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Review

A review on Enzyme Production, Optimization and Assay methods from Microorganisms- with a focus on peptidases

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Abstract

Peptidases, a subclass of enzymes, play an essential role in protein degradation by cleaving peptide bonds based on this they are into six types based on their catalytic mechanisms. They are crucial for various cellular processes, including protein turnover, cell signalling, DNA repair, immune defence, and apoptosis, and are widely studied for their applications in biopolymer degradation, medical treatments, and digestive processes. The Plackett-Burman Design (PBD) is an experimental approach used to efficiently screen multiple variables with minimal experimental runs. PBD has been applied in numerous enzyme production studies, including the optimization of culture media and physical parameters, such as pH, temperature, and inoculum concentration, to improve enzyme yield. Microorganisms such as *Aspergillus niger*, *Lactobacillus amylophilus*, and *Bacillus* species are commonly employed for the production of peptidases. These organisms can produce both intracellular and extracellular enzymes, with the extraction method varying accordingly. Each enzyme type is extracted and quantified using various assays to determine enzyme activity. The assay methods for determining the activity of proteolytic enzymes, collagenase, and glutamyl endopeptidase includes spectrophotometry, colorimetric assays, AMC fluorometry, and thin-layer chromatography were employed. Additionally, enzyme localization was studied by analyzing cell fractions after lysing cells.

Keywords: placket-burmann design, endo peptidases, spectrophotometric assay, colorimetric assay

INTRODUCTION

Enzymes are catalysts that speed up chemical reactions in living organisms without being changed in the process. Peptidases are enzymes that break the bonds in proteins and peptides. There are six types of peptidases, classified by their catalytic mechanisms: serine, cysteine, threonine, aspartic, glutamic, and metallo peptidases [1]

Peptidases are essential for protein degradation, occurring in the cytosol and lysosomes, and play key roles in cell processes like life and death. They also participate in biopolymer degradation, DNA repair, and viral defense [2].

GI proteases help digest dietary proteins in the intestine, breaking them into smaller fragments that can be absorbed [3].

Types of Peptidases

Aspartic Peptidase: Catalyzes peptide bond hydrolysis, important in processes like cheese production, flavour formation, and various medical treatments (e.g., pepsin for ulcers, renin for hypertension, and BACE for Alzheimer's disease) [4].

Cysteine Peptidase: Involved in neuroendocrine regulation and metabolism, it cleaves the amino terminal pyroglutamate [5].

Glutamic Peptidase: Functions at acidic pH and is resistant to certain microbial inhibitors, with a unique catalytic dyad of glutamate and glutamine [6].

Metallo Peptidase: Requires metal ions (e.g., zinc, cobalt) to activate water molecules for hydrolysis [7].

Serine Peptidase: Found in various organisms, including viruses and bacteria, and plays a key role in hu man health and biochemical processes [8].
These enzymes are important for various physiological functions and have medical and industrial applications [9].
The **Plackett-Burman Design (PBD)**, developed in 1946 by R.L. Plackett and J.P. Burman, is a **screening method** used to analyze the impact of design factors on a system and improve decision-making. PBD uses **orthogonal arrays** to estimate key effects in the smallest possible design, allowing for efficient screening of multiple factors with fewer experimental runs.
Key features of PBD:

Saturated Designs: PBD allows for the study of up to 7 factors with fewer observations. For example, 7 factors can be examined using just 8 observations in a **fractional factorial (27-4)** or PBD.

Efficient Use of Runs: PBD requires fewer experiments compared to other factorial designs with the same number of factors.

Projective Property: The design helps separate **main effects** and **interaction effects** in future experiments, making it easier to analyse.

Limitations: PBD doesn't estimate interactions directly and may mask first-order effects due to interactions, making it difficult to assess **lack of fit**.
PBD is a **Resolution III** design, suitable for identifying key factors, though it doesn't estimate factor interactions. It is ideal for **screening** experiments where the goal is to find significant factors with fewer runs.

Steps for Creating a PBD Design:
1. Select the components and define their levels.
2. Choose the responses to measure and generate the design matrix.
3. Randomize and perform experiments.
4. Replicate the design and create a statistical or graphical model.
5. Analyse the effects, interpret results, and draw conclusions.
6. Recommend improvements or higher-resolution designs and create verification products.

PBD is useful for initial stages of experiments where identifying primary factors is the main objective, and it offers a **cost-effective** approach to screening [10]
The Plackett-Burman design efficiently identifies critical variables in complex systems through a series of experiments. It evaluates X−1X-1 variables using XX experiments, where XX is a multiple of 4 (e.g., 8, 12). Dummy variables represent unused or negligible factors, allowing the estimation of experimental error variance. This method simplifies the optimization process by pinpointing significant variables with minimal experiments, enabling informed decision-making. The experimental plan for this method is shown in Table 1. From the Plackett-Burrman design optimized nutrient media selected based on high OD values. For this media physical parameters are optimized includes pH, temperature, inoculum level and incubation temperature. For all individual physical parameters 5-7 combinations are made the parameter which has shown highest OD values is said to optimum

parameter. With all these parameter glucoamylase produced and for this concentration of enzyme is calculated and reported. Organism used is *Aspergillus niger* for glucoamylase production (Figure 1) [15].

Table 1: Experimental plan about Plackett-burmann Design

Trials	Variables							
	A	B	C	D	E	F	G	H
1	H	H	H	H	L	L	L	L
2	H	H	H	L	L	L	L	H
3	H	H	L	L	L	L	H	H
4	H	L	L	L	L	H	H	H
5	L	L	L	L	H	H	H	H
6	L	L	L	H	H	H	H	L
7	L	L	H	H	H	H	L	L
8	L	H	H	H	H	L	L	L

Organisms

Peptidases is produced from various microorganisms as ***Aspergillus niger*** (isolated from potato) was received from the Fungal Culture Bank, University of Punjab, Lahore, Pakistan [11]. **Lactobacillus amylophilus GV6** A facultatively anaerobic, mesophilic, and amylolytic bacterial strain., Isolated from starch manufacturing waste using MRS media. **[12].** ***Bacillus intermedius 3-19, Streptomycin- resistant strain*** carrying the complete gene for glutamyl endopeptidase. ***Bacillus thuringiensis TS2-*** Isolated from a feather dumping site in Sivakasi, Tamil Nadu, India [13]. ***Aspergillus niger MTCC 281*** obtained from MTCC, Chandigarh, India.[14]. These different microorganisms used different nutrient medias and confirmed for enzyme production by various methods.

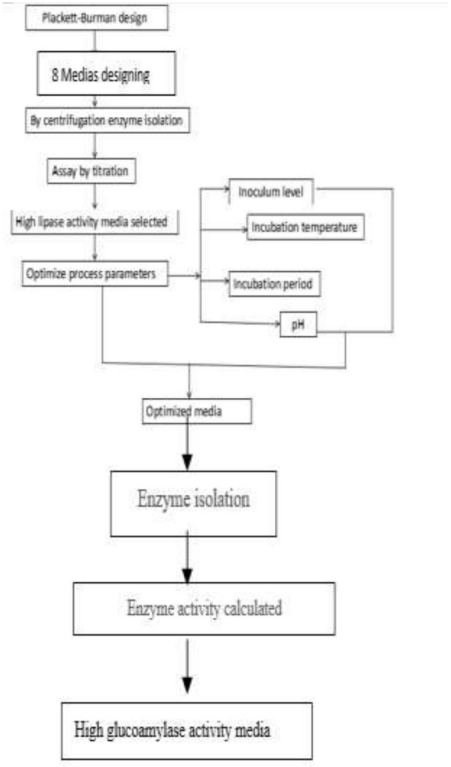

Fig 1: For placket-burmann design

MATERIALS AND METHODS

Culture Media Components

For aspergillus niger includes Malt extract (30 g/L), Agar (15 g/L), Peptone (5 g/L) Distilled water (1 L) in the laboratory stage. In the fermentation stage Media is incorporated with Glucose (various concentrations), Urea

(nitrogen source), KH_2PO_4 $MgSO_4 \cdot 7H_2O$ etc.., these medias are incubated in a using submerged fermentation process and shake flask cultures of 250ml capacity at P^H 5.3 with 120rpm rotational speed for shaking.[11].
For *Lactobacillus amylophilus* GV6 is maintained on MRS medium in laboratory and maintains stage, with few times sub-culturing by using Solid Substrate fermentation process where wheat bran (54.4% starch content), with 83% of the moistening liquid and Calcium carbonate ($CaCO_3$) for neutralizing lactic acid.[12]
For Bacillus intermedius 3-used Peptone (2.0), $CaCl_2 \cdot 2H_2O$ (0.01), $MgSO_4 \cdot 7H_2O$ (0.03), NaCl (0.3), $MnSO_4$ (0.01), pH adjusted to 8.5 used as media components in laboratory stage and for production medium Nitrogen sources- Peptone, yeast extract, tri-ammonium citrate, meat extract, maize steep liquor, and casein hydrolysate, Additional salts- $CoCl_2$, $ZnCl_2$, $FeSO_4 \cdot 7H_2O$, $CuCl_2 \cdot 2H_2O$., Feather basal medium for keratinase production [13].
For Bacillus thuringiensis TS2 used potato dextrose agar (PDA) prepared slants and sub-cultured monthly. Spores from a 7days old culture added to 0.1% Tween-80 solution this suspension used as inoculum. Broken rice of varieties as PONNI, IR-20, CR-1009, ADT-36, ADT-66 obtained from the Indian Institute of Crop Processing Technology, Thanjavur used as substrate for production of enzyme. Ammonium nitrate: 0.5%. Potassium dihydrogen orthophosphate: 0.2%. Sodium chloride: 0.1%. Magnesium sulphate: 0.1%. salt solutions used, Tween-80 (0.1% solution). Phosphate buffer (0.2 mol/l, pH 7.0). Casein (1%), Trichloroacetic acid (10%). Tyrosine standard. Sodium carbonate (0.4 mol/l). Folin and Ciocalteau's phenol reagent (3-fold diluted) are used as additional solutions.[14]

Extraction methods and Processing

For aspergillus niger after incubation, filter the mycelia are cleaned with 0.1% saline water then homogenize in water at 1500 rpm for 30 minutes, followed by centrifugation at 10,000 rpm for 20 minutes and isolate mycelium debris for dry mass measurement. Precipitate the filtrate by Mixing it with ethyl alcohol in a 1:4 ratio and store at 4°C overnight. Centrifuge at 10,000 rpm for 15 minutes to collect precipitate. Dilute pellets in 2 ml of distilled water and refrigerate for later use. These pellets are used for measurement of glucose oxidase activity [11]
L. amylophilus GV6 used for production of lactic acid using statical techniques After incubation, fermented wheat bran was extracted into water. The mixture was filtered through moistened cheesecloth. The lactic acid extract was centrifuged at 8000g for 20 minutes followed by lactic acid quantification [12].
Streptomycin-resistant B. intermedius 3-19 carries the complete gene for glutamyl endopeptidase. Recombinant strains were usually cultivated for location study 21 medium are grown for 24 hours and centrifuged and washed with tris-HCl, suspended in solution containing tris-HCl ,10mM MgSO4 and 1mm EDTA. lysozymes are added to the suspension at concentration 1mg/ml. cell lysis is caused by lysozymes which are incubated for 30mins at 37^{o}C. By which nucleic acid was sedimented and separated with centrifugation. Supernatant was taken as the fraction of cell wall proteins and the enzyme activity was determined. Response surface methodology was employed as efficient technique for optimization of culture medium. Yeast extract is used as nitrogen source, micro elements and vitamins it stimulated in production of endopeptidase production at optimal concentration pf 2% and aeration also increases the production of endopeptidase to maximal. Carbohydrates into culture medium strongly stimulated growth while supressing the biosynthesis. Increase in calcium ion resulted decrease of endopeptidase production as in bacillus intermedium strains (**13**).
In skim milk agar plates were prepared selected and isolated clear zone surrounding the colony to these colonies biochemical tests, glucose tests, fatty acid methyl ester (FAME) analysis, and 16S rDNA sequences were carried out. The bacterium was submitted to NCBI (National Centre for Biotechnology Information) with accession number Bacillus thuringiensis TS2 (FJ377887). Using this bacteria Keratinase activity performed peaked at 96 hours (52.3±1.2 U/mg and 208.5±4.91 U/ml). The lowest keratinase production was determined at 24 hours (specific activity 12.0±0.11 U/mg and total activity 50.6±1.8 U/ml). In research, was extracted from starch industry waste using the MRS medium. The culture was developed and maintained through regular subculturing in MRS medium by Screening the Plackett-Burman design for 15 variables [14].

Enzyme isolation methods

Enzymes can be produced either intracellularly or extracellularly, and the extraction process differs accordingly. Intracellular enzymes are synthesized within the cell and require cell wall disruption for isolation. This can be achieved through mechanical methods, such as sonication, or chemical methods using agents like sodium dodecyl sulphate (SDS) or lysozyme, followed by centrifugation to separate the enzyme. Extracellular enzymes, on the other hand, are secreted into the surrounding medium (broth) and are isolated by filtration or centrifugation, with the enzyme present in the filtrate or supernatant.
A strain of Aspergillus niger which showed production of extra cellular enzyme was employed in the present study to determine the most suitable production medium for the fermentative production of glucoamylase by submerged fermentation process on stationary culture [15].
To isolate enzymatic activities from a diverse library of protein variants expressed in the cytoplasm of *E. coli*, enzymes are released by repeatedly freezing and thawing bacterial colonies grown on a porous master filter. These

enzymes diffuse to an adjacent reaction filter in close contact with the master filter. Reaction substrates can either be immobilized on the reaction filter or on the enzymes themselves, which are subsequently captured on the reaction filter. Detection of the reaction products is achieved using appropriate affinity reagents. Using biotin ligase as a model enzyme, this approach demonstrated its effectiveness. Active enzymes released by the bacteria locally biotinylated immobilized target substrate peptides, enabling the sensitive and specific identification of individual catalytically active colonies.[16].

Skim milk agar medium is used for screening of protease production by fungi, the pure culture isolates were streak on the skim milk agar plates and incubated at room temperature (27°C) for four days. Then the appearance of clear zone in the medium around the colony indicates protease activity. The zones diameter was measured in mm and results are recorded. The enzymatic index (EI) expressed as R/r, which R is the degradation zone diameter and r is the colony diameter. The species that exhibits maximum clear zone selected for further identification. For the identification of isolated fungal species which shows highest production of extracellular protease were done based on macroscopic and microscopic characteristics as shown in isolate 14L3S as Aspergillus sp., isolate 6L1D Rhizopus sp, isolate 5L2S Fusarium sp, isolate 9L2D also Fusarium sp [17].

Assay:

Glucose oxidase activity was assessed using a spectrophotometric method, reducing benzoquinone to hydroquinone at 290 nm with glucose as the substrate [11].

A colorimetric assay was developed to measure proteolytic enzyme activity using DABS-Cl-labelled casein. The labelled casein, stable in powder form and in solution, showed maximum absorbance at 505 nm. Enzymatic reactions were conducted in Tris-HCl buffer with calcium at 37°C, and activity was measured by absorbance changes after stopping reactions with TCA. The assay was optimized for pH 6.53–9.00, exhibited high sensitivity (detecting nanogram levels of trypsin and chymotrypsin), and demonstrated linearity for specific enzyme-to-substrate ratios and incubation times. This method provides a sensitive and reliable alternative to fluorescence and radiolabelling techniques [16].

Collagenase is the endopeptidase enzyme, is studied for hydrolysis of the peptide substrate used is Suc-GPLGP-MCA was evaluated using both AMC fluorometry and thin-layer chromatography. The fluorescence intensity of the AMC was measured at 460 nm with excitation at 380 nm [17].

In the biosynthesis of glutamyl endopeptidase from *Bacillus intermedius 3-19* in recombinant strain of *Bacillus subtilis* has been investigated. The study determined the activity and localization of an enzyme, glutamyl endopeptidase, using Z-Glu-pNA as a substrate. Enzyme activity was measured by incubating the substrate with the enzyme at 37°C and stopping the reaction with sodium acetate buffer. The absorbance at 410 nm was used to calculate enzyme activity, with productivity expressed as specific activity per unit biomass. Growth and protease accumulation rates were also calculated.

To study enzyme localization, cells were lysed with lysozyme, and fractions (cell wall, cytoplasm, and membrane) were separated. Protoplasts were prepared by treating cells with lysozyme and sucrose. Enzyme activity was analysed in supernatants from different cell fractions. Subtilisin activity in various fractions was measured using a specific substrate [18].

CONCLUSION

Enzyme production and optimization have benefited greatly from advanced experimental methods. Techniques like the Plackett-Burman Design (PBD) enable efficient screening by identifying key factors affecting enzyme production with minimal experimental runs. Microbial strains such as *Aspergillus niger*, *Lactobacillus amylophilus*, and *Bacillus* species are cultivated using tailored fermentation processes and nutrient media to maximize enzyme yields. Enzyme isolation techniques, including sonication for intracellular enzymes and filtration for extracellular enzymes, ensure effective extraction. Analytical methods such as spectrophotometry and colorimetric assays are utilized to measure enzyme activity, providing sensitive, reliable data for further optimization and application in industrial and medical fields.

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