**BIOTECHNOLOGY**

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Author : **BIRRU BHASKAR**

Title of the thesis : **DESIGN AND ASSESSMENT OF DYNAMIC PERFUSION BIOREACTOR FOR POLYMER GUIDED BONE REGENERATION**

Guide : **Dr. P. SREENIVASA RAO**

Degree : **Ph. D.**

Student ID No. : **701437**

**ABSTRACT**

 Bone tissue engineering aims to combine scaffolds, stem cells/osteoblasts and physiochemical factors to regenerate healthy bone tissue. The appropriate biomaterial scaffold and physical cues for enhanced bone regeneration is very essential for functional engineered bone graft synthesis. In this study, we synthesized solvent cast/porogen leached composite PLA-PEG porous scaffolds and varied the overall porosity by modulating the PEG: PLA ratio and were characterized with respective to pore size, mechanical strength, *in-vitro* degradation, and cytocompatability of human Mesenchymal stem cells (h MSC’s). We have observed that the increased PEG content in the composite scaffold increase the degradation rate and also larger pores were formed. All the scaffolds were exhibited the pore size in the range of 224-327 μm. The metabolic activity of h MSC’s on scaffolds results were indicated that all the scaffolds are cytocompatible. The cell attachment on the scaffolds also displayed, which proved that cells are well compatible and adhere on the scaffold. To assess their suitability to bone tissue engineering, MLO-A5 murine osteoblast cells were cultured and cell metabolic activity, alkaline phosphatase (ALP) activity and bone-matrix production determined (alizarin red S staining for calcium, direct red 80 staining for collagen). It was found that metabolic activity was significantly higher over time on scaffolds containing PEG, as well as significantly higher ALP activity and mineralized matrix production on scaffolds containing 25% PEG. Porous architecture and cell distribution and penetration into the scaffold were analyzed using SEM and con focal microscopy, revealing that inclusion of PEG increased pore interconnectivity and therefore cell in growth in comparison to pure PLA scaffolds. The results of this study confirmed that PLA-PEG porous scaffolds support mineralizing osteoblasts better than pure PLA scaffolds, indicating they have a high potential for use in bone tissue engineering applications. In the similar way, these composite scaffolds were tested with hMSCs. 10% of PEG showed higher cell viability and bone mineralization compared to 25% PEG and pure PLA scaffolds. 10% PEG contained composite scaffold has been chosen for bioreactor culture studies to elucidate the effect of fluid flow stimuli on osteogenic differentiation of h MSCs.

The importance of physical stimuli in relation to biochemical stimuli in deciding the fate of stem cell differentiation for its (3D) organ development is not yet fully elucidated. This holds especially true in the arena of bone tissue engineering, even though there are encouraging reports on the development of biomaterials and the role of physical cues for bone formation. Thus, there is a great need to establish appropriate *in vitro* culture conditions for directing the cellular response in development of a desired bone graft substitute. Driven by these positive reports and drawbacks, my study aims to comprehend the effect of fluid flow stimuli on bone regeneration using dynamic perfusion bioreactor. We investigated the effects of fluid flow induced shear stress (FFSS) on osteogenic differentiation of human embryonic stem cell-derived mesenchymal progenitors (hES-MP cells) cultured on large polyurethane (PU) scaffolds (30 mm diameter × 5 mm thickness) in osteogenesis induction media (OIM). Cultures subjected to flow contained significantly more metabolically active cells and higher total DNA content, as well as significantly higher ALP activity compared to scaffolds grown in static culture. The h MSCs were cultured on 10% PEG contained scaffolds in custom made perfusion bioreactor. The enhanced ALP, Ca+2 mineralization and collagen formation were noticed under dynamic perfusion than static culture. The results of this study confirmed that PLA-PEG porous scaffolds and dynamic perfusion bioreactor supported for higher bone mineralization, indicating they have a high potential for use in bone tissue engineering applications.

**Keywords:** Composite PLA/PEG scaffolds, Bone tissue engineering, dynamic perfusion bioreactor, human Mesenchymal stem cells (h MSCs), Osteogenic differentiation, physical stimuli, Bone matrix deposition.

 **BIOTECHNOLOGY**

Author : **MS. P.MADHURI**

Title of the thesis : **STUDIES ON L-ASPARAGINASE PRODUCTION FROM NOVEL BACTERIAL ENDOPHYTE OF OCIMUM TENUIFLORUM**

Guide : **Dr. R. SATISH BABU**

Degree : **PH. D**

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**ABSTRACT**

 Bioactive compounds from endophytes have been used to treat various diseases. LAsparaginase is one such endophytic bioactive biocatalyst that gained medicinal importance. In the pharmaceutical industry, L-Asparaginase is used to treat Acute Lymphocytic leukemia (ALL). L-Asparaginase is the first enzyme used to treat leukemia in humans. At present, Elspar® and Erwinase are the commercially available L-Asparaginase drugs in the market. Shorter half-life and side effects remain the drawbacks of the commercially available L-Asparaginase drugs. To overcome the drawbacks, L-Asparaginase has been isolated from endophytes of Ocimum tenuiflorum(Tulasi plant)*.* This medicinal plant, Ocimum tenuiflorum*,* was chosen from NIT Warangal, Telangana, India. L-Asparagine (L-Asn) deamination plays a vital role in ALL treatment. 20 (Bacteria and Fungi) out of 35 endophytes has been screened for L-Asparaginase production using rapid plate assay technique, in which four strains produced high amounts of L-Asparaginase. Based on the maximum pink zone formed around the colony, the novel endophyte was chosen, and it was identified as *Bacillus stratosphericus* from 16S rRNA sequencing analysis.

 The current study focuses on the maximization of L-Asparaginase production besides minimizing the L Glutaminase from *Bacillus stratosphericus,* which was isolated from Ocimum tenuiflorum. With unoptimized medium, the maximum activity observed for L Asparaginase as 7.81 IU/mL and L-Glutaminase as 6.51 IU/mL at 48 h, pH - 7.0, and temperature 25 **°**C. Process optimization studies were performed by traditional, statistical and evolutionary methods. In Traditional optimization, One-Factor-At-a-Time (OFAT) studies were implied to enhance the LAsparaginase activity and minimize the L-Glutaminase activity. It was observed from OFAT studies that the L-Asparaginase activity was increased to 18 IU/mL, and the L-Glutaminase activity decreased to 5.7 IU/mL. Plackett-Burman studies were performed to screen the process variables, which are influencing the enzyme activity. It was observed from Plackett Burman studies that time, temperature, pH, and LAsparagine were identified as significant variables influencing the enzyme activity. Statistical optimization studies were conducted to study the interactive effects of significant variables on enzyme activity. The statistical optimization studies were performed within the desired space using Response Surface Methodology (RSM). It was observed from RSM optimization that the L-Asparaginase activity increased to 25.28 IU/mL, whereas the L-Glutaminase activity diminished to 3.09 IU/mL. Evolutionary optimization studies were implied to find global optimizations, unlike statistical optimizations, where the solution is obtained within the desired space. To find global optimization, Genetic Algorithm (GA) evolutionary algorithm studies were conducted. GA optimization was employed on the quadratic equation obtained from RSM studies to find global optimal solution. The optimal concentrations of LAsparaginase and L-Glutaminase obtained from GA were 29.68 IU/mL and 0.12 IU/mL, respectively. The optimal process variables obtained from GA optimization studies were found to be Incubation time - 55 h, pH - 6.0, and Temperature – 24 °C and L-Asparagine - 2.5 g/L.

 Modelling studies were implied to study the process variable effects on the enzyme activity and to develop the best model for the predictions. In this study, modelling studies by Feedforward Artificial Neural Network (ANN) and Genetic Programming (GP) were employed on significant variables to generate model for *Bacillus stratosphericus* L Asparaginase. Using the experimental data, the network was built with 4 inputs and 2 outputs along with 10 hidden neurons. Levenberg - Marquardt backpropagation algorithm was employed to study the interactions between variables and their influence on L-Asparaginase and L-Glutaminase activity. The predicted enzyme activities were compared with the experimental data. The regression coefficient (R2) value was found to be 0.99419 from ANN, and it was higher in comparison to R2 obtained from RSM. The experimental data obtained from RSM was further studied by an evolutionary algorithm Genetic Programming (GP) to generate model based on Darwin’s survival of fittest theory. GP does not require prior knowledge of the data sets, where the results are represented in the form of trees. Multi Gene Genetic Programming (MGPP) is a variant of GP used to solve non-linear mathematical models. The model equation obtained from the GP analysis is represented in the form of the tree. Each tree represents a single gene. Best fit individuals obtained at each generation by using genetic operators were selected to get better R2. The predicted and experimental data showed good significance with R2=0.99956. From purification studies, it was understood that the *Bacillus stratosphericus* L-Asparaginase is a homotetramer with an individual molecular weight of 35 kDa. Purified L-Asparaginase sequence has been tailored using MALDI/TOF (Applied Biosystems). Due to the unavailability of the crystal structure, *In silico* studies were performed to develop model, Docking and Molecular Dynamics (MD), and simulation studies were performed to find the interactions and stability of the enzyme-ligand complex. The homology model was developed by using MODELLER 9.15v as the endophyte lacks crystal structure of L-Asparaginase enzyme and validated by dint of quality index tools. Docking studies were performed using iGEMDOCK 2.1v. In comparison, free energy binding efficiency of receptor towards L-Asparagine (L-Asn) is good with lesser energy -71.6 kcal/mol in comparison to L-Glutamine (L-Gln) having -67.7 kcal/mol. To find the stability of the docked complexes in dynamics environment, MD and simulation studies were performed using GROMACS V 4.6.5. The trajectory analysis for 10 ns shows the better RMSD, RMSF, Rg, and the average number of hydrogen bonds for complex 1 (L-Asparaginase + L-Asn docked complex). Hence, complex 1 was found to be more stable than Complex 2 (L-Asparaginase + L-Gln docked complex). It was observed that the *in silico* results are in agreement with the *in vitro* results. The anti-tumor property of the purified *Bacillus stratosphericus* L-Asparaginase was studied by MTT cytotoxic assay on U-937 cell lines. From MTT assay, it was observed that the *Bacillus stratosphericus* L-Asparaginase has anti-tumor properties towards leukemic cell lines.

**Keywords**: L-Asparaginase, Acute Lymphoblastic Leukemia, Endophytes, OFAT, Plackett-Burman, Response Surface Methodology, Artificial Neural Network, Genetic Programming, Genetic Algorithm, Purification, MTT Assay, Sequencing, Homology Modelling, Docking, Molecular Dynamics and Simulations.

 **BIOTECHNOLOGY**

Author : **Ms. P. MANASA**

Title of the thesis : **ENHANCED BIOETHANOL PRODUCTION FROM CELLULOSIC BIOMASS USING IMMOBILIZED YEAST CELLS**

Guide : **Dr. NARASIMHULU KORRAPATI**

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**ABSTRACT**

Lignocellulosic materials are important raw materials for biofuel production because of their resource, in comparison to other types of biomass. The pre treatment techniques alter the chemical structure of lignocellulosic substrates to improve hydrolysis step. To determine the overall efficiency of the designed process to convert lignocellulosic carbohydrate to ethanol, it is important to determine the composition of the lignocellulosic materials. Sunn hemp, a non-woody fiber crop was chosen to check the effect of ultrasound on its recalcitrant structure to enhance its enzymatic hydrolysis to liberate fermentable sugars. In this work, the effect of ultrasound-assisted alkaline pre treatment on *Crotalaria Juncea* biomass was studied in terms of cell wall carbohydrate composition and reducing sugar release by enzymatic hydrolysis.

 A maximum cellulose yield of 63% was achieved when biomass was treated with 10 minutes sonication, 1% NaOH at 121oC for 20 minutes. After ultrasound-alkaline pretreatment, crystallinity index of cellulose and solubilization of pentose and lignin increased from 39% to 53% leading to a significant increase of enzyme accessibility to cellulose in order to release reducing sugar. Morphological observations showed the erosion on the biomass before and after ultrasound pretreatment. Response surface methodology (RSM) was chosen to study the individual effect of variables on enzymatic hydrolysis and their interdependence effect. The quantity of reducing sugars released was optimized using RSM; experimental results validated at optimized process variables and gave 5.17 mg/ml of glucose and 2.08 mg/ml of xylose.

 Cellulase enzymes were used for hydrolysis of lignocellulosic biomass into fermentable sugars. Although cellulase enzyme has been notably considered for industrial application, but the application is limited due to their lack of reusability and stability at temperature. Enzyme immobilization was used for enhancing enzyme stability and reusability. In the present work, ferrite nanoparticles were synthesized and cellulase enzyme from *Trichoderma reesei* was immobilized through covalent binding. The synthesized ferrite particles were characterized by XRay Diffractometer. The size of the ferrite particles was observed in the range of 100nm to 121nm. Successful binding between the ferrite particles and cellulase enzyme was validated using FTIR analysis. Maximum binding around 74% immobilization occurred at 4mg/ml of ferrite loading to enzyme concentration of 20units at 60˚C, pH 5. Enzyme saccharification on ultrasound-assisted alkaline pretreated biomass showed 72% yield with free enzyme and 53% with immobilized enzyme. Fermentation of hexose and xylose sugars present in the hydrolyzate was performed using hexose and pentose adapted microorganisms. Mixed fermentation of *Saccharomyces* *cerevisiae*3288 and pentose yeast *Pichia stipitis*3507 showed higher ethanol yield compared to axenic culture. After fermentation and purification, the enhanced production of bioethanol was found to be ± 0.56 lit per kg.