogenesis activity Chick Ex-Ova CAM assay was performed and confirmed that purified glutaminase showed good potential for anti-angiogenesis which suggests that it may also exert this characteristic in the treatment of cancer. The angiogenesis is the mechanism of formation of new blood vessels from existing vascular endothelium. Transformed cells/tissue also require additional supplies of nutrients and oxygen which is supplied by existing blood vessels in order to develop and advance.

3.8.4. Antioxidant activity of Glutaminase by ABTS Assay:

The antioxidant profile of glutaminase at various concentrations was evaluated by measuring the absorbance and % of inhibition. It was observed that glutaminase exhibited good antioxidant activity (70.62%) with 680.55 µg/ml IC_{50} value as compared to the standard Ascorbic acid (80.41%) with 77.12 µg/ml IC_{50} value (Figure 10).

Free radicals and ROS are found in biological systems. Antioxidants possess the ability to neutralize free radicals where these free radicals that can oxidize lipids, proteins, and nucleic acids, and turns them into a potential reason for degenerative diseases. Antioxidants serve a number of purposes as dietary supplements in prevention of disease or disorder such a cancer, myocarditis, anemia, diabetes etc. (Mousumi and Dayanand 2013; Tiwari, 2001). By product of this glutaminase enzyme, such as glutamic acid which serves important precursor for the synthesis of glutathione, worked as strong antioxidant agent *in vivo* (Nahar et al., 2017).

4. Conclusion:

The microbial flora of nature is continuously evolved to sustain in the different living habitats. They acquire the outstanding ability to produce their own metabolites to survive in their local and re-ordering their environments. The metabolites which have produced during these remoulded inhospitable conditions may have some fantastic bioactivities against these altered conditions and physiologies either natural or manmade. L – glutaminase is one of the such product and proved its pharmacological and industrial importance through various biological activities which includes antioxidant, cytotoxicity against cancer cells, ant angiogenesis, flavour production, amino acid production. etc…. Exploitation of natural sources to obtained industrially important product is need of time which ultimately results in reduction of cost and pollution problem. In present study use of agro – industrial source like wheat bran and halophilic microorganism *Bacillus halotalerance* PHN 1 gives good results

for the enzyme production under solid state fermentation which is then further optimized by statistical methods of optimization.

Declaration of Competing Interest

Authors declarer no any kind competing interests.

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Figure legends:

Figure - 1: OFAT – Screening of different sugars as C source for medium optimization: Error bars indicate standard deviation at $P < 0.05$

Figure - 2: OFAT – Screening of different N_2 source for medium optimization: Error bars indicate standard deviation at $P < 0.05$.

Figure – 3: The 3D surface and contour plots of interacting variable **A and B** for Mannitol and Wheat bran showing minimum impact on L - glutaminase activity. **C and D** for NaCl and Wheat bran showed larger effect on L -glutaminase activity. **E and F** for $MgSO_4$ and Wheat bran showed positive impact on L- glutaminase activity.

Figure – 4: The 3D surface and contour plots of interacting variable **A and B** for Mannitol and NaCl showing minimum impact on L - glutaminase activity. **C and D** for NaCl and $MgSO_4$ showed larger effect on L -glutaminase activity. **E and F** for Mannitol and $MgSO_4$ showed positive impact on L- glutaminase activity.

Figure – 5: % inhibition of HepG2 Liver Cancer cell line by crude and purified enzyme using MTT assay

Figure – 6: Impact on HepG2 Liver Cancer cell line by crude and purified enzyme using MTT assay

Figure – 7: % inhibition of MCF7 breast cancer cell line by crude and purified enzyme using MTT assay

Figure – 8: Impact on MCF7 breast cancer cell line by crude and purified enzyme using MTT assay

Figure – 9: Anti-angiogenesis activity of purified glutaminase enzyme showing good extent of anti-angiogenesis activity compared with control

Figure – 10: Antioxidant activity of glutaminase by ABTS Assay

Table:

Table - 1: Plackett Burman experimental design parameter set up for variables.

Table- 2: Experiments Run and obtained result by Plackett Burman design

Table - 3: Coded variable for CCRD of selected variable

Table - 4: Experiments Run and obtained result by CCRD of selected variables

Table – 5: ANOVA for first order model in PB design

Table - 6: Analysis of variance (ANOVA) for the experimental results of CCRD a quadratic model for optimization of glutaminase production.