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MICROBIAL PRODUCTION OF BIOPLASTICS

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Abstract

Current article focus over review of biopastic such as PHA, PLA from lactic acid. However microbial production is more safer and can save money time and labor wih maximum purity.

Keywords: *PHA, LACTIC ACID, BIOPLASTIC*

1.1 Introduction:

The term bioplastics was coined by European Bioplastics, a European umbrella organisation for bioplastics. Bioplastics are biodegradable, bio based or both (European Bioplastics, 2016)

Chemically *unrelated products that are created by microorganisms (or a portion of them) under various environmental circumstances are included in the category of "***biomaterials." The bioplastics** *family is a significant group of biomaterials.*

Currently, only around 1% of the annual plastic production in the world is bioplastics which is 300 million tons.(Sabbah and Porta 2017).

These polyesters have physico-chemical characteristics similar to petrochemical plastics and are found abundantly throughout nature. They build up intracellularly in microorganisms in the form of storage granules. Typically, these polymers are created from **hydroxy-acyl-CoA derivatives using several metabolic processes.** *The monomer composition, macromolecular structure, and physical characteristics of bioplastics vary depending on their microbial source. They are all largely biodegradable and biocompatible, which is really intriguing from a biotechnological perspective.*

1.2 Microbial production of bioplastics

is an innovative and sustainable approach to produce biodegradable plastics using microorganisms.

Bioplastics are a type of plastic derived from renewable biomass sources, in contrast to traditional petroleum-based plastics that are non-renewable and contribute to environmental pollution.(Rujnić-Sokele and Pilipović 2017).

1.3 Classification

Bio-based plastics are further classified into three categories: Modified natural polymers, synthesized bio-based polymers from synthesized bio-based monomers and bioplastics from waste.

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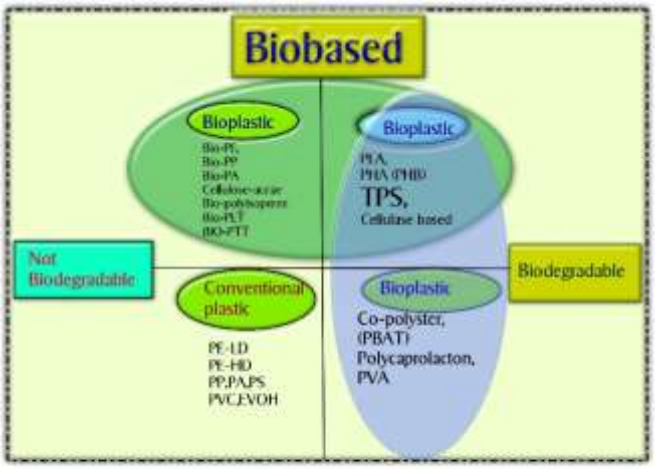

Material coordinate system of bioplastics (European Bioplastics, 2011; UK National Info Point, 2014). EVOH: ethylene vinyl alcohol; PA: polyamide; PBAT: polybutylene dipate terephthalate; PE: polyethylene; PE-HD: high density polyethylene; PE-LD: low density polyethylene; PET: polyethylene terephthalate; PHA: polyhydroxyalkanoate; PHB: polyhydroxybutyrate; PLA: polylactic acid; PP: polypropylene; PS: polystyrene; PTT: polytrimethylene terephthalate; PVA: polyvinyl alcohol; PVC: polyvinyl chloride; TPS: thermoplastic starch.

Figure 1 Common bio-based bioplastic

The five most common types of bioplastics include:

1. **Starch-Based.** *Simple bioplastic derived from corn starch. (Iyer et al. 2023; Waldrop 2021)*
2. **Cellulose-Based.** *Produced using cellulose esters and cellulose derivatives. (Sabbah and Porta 2017).*
3. **Protein-Based.** *Produced using protein sources such as wheat gluten, casein and milk.(Pooja et al. 2023).*
4. **Bio-derived Polyethylene.** *bio-derived monomers monoethylene glycol. (Rosenboom, Langer, and Traverso 2022).*
5. **Algae based. E.g. spirulina** *(Iyer et al. 2023).*
6. **Aliphatic Polyesters.** *Bioplastic can be produced from various plan pars. (Thomas 2023).*
7. **Polybutylene Succinate (PBS)** *(Barrino et al. 2023).*
8. **Microbial production of bioplastics** *typically involves the use of microorganisms, such as bacteria or fungi, to convert simple carbon sources into polymer compounds. (Filiciotto and Rothenberg 2021)*

The two main types of bioplastics produced through microbial processes are:

1.4 Polyhydroxyalkanoates (PHA):

Polyhydroxyalkanoates are a group of biodegradable polyesters that are synthesized and accumulated by certain microorganisms as intracellular carbon and energy storage materials. PHAs have properties similar to conventional plastics and can be used in various applications, such as packaging materials, disposable items, and agricultural films. The most common PHA-producing bacteria are Cupriavidus necator (formerly known as Ralstonia eutropha) and various species of Pseudomonas.

1.5 Polylactic acid (PLA): *Polylactic acid is a biodegradable and bioactive thermoplastic derived from renewable resources, such as corn starch or sugarcane. Poly lactic acid (PLA), one of a variety of biodegradable plastics, is not only generally accessible but also secure to degrade after use without harming the environment. In terms of several qualities suited for industrial use, including as mechanical, physical, biocompatibility, and processability, PLA is also on par with other common polymers like PP and PET. As a result of these qualities, PLA has emerged as the biopolymer most frequently utilized in a variety of sectors, including packaging, agriculture, and the automobile industry. The global PLA market has steadily increased thanks to its greater demand. In reality(Taib et al. 2023). One of the biodegradable and renewable thermoplastic biopolymers is polylactic acid (PLA), which is compostable. Producing fibers, flexible nonwovens, strong and durable materials (100,000 Da or above) can be done using high-molecular-weight PLA. The techniques used for food packaging, food industry waste, biopolymers' classification, the synthesis of PLA, the significance of PLA attributes for food packaging, and technologies for processing PLA in food packaging*

Microbial fermentation processes *involve converting sugars derived from these renewable sources into lactic acid, which is then polymerized to form PLA. The bacteria Lactobacillus and Lactococcus are commonly used for PLA production.*

The process of microbial production of bioplastics involves the following steps:

Substrate selection:

Microorganisms require a carbon source to produce bioplastics. Common substrates include sugars derived from crops or agricultural waste.

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Microorganisms require a carbon source to produce bioplastics. Common substrates include sugars derived from crops or agricultural waste.

Microorganism selection:

Specific strains of bacteria or fungi are chosen for their ability to efficiently produce the desired bioplastic polymer.

Fermentation:

The selected microorganisms are grown in a bioreactor under controlled conditions, and the carbon source is provided as a feedstock.

Bioplastic accumulation:

During the fermentation process, the microorganisms convert the carbon source into bioplastic, which accumulates within the cells.

Harvesting and extraction:

After a sufficient amount of bioplastic has been produced, the cells are harvested, and the bioplastic is extracted and purified.

Processing:

The extracted bioplastic can be further processed into various forms, such as pellets or films, for use in different applications.

Advantages*:*

Microbial production of bioplastics offers several advantages, including reduced dependence on fossil fuels, reduced greenhouse gas emissions, and the potential for biodegradability, which can help alleviate plastic waste and pollution problems.

Challenges in bioplastic production

However, challenges remain in terms of cost-effectiveness, scalability, and competing with well-established petroleum-based plastics in the market. Researchers and industries are continually working on improving the efficiency and viability of microbial bioplastic production to make it a more sustainable alternative to conventional plastics.

Bioplastics are plastics derived from renewable biomass sources such as plants, bacteria, and algae. *One common bioplastic is polyhydroxyalkanoates (PHA). The metabolic pathway for PHA production in bacteria involves several steps:*

Substrate Uptake: *The bacteria take up renewable carbon sources, such as sugars or lipids, from the environment. These carbon sources serve as the building blocks for bioplastic production.*

Glycolysis: *In the glycolytic pathway, glucose or other sugars are broken down into smaller molecules, producing energy (ATP) and precursor molecules (e.g., acetyl-CoA).*

Fatty Acid Biosynthesis: *In some bacteria, the acetyl-CoA generated from glycolysis is converted into fatty acids through the fatty acid biosynthesis pathway.*

2. PHA Biosynthesis:

The fatty acids or other precursors are then converted into polyhydroxyalkanoates through a series of enzymatic reactions. This step involves enzymes such as **PHA synthase**, *which polymerizes the monomers to form the bioplastic.*

Inclusion Body Formation: *PHA is accumulated as inclusion bodies inside the bacterial cells. These inclusion bodies can be harvested and processed to obtain the bioplastic material.*

Environmental Factors: *Various environmental factors like nutrient availability, temperature, and pH can influence the efficiency and yield of the bioplastic production process.*

It's important to note that the specific metabolic pathway and the intermediates involved may vary depending on the type of bioplastic being produced and the microorganisms used in the process. Additionally, different bioplastic production methods might involve slightly different pathways or steps.

Lactic acid fermentation

Homofermentative process

Homofermentative bacteria convert glucose to two molecules of lactate and use this reaction to perform substrate-level phosphorylation to make two molecules of ATP:

glucose + 2 ADP + 2 Pi → 2 lactate + 2 ATP

Heterofermentative process

Heterofermentative bacteria produce less lactate and less ATP, but produce several other end products:

glucose + ADP + Pi → lactate + ethanol + CO2 + ATP

Examples *include Leuconostoc mesenteroides, Lactobacillus bifermentous, and Leuconostoc lactis.*

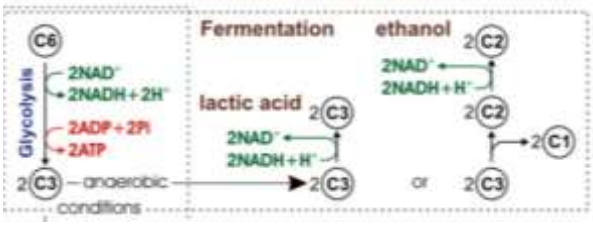

Figure 2 metabolic pathway

Ref: wikepedia.

Conclusion

Microbial production of lacic acid has much scope oday and can

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Blood Stain Pattern Analysis: A Comprehensive Review of Methods, Reliability of Computerized Analysis, and Future Advancements

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Abstract

Bloodstain Pattern Analysis (BPA) stands as a precious tool in the crime scene investigation and reconstruction, providing invaluable insights into the circumstances surrounding bloodshed. This comprehensive review delves into the profound significance of BPA, charting its evolution over time while spotlighting recent breakthroughs and identifying potential areas for further research and development, especially within the domain of digital technology.

The fundamental essence of BPA lies in meticulously analyzing the form and dispersion patterns of bloodstains found at crime scenes., which aids investigators in comprehending the deposition of blood on evidence and shedding light on the movements and positions of the individuals and objects involved during the incident. Notably, BPA facilitates differentiating between accidents, homicides, and suicides, as well as identifying bloodstains left by criminals, thus playing a crucial role in ascertaining the circumstances surrounding an incident. Elements like blood velocity and the nature of the impacted surface significantly influence the size and shape of bloodstains, imparting crucial clues for an accurate crime scene reconstruction.A noteworthy application of BPA is in impact spatter analysis on hands, which holds importance for forensic ballistic examiner to recognize the firearm. Studies are discussed, related to sophisticated image processing and computerized techniques for BPA to scrutinize their reliability and accuracy. Cutting-edge advances have been witnessed in the field, including the application of Raman spectroscopy, automated methodologies, and the utilization of software programs like the FARO Scene program. These advancements have substantially elevated the efficacy and capabilities of BPA, empowering forensic investigators with enhanced analytical tools. Despite the remarkable strides made in blood spatter pattern analysis, the review underscores the abundant potential for continued research and development. In particular, refinements in methods for dating dried blood pattern and the evolution of automated techniques for crime scene reconstruction are prime avenues worthy of exploration.

Keywords: *Blood, Spatter Pattern, Blood Spatter Pattern Analysis (BPA), Computerized examination, Automated analysis, Impact pattern, Recent Advancements*

1.1 Introduction:

Bloodstain pattern analysis (BPA), is a technique used to reconstruct the events that led to bleeding. By understanding how blood ended up on a wall or other surface, it is feasible to ascertain whether a crime has occurred and if the blood present can serve as evidence of that crime. Bloodstains are classified into different types, each providing a clue to the crime scene. Three primary categories of bloodstains include passive, transfer, and impact stains (1,2)

BPA offers investigators valuable insights in various aspects. Bloodstain pattern analysis is instrumental in identifying the points of convergence and origin of bloodstains, discerning the direction and type of impact responsible for the bloodstain or spatter, and understanding the mechanisms that generated patterns. Additionally, this analysis aids in comprehending the deposition of bloodstains on evidence, as well as potentially revealing the positions of the victim, assailant, or objects at the scene and their potential movement after the bloodshed occurred. By examining spatter patterns, transfers, and other indicators, analysts can classify seemingly haphazardly distributed bloodstains at a crime scene, and reconstructing the sequence of events that transpired after the bloodshed occurred. Moreover, bloodstain pattern analysis can corroborate or challenge statements provided by the accused, offer supplementary factors for estimating the postmortem interval, and correlate with other pertinent laboratory and pathology findings (3).

The form of a bloodstain pattern is significantly impacted by the force applied to propel the blood and the characteristics of the surface upon which it lands. In criminal investigation, the primary responsibility of a bloodstain pattern analyst is to aid in reconstructing events of a reported incident that might

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have resulted in the presence of stain patterns at a crime scene, including those on clothing items discovered at the location. When blood evidence is collected and stored correctly, it can establish a compelling link between an individual and a criminal act. This particular form of physical evidence demands that the analyst possesses the capacity to identify and comprehend patterns to ascertain how they came into existence. Utilizing the size, shape, distribution, and placement of bloodstains, analysts formulate conclusions regarding the precise sequence of events, as well as potential occurrences, during the incident (1) Suppose the cause of death is established as blunt force trauma to the victim's head. In that case, the blood spatter's pattern and volume should align with the notion of a blunt instrument striking the individual's head once or multiple times. When the spatter is blood that has been expelled from the body, the analyst will review pathologist reports to identify any injuries that might result in blood being present in the victim's nose or respiratory system. If the analyst does not find any blood in these areas, it may allow them to eliminate expiration as a potential cause (2).

Types of Tests for Detection of Bloodstain

Before blood can be classified and analysed, it must first be discovered and verified. The reason analysts often rely on the ABC Approach (Appearance, Behaviour, and Context) to bloodstain verification is due to the impracticality of analysing the entire scene for the presence of blood. In cases where chemical tests are required, their application depends on whether the suspected blood is visible or not. Detecting blood can be challenging, particularly when offenders attempt to conceal it by employing cleaning solutions (4)

To further examine blood stain first we have to identify if it is blood or not. There are various methods for detection of blood. The Kastle-Meyer test is a presumptive test that employs the chemical indicator phenolphthalein to potentially detect the presence of haemoglobin (4,5). Although the Kastle-Meyer test is quick, it cannot be directly applied to a bloodstain as it can potentially destroy DNA evidence. As a result, it is most effective to utilize the Kastle-Meyer test when there is an ample amount of blood evidence available(4). Hydrogen peroxide can be employed independently as a valuable method for detecting blood. When hydrogen peroxide comes into contact with blood, the catalase enzyme present in blood facilitates the production of oxygen bubbles. This reaction is classified as an oxidation process, resulting in the formation of visible white foam, making it a discernible indication of presence of blood. Despite its limited sensitivity, hydrogen peroxide remains beneficial due to its cost-effectiveness and its non-interference with DNA, even after being exposed for up to one month (4–6).

Hydrogen peroxide is used to treat both visible and difficult-to-detect blood. It has the capability to alter the color of blood, and the contrast is enhanced by the white foam it produces, making it useful for detecting blood on dark or difficult-to-see surfaces. However, hydrogen peroxide is not practical for detecting small bloodstains because it cannot detect minute amounts of blood. Infrared imaging is an effective technique for detecting bloodstains on black surfaces, and it can detect even the smallest traces of blood. Luminol is widely employed as one of the most prevalent techniques for blood testing. The phenomenon of light emission is observed when a solution containing luminol (also known as 5-amino-2,3-dihydro-1,4-phthalazine-dione or 3-aminophthalhydrazide) and hydrogen peroxide is applied to dried bloodstains(7). Upon reacting with haemoglobin, luminol emits a blue-tinted chemiluminescence (light) in the absence of external light(7). Luminol has demonstrated efficacy in detecting blood covered with many layers of paint and can even identify blood in damaged samples while preserving DNA. Due to its high sensitivity, luminol is the most practical blood testing method. Nevertheless, the outcomes of luminol testing can be influenced by factors such as age of luminol, its preparation, storage conditions, and the technique used for chemiluminescence measurement, which may result in inconclusive or unsatisfactory findings. In a particular study, it was discovered that even blank samples emitted low levels of chemiluminescence. Further research holds the potential for developing a more cost-effective, sensitive, and reliable identification technology (4,8).

Types of Blood Stain Patterns

According to research, bloodstains can generally be classified into seven categories: passive, projected, impact, arterial spurt, cast-off, wipe patterns, and transfer bloodstains. Each category provides valuable information to investigators about the events that led to the bloodshed (2).

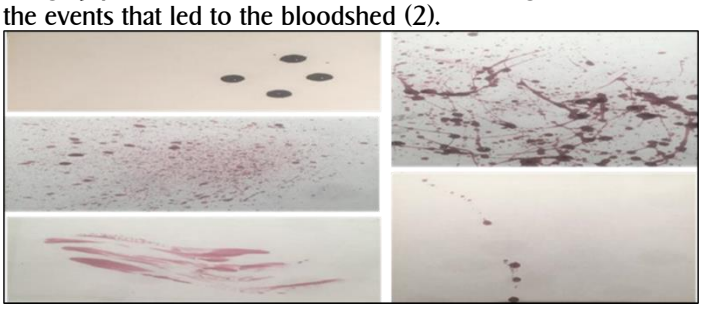

Fig.-1: Frequently encountered types of blood spatter and transfer observed(4).

In Fig. 1 (Top Left) Low-velocity drops caused by gravity can be readily distinguished by their round and smooth edges. (Left middle) A thin mist of blood, usually as a consequence of gunshot wounds, produces high-impact velocity spatters. (Left bottom) Swipes are a type of bloodstain that can result from various factors, including contact with hair and hands. (Bottom Right) A blend of big and tiny drops arises from blunt-force injuries in medium-velocity impact splatter. (Top right) Medium-velocity spatters are often accompanied by cast-off blood patterns due to the blunt-force impact. Cast-off occurs when blood flows off a murder weapon, like a hammer or bat(4).

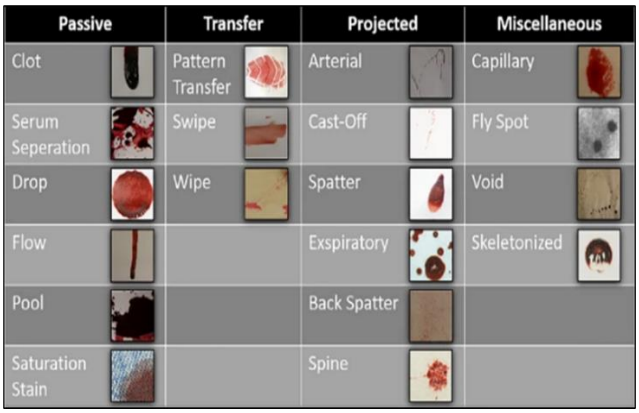

Fig.-2: One of the prevalent classification systems for bloodstain patterns that distinguishes between passive, transfer and projected traces created actively. Traces that are challenging to categorize are placed in miscellaneous category (9).

During the scrutiny of bloodstain patterns on the hands, the forensic examiner attaches crucial significance to impact spatter. When blood is impacted, it can generate either forward or back-spatter stains, as the force causes the blood to break into smaller droplets. Back-spatter patterns occur during shooting incidents when blood droplets are expelled from the entry wound and impact the front areas of the trigger hand, weapon, and supporting hand. These spatter patterns are classified as "medium to high velocity spatter." Rounded spatter marks are created when droplets impact a surface perpendicularly, while elongate marks, and "exclamation stain," occur due to low-angled impacts (10).

Examination Methods and Computerized Analysis Reliability

BPA, a specialized discipline, focuses on examining bloodstains discovered at crime scenes involving violence. The application of force on a body causes blood to fall passively, leading to the creation of spatter marks. In 1895, Dr. Eduard Piotrowski from the University of Krakow in Poland conducted the pioneering and systematic study on blood spatter. He used white sheets and dead rabbits to conduct experimental research and concluded that bloodstain patterns often reflect the second strike, not the initial blow. This led to further studies in BPA (11,12). The image analysis approach offers an automated alternative that can potentially be utilized at crime scenes. Professionally educated forensic specialist can analyse individual blood spots using a well-established but exceedingly time-consuming procedure. Upon impact, this procedure computes the body's 2nd dimension position on the floorplan. Comparison trials were conducted to confirm the algorithm's accuracy(13)

(10) analysed the bloodstain patterns on the deceased's hands, the position and orientation of the hands, and consequently the firearm, were determined. For this purpose, blood spatter stains on the hands were directly examined at the scene in five suicide cases involving gunshots. Close inspection of the spatter markings provided forensically relevant findings about forensic reconstruction in all cases. Hence, in shooting incidents, it is recommended to investigate and document blood-spatter findings on the hands before examining them for gunpowder residue or preparing them for transportation.

Researchers conducted a study to explore the attributes of aerosols of blood generated during physiological experiments. The participants exhaled small aerosols under various conditions, such as through straws, spitting, and making mouth sounds. Additionally, the study involved a semi-prone participant spitting through a haemorrhaging mouth or snorting through single nasal orifice. Each action resulted in the production of numerous droplets of varying sizes, with the majority being less than 1 mm in diameter. The characteristics of exhaled aerosol of blood are elucidated by utilizing the fluid mechanics and the established mechanics of airflow-induced aerosol (14). The study of formation of blood aerosol in different situations will in turn help in knowing cause of blood spatter pattern in medicolegal cases. In a study saliva test methods were compared to know the suitable method for identifying coughed or spat out blood spatter. Among the various test kits used for stain identification, the RSID (Rapid Stain Identification)-kit demonstrated the highest sensitivity and remained unaffected by the presence of blood, even in cases where saliva was mixed with blood. However, The RSID-Saliva test results for detecting saliva in blood must be interpreted within a 10-minute timeframe. Both the starch gel diffusion method and the Phadebas sheet exhibited low sensitivity, and the testing process was time-consuming (15).

(16) formulated a theoretical model to anticipate and comprehend blood-spatter patterns that occur as a consequence of a gunshot. The occurrence of rearward spatter of blood is associated with the Rayleigh-Taylor instability in blood expelled into the surrounding air. This enables the assessment of the initial distribution of sizes of drops and velocities. The movement of multiple droplets in the air is examined by employing governing equations that consider the gravity and air drag. The model's forecasts are compared with experimental data concerning back spatter generated by a gunshot impacting blood soaked in sponge.

In BPA, the analyst might need to make estimations regarding whether a particular stain over a fabric originated from a specific location, serving investigative purposes. Experiments have demonstrated that the maximum distance a blood droplet can travel exhibits significant variation, ranging from under 1 meter to over 10 meters. It is recognized that interpreting stains on porous materials, like textiles, is more challenging compared to stains on non-absorbent surfaces due to the presence of wicking. Various fluid dynamic spatter tests have been conducted and a fluid dynamics model has been developed to characterize blood droplet trajectories. The tests were carried out using pig blood, which has well-understood properties. Upon comparison, the experimental results were found to align with the numerical predictions, demonstrating agreement between the two. The findings are displayed in a chart applicable to crime scene reconstruction, designed for user-friendliness and requiring only basic knowledge of fluid dynamics to interpret effectively (17)

Cast-off splatter patterns are recognized by linear trails of circular stains. These unique patterns emerge when centrifugal forces expel droplets from a swinging object coated in blood or another liquid. In this paper, a technique for reconstructing swing or movement of the object is introduced. The method relies on Euclidean geometry and

stain analysis, incorporating a three-dimensional statistical probability zone to depict the reconstructed swing. Reconstruction method can reconstruct multiple intersecting or adjacent swings, as demonstrated by simple numerical examples. The study also assesses the reconstruction method's robustness, spatial convergence, and computing time (18).

Under laboratory circumstances, an experiment was undertaken to better understand blood stain production using Awlata dye on university grounds. The researchers employed Awlata (Alta), an Indian dye commonly used for womens, to create synthetic bloodstains. This allowed them to gain insights into the formation of bloodstains at various heights and their connection to spines and satellite stains. As a result, a link between the production of blood stains and height was discovered. This discovery utilizing fictitious blood stains might aid future research (1)

Future Advancements

In the study, researchers examined the precision and consistency of origin estimation by utilizing the FARO (Frasier and Raab Orthopaedics) Scene program and the FARO Focus3D laser scanner. They generated five impact patterns for each of three combinations of distances from the floor (designated as z) and the front wall (designated as x). A total of fifteen spatters were generated using a custom impact rig, and subsequently, they were scanned with the laser scanner, photographed using a DSLR camera, and processed with the Scene software. This method was found to be accurate and reproducible, with results comparable to those produced by other digital software. Determining the point of origin of an impact pattern is a crucial aspect of crime scene reconstruction (19). A novel approach is formulated to enhance the accuracy of determining the area of origin. Traditionally, it is based on the assumption of straight trajectories for spattered drops, lacking a precise method to evaluate the level of uncertainty in the resulting X, Y, and Z coordinate estimation. The research focuses on addressing these two issues. The newly developed method incorporates principles from fluid dynamics and statistical uncertainty. The study's spatter patterns are accessible in a high-resolution open-access dataset, enabling comparisons with various reconstruction or stain selection approaches to the one described in this research (20).

A study has proposed a patented technique for dating drying blood pools for forensic applications. This technique is based on the morphological changes that occur in the blood pool as it dries. The study also discusses the evaporation process of gels, which exhibit similar disordered fracture patterns to blood. The objective is to identify dependable patterns capable of offering insights into the evolution of a blood pool over time. An empirical model is formulated, establishing a connection between the final dried blood patterns and the mechanism responsible for their creation. This model holds potential significance in the field of forensic bloodstain pattern analysis. Additional investigations are necessary concerning pools of diverse shapes and sizes, considering the effects of drying at various temperatures and humidity levels. The research could lead to the development of a model for blood pool evaporation, which could be used to track the drying front on a crime scene using a reference table(6). Raman spectroscopy offers a non-invasive method for analysing bloodstains and detecting specific kinetic changes in aged bloodstains for a period of up to two years. A novel classification modelling approach was utilized to initially distinguish bloodstains as blood rather than any other bodily fluid. The overall success rate for correctly identifying all stains as blood was 89%, and there was a perfect 100% identification of blood for stains aged up to one month. The spectral changes observed over time were in line with well-known biochemical processes that naturally occur as blood ages. These differences were significant enough to enable differentiation and predictions of time-since-deposition on a time scale ranging from hours to years(21)

Automated methods are gaining traction as a prominent research focus in the domain of forensic science. In a particular study, it was revealed that 20% of blood pattern analysts made erroneous identifications when tasked with identifying a blood spatter. Nonetheless, it was evident that the majority of analysts who made incorrect identifications were inexperienced and had limited training or expertise in bloodstain pattern analysis. Although professionals may not be immune to errors, there is a distinct correlation between expertise and accuracy when identifying blood spatter. Mistakes in recognizing blood spatter evidence are prevalent when the crime scene exhibits overlapping stains, rendering each individual stain challenging to differentiate (22,23). Automated method has been developed to classify bloodstain spatter patterns into two categories: gunshot and blunt impact. This approach employs machine learning techniques to categorize the data and relies on a dataset of 94 publicly available blood spatter patterns. In the study, a random forests approach was utilized to determine the most influential features for classification. The findings indicated that classification accuracy declines as the distance between the blood source and the target surface increases. At distances of 30 cm, the model attained a remarkable accuracy of 99%. It demonstrated a slightly lower accuracy of 93% at distances of 60 cm and 86% accuracy at distances of 120 cm. The presence of the muzzle gases can also affect classification accuracy, as shown by results from ten additional backspatter patterns (24). The examination featured a specific case study in which a man experienced a shooting incident followed by decapitation, resulting in a varied assortment of blood spatter patterns. These patterns encompassed high-velocity impact spatter from the bullet, arterial spurts arising from the decapitation, and low-velocity drips and smears indicative of dragging. Due to this, crime scenes can exhibit a combination of various blood spatter types. In one study, an image-processing method was employed to differentiate blood spatter into local and globular components, assigning each a distinct numerical value. In the future, statistics could be employed to promptly differentiate between different bloodstain patterns based on this data, but further research is required to explore additional types of blood spatter. Another method that allows analysts to examine more accurate and detailed crime scene images is multi-resolution 3D scanning(22,23).

Discussion

In the field of bloodstain pattern analysis, the crucial task is to determine the source and properties of bloodstains, as this plays a vital role in comprehending the events that transpired

at the crime scene. Nevertheless, conducting a comprehensive analysis of the entire crime scene for the presence of blood is impractical. Therefore, the ABC Approach (Appearance, Behaviour, and Context) is employed as a valuable tool to concentrate on potential bloodstains that require verification(4). To examine bloodstains, first, we must detect if they are indeed blood. The Kastle-Meyer test detects haemoglobin but risks DNA destruction. Hydrogen peroxide is cost-effective and doesn't interfere with DNA but has limited sensitivity. Luminol is highly sensitive and widely employed, detecting minute traces even in low-light conditions(5,6).

Categorizing bloodstains into passive, projected, impact, arterial spurt, cast-off, wipe patterns, and transfer types provides vital data for reconstructing crime scenes(2). Forensic examiners give particular significance to impact spatter patterns when investigating bloodstains on the hands of shooting victims(10). It is recommended to prioritize examining and documenting blood spatter patterns on hands before checking for gunpowder residue or transporting evidence(10). Understanding exhaled blood aerosol characteristics is essential for interpreting spatter patterns in medicolegal cases. Fluid mechanics and airflow-induced aerosol mechanics knowledge help determine the origin and pattern of blood spatter in forensic investigations(14). The RSID-kit shows high sensitivity in detecting coughed or spat-out blood spatter, remaining unaffected by blood presence, even with saliva mixed in. Interpret RSID-Saliva results promptly within 10 minutes. However, the starch gel diffusion method and Phadebas sheet exhibit low sensitivity and are time-consuming(15).

Interpreting bloodstains on porous materials like textiles poses wicking challenges. Fluid dynamic spatter tests resulted in an accurate model for blood droplet trajectories, aiding crime scene reconstruction. Experimental and numerical results validated the model's effectiveness(17). A user-friendly chart based on these findings helps investigators interpret blood spatter patterns effectively with basic fluid dynamics knowledge(17). Cast-off splatter patterns, recognized by linear trails of circular stains, indicate droplets expelled from a swinging, blood-coated object. Euclidean geometry and stain analysis reconstruct the object's swing or movement, enhancing crime scene analysis(18).To study bloodstain formation at different heights and their relation to spines and satellite stains, researchers used Awlata (Alta), an Indian dye simulating synthetic bloodstains. This experiment revealed a connection between bloodstain production and height, opening avenues for further research with simulated bloodstains(1).

FARO Scene program and FARO Focus3D laser scanner show an accurate and consistent origin estimation, comparable to other digital software(19). A proposed patented technique for dating drying blood pools in forensics has significant potential, establishing a connection between final dried blood patterns and their creation mechanisms(6). Further investigations should explore pools of different shapes and sizes, considering drying effects at various temperatures and humidity levels. This research could lead to a model, aiding crime scene investigations with a reference table to track the drying front(6). Raman spectroscopy offers a non-invasive approach to analyse bloodstains and detect specific kinetic changes in aged bloodstains for up to two years(21). A novel classification modelling approach effectively distinguished bloodstains from other bodily fluids with an 89% overall success rate. For stains aged up to one month, blood identification was 100% accurate, showing great potential for enhancing forensic investigations' efficiency(21). Automated methods are gaining momentum in forensic science research, aiming to improve accuracy and efficiency. One study found that 20% of blood pattern analysts made erroneous identifications when identifying blood spatter(22,23). To address this, an automated machine learning method was developed to classify bloodstain spatter patterns into two categories: gunshot and blunt impact. Using a dataset of 94 publicly available blood spatter patterns, this approach achieved remarkable 99% accuracy at 30 cm distances between the blood source and target surface(24). In another study, an image-processing method differentiated blood spatter into local and globular components, assigning distinct numerical values. Statistics could promptly differentiate between various bloodstain patterns based on this data, showing promise. Further research is needed to explore additional types of blood spatters(22,23). Multi-resolution 3D scanning offers valuable crime scene images for accurate analysis. This technology provides a comprehensive perspective, enhancing the understanding of blood spatter patterns(22,23).

In conclusion, BPA forms an essential role in criminal investigations, offering invaluable insights into the events surrounding bloodshed (1,10,17). By examining the size and arrangement of blood spatter stains, BPA can assist in establishing the positions of individuals involved in a crime scene, the type of weapon utilized, and distinguish between various incident types, including accidents, homicides, and suicides. The integration of cutting-edge digital software, such as the FARO (Frasier and Raab Orthopaedics) Scene program and 3D imaging techniques, has primarily augmented BPA's capabilities in recent times (20). The studies related to blood aerosols formed in various situations will help in finding cause of Blood spatter pattern. Although considerable strides have been made in qualitative and quantitative BPA utilizing image processing and computerized methods, there remains a necessity for further exploration and validation of automated approaches(22). Additionally, research efforts should be directed towards refining methods for dating dried blood to enhance the temporal accuracy of crime scene reconstruction using BPA (13). Establishing of reliability and precision in the mathematical models for BPA with help of further research is essential to bolster their applicability in forensic investigations. With the relentless advancement of technology, the future of BPA holds promising potential, especially within the realm of digital platforms. Uninterrupted research and development endeavours will assuredly refine and broaden BPA's scope, yielding even more comprehensive and precise outcomes in crime scene analysis. In essence, the study of bloodstain patterns continues to evolve as a dynamic field with ongoing prospects for progress and innovation.

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Economical Cellulase Production under Optimized condition in Batch condition using Water Hyacinth (WH) Waste

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Abstract

Currently research attempted for enhanced cellulase production using Water hyacinth waste. We found that . Trichoderma reesei selected with WH for cellulase production. Optimization the effective conditions for production of CMCase temp-40 ºC, pH-5, tween80 3%, WH7.5%, nitrogen source 1% as peptone, incubation time 7days, inoculum 5% at rotation 100 rpm for FPase production only two condition was difference inoculum was higher (10%) and rotation speed was 150 rpm. Production of cellulase for CMCase was 52% more increase activity observed after media optimization and similar for FPase was 84% increase activity observed after media optimization.

Keywords: *CMCase, FPase, Cellulase, optimization, DOE, Trichoderma reesei selected, Biofuel*

1.1 Introduction:

Constant depletion of fossil fuel is of great concern. Therefore only alternative is large scale production of biofuel to meet its demand for blending purpose. High Cellulose available from various agricultural waste around the Globe (around 10^{12} tons of the total annual biomass) may be an ideal substrate for its conversion into bioethanol (Bhatia and Johri 2015). Unfortunately, conversion of crystalline cellulose into amorphous one is a major hurdle due to presence of recalcitrant, which requires judicial selection of pretreatment techniques. Cellulase attacks in stepwise manner for degradation of cellulose first into disaccharides (cellobiose) which is further degraded by beta glucosidase (cellobiases) into monomeric form.

Currently research attempted for enhanced cellulase production using Water hyacinth waste

Reviews of literature

Pre-treatment is one of the important criteria in reducing the lignin content present in lignocellulosic biomass (Bhatt & Shilpa, 2015; Jung, Kim, Kim, & Kim, 2013). Chemical pretreatment have been done by various workers like inorganic chemicals such as HCl and NaOH and organic chemicals such as lactic acid, citric acid e.t.c. (Anita Singh and Bishnoi 2013). The feature of an effective pre-treatment is, low degradation of hemicellulose, low inhibitor formation after cellulose hydrolysis, low energy requirements and cost-effective (Martinez et al. 2001). There are three types of preferred pre-treatment strategies such as 1- physical, 2-chemical, 3-physicochemical and biological pre-treatment. Most often physical pre-treatment such as breaking of larger cellulose particle is essential before application of other methods such as chemical or biological. Some chemical methods such as Alkaline pre-treatment is preferred over acid pre-treatment on the basis of its capability to dissolve lignin rapidly at room temperature, with less degradation of hemicellulose (Kumar, Singh, and Ghosh 2009) and thus high recovery of glucose yield (Nigam 2002).

In one report cellulase production have been done by using waste WH from Rhizopus oryzae MTCC 9642 in submerged and solid state fermentation and media condition were optimized. In optimised condition FPase activity reported in submerged fermentation was substrate concentration (w/v) 1.25%, pH 7.32, and temperature 25 ºC while in SSF mode the substrate concentration. 0.5%, pH 6.0 and temperature 18 ºC (Karmakar and Ray 2011).

In an another work Deshpande et al., 2009 optimised the cellulase production by Trichoderma reesei from water hyacinth. In the optimised condition cellulase activity reported

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was 0.22 ± 0.04 IU/ml (approximately 73.3 IU/g cellulose) after 15-days with specific activity of 6.25 IU/mg protein. Saccharification rate of this enzyme was around 28.7% (1% water hyacinth) with 1.2 IU/g cellulase enzyme. With mixture of microbes such as T. reesei QM 9414 mutant and P. chrysosporium, high cellulase production was reported with WH as compared to wheat bran in SSF mode where liquid/solid ratio was 2.5 with 10 days incubations. However beta glucosidase ceased using WH as a substrate.(Deshpande, Nair, and Khedkar 2009). Cellulase production has been reported by various cellulolytic microbes as briefed in Table 2.2. One of the extensively used fungi for cellulase production is T. reesei. Tangnu,1981 reported production of both cellulases and hemicellulase using Trichoderma reesei RutC-30in different cellulose concentrations (1, 2.5, and 5.0%) in submerged conditions (Tangnu, Blanch, and Wilke 1981). Devi and Kumar (2012) isolated celluloytic microbes from local industrial wastes like paper, timber and saw mills e.t.c which resulted in screening of Trichoderma reesei from paper waste. The maximum enzyme was produced was 3.9 IU at 45°C and pH 5 after 7th day. (Devi and Kumar 2012,).

Table 1 Cellulase producing fungal strain

Fungal strain	Reference
P.chrysogrnum	(Chinedu et al. 2007)
A.heteromorphus	(Ajay Singh, Kuhad, and Ward 2007)
Botsysatra	(Shoemaker and Brown 1978)
A.fumigotus	(Hamilton and John Wase 1991)
Chaetomium sp.NIOCC36	(Ravindran, Naveenan, and Varatharajan 2010)
Fomitopiss sp. RCK 2010	(Deswal, Khasa, and Kuhad 2011)
Scopulariopsis	(Kodali & Pogaku, 2006)
Neuraspora	(Sharada, Venkateswarlu, Venkateshwar, & Rao, 2013)
Tricothecium roseum	(Shanmugam, Mani, & Narayanasamy, 2008)
A.candidus	Milala et al.,2009 Milala,M.A., Shugaba,A., Gidado,A., Ene,A.C. and Wafae,J.A., 2005. Studies on the use of agricultural wastes for cellulase enzyme production by Aspergillus niger. Res. J. Agr. And Bio. Sci., 1(4): 325-328.
A.japonicus	Sharma et al., 1985 Sharma,A., Milstein,O., Vered,Y., Gressed,J. and Flowers,B.M., 1985. Effect of aromatic compounds on hemicelluloses degrading enzymes in Aspergillus japonicas. Biotechnol. Bioeng., 27: 1095-1101.
T.aureoviride	Mercedes et al.,2001 93. Mercedes Zaldivar, Juan Carlos Velasquez, Ines Contreras, Luz Maria Perez., 2001. Trichoderma aureoviride 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and / or biocontrol. Electronic Journal of Biotechnology, 4(3): 0717-3458.
Pesalotia	Metha et al.,1975 Metha,P., Vyas,K.M. and Sakeena,S.B., 1975. Effect of native carbon sources and pH on the cellulases of Alternaria solani and Aspergillus terreus. Science and culture, 41: 401.
Stachy	Mandels et al.,1976 Mandels,M. and Sternberg,D., 1976. Recent advances in cellulases technology. J. Ferment. Technol., 54(4): 267-286.
Polmarum	Kassim et al., 1983 Kassim,E.A., 1983. Cellulase enzyme from Aspergillus niger. J. Fac. Sci. Riyadh Univ., 16.
Trichoderma reesei	Muthuvelayudham, et al.,2006 Muthuvelayudham, R. and Viruthagiri, T., 2006. Fermentative production and kinetics of cellulase protein on Trichoderma reesei using sugarcane bagasse and rice straw. Afr. J. Biotechnol., 5 (20): 1873-1881.
A.niger	Gokhle et al.,1984 Gokhale,D.V., Puntamberkar,U.S., Vyas,A.K., Patil,S.G. and Deobagkar,D.N., 1984. Hyper production of β-glucosidase by an Aspergillus species. Biotechnol. Lett., 6: 719-722.
Tricothecium reseum	Prashath et al., 2008 Prashanth Shanmugam, Madhumathi Mani and Mathivanan Narayanasamy., 2008. Biosynthesis of cellulolytic enzymes by Tricothecium roseum with citric acid mediated induction. Afr. J. Biotechnol., 7(21): 3917-3921.
Merulius	Takao et al.,1985 Takao,S., Kmagata,Y. and Sasaki,H., 1985. Cellulase production by Penicillium purpurogenum. J. Ferment. Technol., 63(2): 127-
Aoryzar	Adeleke et al.,2012 Adikanae,H.V.and Patil,M.B., 1983. Cellulase production by Fusarium solani. Indian Bot. Rep., 2(1): 97-98.

2. Methodology

Culture collection

T. reesei (MTCC No.164) cultures were procured from IMTECH, Chandigarh. As per directions, the T.reesei culture was revived on Potato Dextrose Agar (PDA) at 23ºC.

Sample collection and pretreatment

Water hyacinth leaves were collected from the local pond in Phagwara and washed properly with distilled water and then sun dried leaves crushed to make a powder.

a) Steam explosion (Physical treatment)

The WH powder was autoclaved for different time intervals i.e. for 20 and 30 minutes using distilled water at 121°C at 15 psi and pressure was released by sudden opening of valve.

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B) Chemical pre-treatment

(I) Sodium Hydroxide Pre-treatment

Different concentrations (1, 2, 4, 6, 8 and 10%) of Sodium hydroxide was used with 10 grams of WH powder and allowed to stand at 37°C for 4 hr and was autoclaved at 121°C for 30 min with sudden release of pressure. The solution was washed and filtered to collect the powder with tap water until neutral pH was reached, again filtered and dried at 65°C.

(II) HCl Pre-treatment

Different concentrations of HCl were used in the range 1, 2, 4, 6, 8 and 10 % with 10 grams of WH powder at 37°C for 4 hrs and then autoclaved at 121°C, 15psi for 30 mins. The powder was collected washed extensively with tap water until neutral pH was reached, and sample were filtered and dried at 65°C.

(III) Lactic acid Pre-treatment

Different concentrations of Lactic acid were used in the range 1, 2, 4, 6, 8 and 10 % with 10 grams of WH powder at 37°C for 4 hrs and then autoclaved at 121°C, 15psi for 30 mins. The powder was collected and washed extensively with tap water until neutral pH was reached, and then filtered and dried at 65°C.

SSF fermentation

The fungal spores of T. reesei grown on PDA plate for 6 days were inoculated in the production media as described by Mandel (1957). The 10% w/v of pre-treated WH powder was added to 250 ml Erlenmeyer flasks with fungal culture T. reesei.

Optimization of factors for Cellulase production

Taguchi statistical methodology has been applied for Design of experiments with selected eight variables viz. effect of temperature, pH, time of incubation, inoculum level, Nitrogen source, WH concentration, Tween-80, and revolution per minutes on cellulase production using the software Qualitek 4. The M18 array were fit for selected variable and 18 experimental design were obtained by Qualitek-4 software (Nutek Inc., MI).

In brief, we can describe Taguchi Optimization methodology in five simple steps.

1- Selection of factors and their concentrations level (variables)

2- Selection of proper orthogonal array.

3- Setting of experiments as per design of experiments and getting results in three trials.

4- Statistical analysis ANOVA for determination of the main influencing factor.

5- Prediction of optimum condition and validation of results by actual experiment.

Determination of significant factors: *The factors showing difference in levels shows significant influence on cellulase production were determined based on level difference. In addition the interaction between two factors gives a better insight into the overall process analysis. Any individual factor may interact with any or all of the others factors creating the possibility of presence of a large of interactions. This kind of interaction is possible only in Taguchi DOE methodology. Analysis of experimental data and prediction of performance phase 3. The result obtained were analysed was based on the S/N ratio ANOVA. The result obtained after the data processing by Qualitek-4 software.*

Analysis of sample

Sample were analysed from supernatant obtained after centrifugation of hydrolysate at 2000 rpm for 15-20 min at 4°C and the cell free culture supernatant fluid was further used for cellulase assay.

a) FTIR analysis

2 mg of untreated and pre-treated solid mass of WH were used for FTIR analysis (Lovely Professional University). WH samples were prepared by mixing with 200mg of spectroscopic grade KBr. Untreated WH powder was used as a standard for FTIR analysis against treated samples.

b) Anthrone Assay (Cellulose estimation)

Cellulase estimation was carried out as per protocol of Updegraff (1969).

c) Enzyme Assay

CMCase and FPase activities *assayed were by the method reported by Ghose 1987 using CMC as substrate and Whatman No.1 filter paper as substrate respectively. According to International Union of Biochemistry "One international unit of enzyme (1IU) corresponds to the amount of enzyme required to release 1 micromole of reducing sugars per minute during the hydrolysis reaction"*

I) CMCase assay

For CMCase assay 1 ml of diluted enzyme solution was added to 1% carboxymethyl cellulose solution The reaction mixture was incubated for 30 minutes at 50°C and 3 ml of DNS reagent was added .The test tubes were boiled for 15 minutes and 1ml of 40% sodium potassium tartrate was added. The test tubes were cooled and absorbance was measured at 550 nm.

II) Estimation of reducing sugars

Reducing sugars concentration was estimated by a method reported by Miller 1959.

Results and Discussion

Pre-treatment studies

The Dried WH powder was treated with 8% of NaOH, HCl and Lactic acid. Water hyacinth powder after pre-treatment process shown in figure 1.(a & b)

Fig 1: (a) Water hyacinth (leaves powder) (b) Water Hyacinth powder treated with different conc. of NaOH

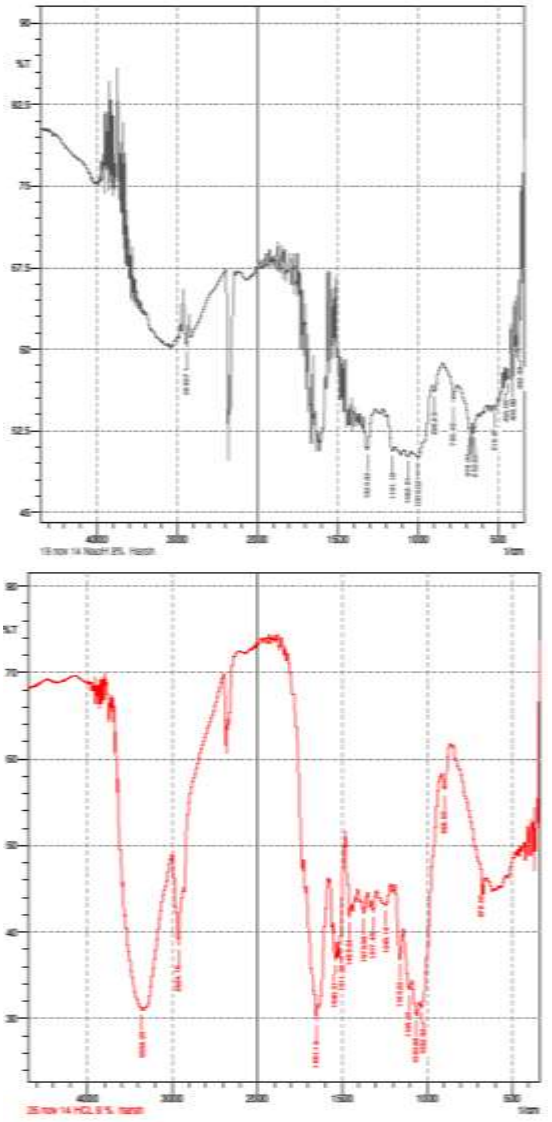

Fig. 2 (a) 8% NaOH
Fig. 2 (b) 8% HCl

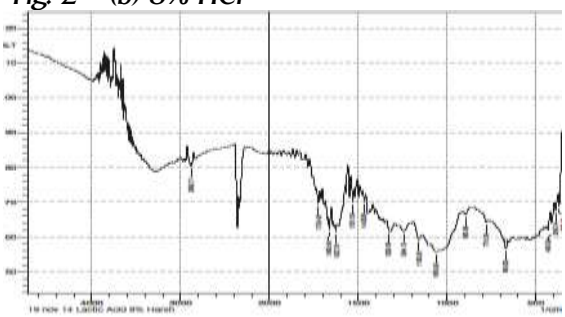

Fig. 2 (C) 8% Lactic acid

6.1 Optimization of parameters for Enzyme production

The parameters selected in this work were temperature, pH, Tween 80, Water hyacinth, Nitrogen source, Time of incubation, Inoculum level and Rotations was shown in table 3. On the basis of selected factor L18 design were obtained. Shown in Table 3 and table 4.

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Table 2: Factors selected for optimization of media.

Factors	Level 1	Level 2	Level 3
1. pH	*3*	*5*	*7*
2. Temperature (°C)	*30*	*40*	
3. Nitrogen source	*Yeast extract*	*Peptone*	*Beef extract*
4.WH (%)w/v	*7.5*	*10*	*15*
5. Tween 80 (%) v/v	*0.5*	*1.5*	*3*
6. Inoculum conc. (%)	*5*	*7*	*10*
7. Rotation (rpm)	*0*	*100*	*150*
8. Incubation time(days)	*3*	*5*	*7*

Table 3: Design of experiments

Trial No.	pH	Temp. (°C)	Nitrogen source (1%)	Waterhyacinth (%w/v)	Tween 80 (v/v %)	Inoculum (%)	Time of incubation	Rotation (rpm)
1	3	30	YE	7.5	0.5	5	3	0
2	5	30	Peptone	10	1.5	5	7	0
3	7	30	BE	15	1.5	10	3	0
4	7	30	Peptone	7.5	3	5	5	100
5	3	40	Peptone	15	0.5	7	5	0
6	7	40	BE	15	0.5	5	7	100
7	5	40	BE	7.5	3	7	7	0
8	3	40	YE	7.5	1.5	10	7	100
9	3	30	Peptone	15	3	10	7	150
10	7	30	YE	10	0.5	7	7	150
11	3	40	BE	10	3	5	3	150
12	7	40	YE	10	3	10	5	0
13	5	40	Peptone	10	0.5	10	3	100
14	5	40	YE	15	1.5	5	5	150
15	5	30	YE	15	3	7	3	100
16	5	30	BE	7.5	0.5	10	5	150
17	3	30	BE	10	1.5	7	5	100
18	7	40	Peptone	7.5	1.5	7	3	150

YE= Yeast extract, BE= Beef extract

Main effects determination by level difference (Average effects of factors and interaction)based on S/N ratio analysis

Column #/Factors	Level 1	Level 2	Level 3	L2-L1
1. Temperature	*30.871*	*30.303*		*-.568*
2.pH	*28.598*	*32.321*	*30.843*	*3.722*
3.Nitrogen source	*31.357*	*29.834*	*30.571*	*-1.523*
4.WH(%)w/v	*30.948*	*29.483*	*31.331*	*-1.465*
5 Tween 80 (%) v/v	*31.144*	*29.539*	*31.079*	*-1.605*
6. Inoculum conc.(%)	*30.264*	*30.395*	*31.103*	*.131*
7.Rotation rpm	*30.237*	*30.133*	*31.391*	*-.104*
8. Incubation time (days)	*30.203*	*31.281*	*30.277*	*1.077*

#	Interacting factor pairs (order based on SI)	Columns	SI(%)	Col	Opt
1	*Inoculum conc.(%X rotation (rpm)*	*6X7*	*96.04*	*1*	*[1, 2]*
2	*Nitrogen source X Tween 80*	*3X5*	*60.13*	*6*	*[1, 2]*
3	*Tween 80 X Incubation time*	*5X8*	*56.75*	*13*	*[3, 2]*
4	*Nitrogen source X Incubation time*	*3X8*	*56.16*	*11*	*[3, 2]*
5	*Temp X Inoculum conc.*	*1X6*	*51.1*	*7*	*[1, 1]*
6	*WH(%) X Tween 80*	*4X5*	*49.23*	*1*	*[3, 1]*
7	*WH(%) X Inoculum conc.*	*4X6*	*48.34*	*2*	*[1, 2]*
8	*Inoculum Conc. X incubation time*	*6X8*	*39.66*	*14*	*[1, 2]*
9	*pH X Incubation time*	*2X8*	*34.07*	*10*	*[2, 2]*

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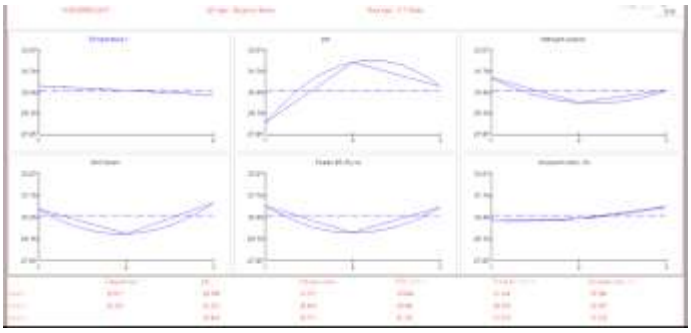
10	Tween 80 X Inoculum conc.	5X6	32.69	3	[1, 1]
11	pH X WH(%)	2X4	32.08	6	[2, 3]

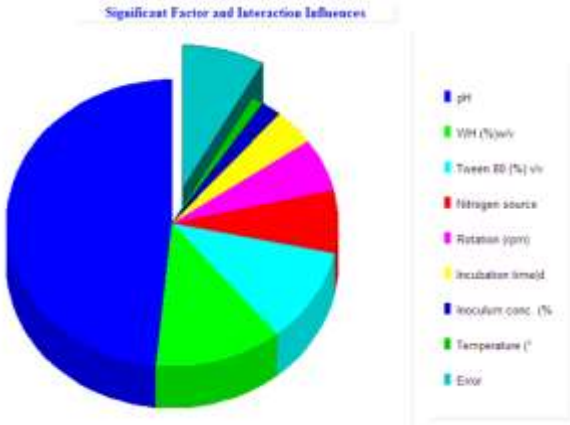
Col #/Factor	DOF(f)	Sum of Sqrs.(S)	Variance(V)	F-Ratio (F)	Pure Sum (S')	Percent P%
1. Temperature	1	1.448	1.448	3.592	1.045	1.224
2.pH	2	42.172	21.086	52.313	41.366	48.468
3.Nitrogen Source	2	6.957	3.478	8.63	6.151	7.207
4.WH(%)w/v	2	11.412	5.706	14.157	10.606	12.427
5.Tween 80 (%) v/v	2	9.908	4.954	12.291	9.102	10.665
6. Inoculum conc.(%)	2	2.442	1.221	3.029	1.636	1.917
7.Rotation rpm	2	5.849	2.924	7.256	5.043	5.909
8. Incubation time (days)	2	4.348	2.174	5.394	3.542	4.15
Other /Error	2	0.805	0.402			8.033
Total	17	85.346				100.00%

Col #/Factor	DOF (f)	Sum of Sqrs.(S)	Variance(V)	F-Ratio (F)	Pure Sum (S')	Percent P%
1. Temperature	-1	-1.448		POOLED	(CL=84.35%)	
2.pH	2	42.172	21.086	52.313	41.366	48.468
3.Nitrogen Source	2	6.957	3.478	8.63	6.151	7.207
4.WH(%) w/v	2	11.412	5.706	14.157	10.606	12.427
5.Tween 80 (%) v/v	2	9.908	4.954	12.291	9.102	10.665
6. Inoculum conc.(%)	-2	-2.442		POOLED	(CL=81.33%)	
7.Rotation rpm	2	5.849	2.924	7.256	5.043	5.909
8. Incubation time (days)	2	4.348	2.174	5.394	3.542	4.15
Other /Error	5	4.695	0.939			11.174
Total	17	85.346				100.00%

Col #/Factor	Level Description	Level	Contribution
1. Temperature	30	1	0.283
2.pH	5	2	1.733
3.Nitrogen Source	YE	1	0.769
4.WH(%)w/v	15	3	0.743
5.Tween 80 (%) v/v	0.5	1	0.556
6. Inoculum conc.(%)	10	3	0.515
7.Rotation rpm	150	3	0.803
8. Incubation time (days)	10	2	0.693
Total contribution from all factors 6.094			
Current grand average of performance... 30.587			
Expected result at optimum condition..... 36.682			



Effect of Rotation on Cellulase production



6.4 Optimum Conditions for Cellulase Production

a) Effect of temperature:

The effect of temperature for optimization of CMCase and FPase two temperature conditions were selected (30^{0}C & 40 ^{0}C). From Figure 10 (a), it can be observed that by increasing the temperature from 30^{0}C to 40 ^{0}C cellulase production increases. Table 6 had shown the main effect of temperature on CMCase production, which was highly effected by temperature whereas FPase production was less affected by temperature. From Table 6 it can be observed that the increase of temperature leads to increase in cellulase production. Result of ANOVA was shown in Table 9. Which includes the sum of squares(s), variance (v), F-ratio (F), Pure sum (s') and percent (P). For temperature at Degree of factor 1, sum of squares 9.2, variance 9.2, F-ratio12.2, the percent contribution obtained was 21 % for CMCase whereas for

FPase at Degree of factor 1, sum of squares 0.9, variance 0.9, F-ratio 3.4, the percent contribution obtained was 5.3 %. Thus it can be concluded that temperature is one of the most influencing factor for CMCase production. While FPase production is effected by the temperature. Similar work was done by the **Despande *et al.,*** *2009; he reported the maximum cellulose activity at temperature 30ºC. Temperature has a great influence on enzyme production. If temperature is too high, microorganisms grow faster but enzyme production is low and if temperature is low, microbial growth is slow, resulting in long production cycle, so optimization of temperature is a necessity.* **Guowei *et al.,* 2011** *in their study used different incubation temperature (26°C, 28°C, 30°C, 35°C and 40°C.) for enzyme production. The activity of CMCase and FPase first increased up to certain value and then decreased. The activity of CMCase increased from 236.09U/g at 26°C to377.20U/g at 30°C and then decreased to 21.61U/g at 40°C. The activity of FPase increased from 37.33U/g at 26°C to 92.16U/g at 30°C and then decreased to 5.48U/g at 40°C.The optimal incubation temperature was 30°C for CMCase and FPase.* **Nochaure *et al.,* 1993** *studied the effect of temperature on cellulase enzyme production by the A. Niger and Trichoderma reesei in the range of 20 to 85 ± 2°C.The optimal temperature for exoglucanase (1.95 U/mL) and endoglucanase activity (1.88 U/mL) A. niger was 40°C and 50°C, while the optimum temperature for β-glucosidase activity was between 45°C and 55°C.*

b) Effect of pH

The pH selected 3, 5, &7 pH is one of the most important contributor factors after the temperature in cellulase production. On increasing the pH from 3-5, there was a sharp increase in the enzyme production, after further increase in pH from 5-7 enzyme production ceases rapidly. This can be conformed from Fig.10 (b) pH interacts with many factors such as (SI%38) especially with rotation for CMCase production while for FPase,pH interacts with WHwith (SI%56). High dissociation of ions during rotation helps in more mass transport. According to Table 7 FPase productions were highly dependents on nitrogen source, temp and substrate concentration. For CMCase the pair of factors that affects production are Tween80 (SI%12), incubation time (SI%25).As per ANOVA analysis, pH is second highest contributor factor in CMCase production and first highest contributor factor in FPase production with F-ratio 6 & 5.2 respectively. This data can be conformed from Fig 11. pH plays an important role in enzyme production since the activity of the enzyme depends on optimum pH, so optimization of pH is one of the important factor for enzyme production. The influence of pH on enzyme production was studied by **Chung *et al.,* 2010.***They took different pH in the range from 3.0-9.0. Their results showed maximum production of exoglucanase was 1.76 & 2.18 U/mL, while endoglucanase was 1.25 & 1.95 U/mL, and β-glucosidase was 1.44 & 1.71 U/mL by Aspergillus niger and Trichoderma reesei at pH 6-7 and In another study by* **Liu *et al.,* 2007** *where they used waste from vinegar industry as a substrate for production of cellulase by Trichoderma choningii AS3.4262. The Fpase activity obtained was 6.90 IU/g of SDM (substrate dry matter) and CMCase activity 23.76 IU/g. Similar work had been performed by* **Liu *et al.,* 2007** *cellulase were produced by using T.choningii AS3.4262. The activity of FPase and CMCase was reported as 6.90 IU/g and 23.76 IU/g after 48 hrs with pH 5.0.* **Karmakar *et al.,* in 2011** *reported cellulase production using WHusing Rhizopus oryzae MTCC 9642 in submerged and solid state fermentation. Various parameters has been studied such as substrate conc., temp and pH were optimized. The best FPase activity from submerged fermentation was at substrate conc. 1.25%, pH 7.32 and temp 25 ºC while as in SSF mode the best enzyme production was obtained at substrate conc. 0.5%, pH 6.0 and temp 18 ºC.*

c) Effect of Tween 80

Effect of Tween 80 was studied at 3 levels 0.5%, 1.5% and 3% as shown in Fig 10 (c).After addition of 1.5% of Tween 80, CMCase production ceases while FPase production does not .This may be due to effect of other factors interacting with Tween 80 such as rotation speed (SI % 54), inoculums percent (SI% 49), nitrogen source (SI%46). (Table 7). Similar work had done by the **Sharhriarinour *et al.,* 2011***. He studied the effect of Tween 80 on cellulase production, according to results cellulase production was enhanced with addition of Tween 80 in culture (Tween 80 at a conc. of 2 ml/l). Tween 80 is the surfactant which increases mass transport by reduction in viscosity. As a result, at lower concentration, this may be an important additive, whereas at higher concentration, this may not be supportive for high enzyme production because of toxicity and reduced mass transport due to bubble formation.*

d) Effect of water hyacinth

Effect of Water hyacinth was studied at concentration 3 levels was 5%, 7-5% & 10% shown in Fig 10 (d). On increasing the conc. of WHfrom Level1 to Level 2 (7.5-10% w/v), there is a rapid increases in CMCase and FPase production, after that the enzyme production decreases. Water hyacinth interacts with other factors, which is depicted in Table 7. The other interacting factors are incubation time (SI%44) and (SI%25) for CMCase and FPase respectively. Nitrogen source (SI %23), (SI%31) for FPase, with Tween 80 (SI%60 & SI %42), WHand inoculum (SI%82 & 29). This indicates that for hydrolysis of water hyacinth, the most active enzyme is CMCase which requires high percentage of inoculum. Other factors which effects hydrolysis of WHis incubation time (at least 5 days) to start the process. Since enzyme is protein which requires high %age of nitrogen supply, thus effect of nitrogen is around (SI%23) in CMCase while as in FPase it is (SI%31). Water hyacinth (Eichhornia crassipes), an aquatic weed creates a lot of ecological and socio-economic problems to water bodies **(Takasawa *et al.,* 1986)***.Various workers has used WHfor production of fuel and chemicals* **(Burton *et al.,* 2005).** *Thus, WH(Eichhornia crassipes) may prove a highly economical substrate for cellulase production* **(Osei-agyemang *et al.,* 2002).** *According to various reports, WHhas been found rich in hemicellulose followed by cellulose and other components impregnated with the lignin. Water hyacinth may prove highly beneficial for enzyme production since they are rich in protein and various nitrogen sources besides carbohydrates* **(Ghosh, 1981)***. Thus beside cellulases, they are also good substrate for ethanol and organic acids production* **(Zha *et al.,* 2008).** *Another advantages is WHas economical substrate & that they are*

readily available round the year.According to **mukhopadhyay *et al.,* 1999** *increase in WHaround 2.6%.There was 4% increase in cellulase production and ratio of β-glucosidase to FPase was higher 6%. Ammonium sulphate for pepton was best nitrogen source for cellulase production* **Despande *et al.,* 2009** *conducted work for cellulase production by using Water hyacinth as a substrate. Parameter studied for substrate pre-treatment was substrate concentration, initial medium pH, mode of incubation, temperature. Maximum cellulase activity reported was 0.22IU/mL after 15 days. Mass transport of WH(from external medium to cell), the most important factor observed was rotation, incubation time, Tween 80 which collectively is responsible for giving the high result of CMCase production, while almost similar condition prevails for FPase production. Further various fungal strain Aspergillus flavus, Aspergillus niger, Trichoderma reesei MTCC164 have been reported for cellulase production* **(Ismail *et al.,* 1995)**

e) Effect of nitrogen source

Different nitrogen sources were used for cellulase production such as yeast extract, beef extract, peptone at 1% (w/v).Selection of organic nitrogen was based on the fact that at very low concentration nitrogen has different effect over enzyme production. From Fig 10(e) all three nitrogen have almost equal effect thus no detectable change has been observed by changing from Level 1 to Level 3.CMCase production is positively affected by organic addition of nitrogen (0.247) while FPase production is effected by besides main effects there are various other factors that affect nitrogen utilization and mass transport as observed in Table 7. **Ali *et al.,* 2008**, *used WHblend for production of cellulases using mixture of Microbes such as A. niger and A. nidulans in Czapek-Dox medium. According to their report, the maximum enzyme activity was found at following conditions such as temp 35 ºC, pH 7.0, sodium nitrate was found to be as best nitrogen source and 7 & 3 days under static and shaker conditions respectively for A. niger and at 30 ºC, pH 7.0, sodium nitrate as nitrogen source and 7 & 4 days under static and shaker conditions respectively for A. nidulans. Similar work had done by the* **Leynd *et al.,* 2002** *repored maximum production of cellulase enzyme by using 1.0% peptone, beef extract and exoglucanse produced was 1.79 μ/ml while endoglucanse was 1.48 μ/ml and β-glucosidase was 1.92 μ/ml by T. ressei.For CMCase production, incubation time, rotation at (SI%51 and 49), Tween 80 46% for CMCase production. Nitrogen accumulation is effected by similar condition for FPase production at different SI conditions. ANOVA analysis confirmed the fact that organic nitrogen has 4% of overall contribution of different factors at F-ratio 2.1, while for FPase very low effect of organic nitrogen has been observed .this can be conformed form Fig 11.*

f) Effect of incubation time.

The effect of incubation time was selected for study was 3, 5 & 7 days in hydrolysis of WHincubation time plays an important role. On increasing the time from level1 and level 2 in both the enzyme CMCase and FPase. Individually there is less effect of incubation time in CMCase production (as depicted by Fig 10(f), from Table 7). It has been observed that incubation time is also involve not only in hydrolysis of WH(44% SI) but also accumulation of organic nitrogen at (SI %51) other factors such as pH, Tween 80 has little contribution towards enzyme production (SI%25).Almost similar condition prevails for CMCase and FPase production.

Devi and Kumar, 2012, *optimized the cellulase production from Bacillus cereus MRK1 and performed its Bio stoning activity. Different factors such as incubation period, temperature, pH and effect of carbon and nitrogen sources were optimized for maximum yield of the enzyme. Initial optimization process showed pH 8, 32 °C, xylan and yeast extract favouring enzyme production. The test strain showed its ability to secrete cellulase around 102 IU/ml when it was grown in paper sludge supplemented medium. Cellulase production was studied by* **Omojasola *et al.,* 2008** *using pineapple waste as substrate using A. niger, Trichoderma longibrachiatum, and Saccharomyces cerevisiae as inoculum. The various factors optimized were time, pH, substrate conc. inoculum size and temp. Out of the above mentioned cultures, Trichoderma longibrachiatum produced higher amount of glucose (0.92 mg/0.5 ml) at pH 4.5 and temperature of 45ºC on day 7th day of fermentation.*

Deaming *et al.,* 2008 *studied the cellulase enzyme activity from T. reesei up to 6 days. The maximum yield of exoglucanase, and endoglucoanase activity was obtained after 5 days. After ANOVA analysis Table 8 incubation time has 5.8% contribution of factors F-ratio 2.57 for CMCase production while for FPase; contribution is almost 15.9% at F-ratio 4.7 this can be conformed from Table 9.*

g) Effect of Inoculum (%)

The effect concentration of inoculums selected was 5, 7 & 10 for CMCase and FPase production, with the increase in concentration of inoculums had little effect on production shown in Fig 10 (g). According to ANOVA Table 8, it contributes only 0.7 % for CMCase and around 5% for FPase and which can be conformed from Fig 11. **Shikai *et al.,* 2013** *conducted a similar work, result shown that when the concentration of inoculums was increased then the activity of CMCase assay increased from 355.06 U/g at 0.5% to 386.47 U/g at 2.5%.*

h) Effect of rotation

The individual effect of rotation condition selected for study was 0, 100 and 150 rpm on enzyme production. In Fig 10 (h) on increasing the rotation speed, continuous increase in CMCase production from level 1 to level 3 while for FPase after level 2 is a sharp decrease in FPase production. Rotation has positive effect over the carbon substrate utilization, dissolution of various media components and helps in efficient aeration and nutrient transport. It also helps in maintenance of uniform pH and temperature. Besides this, they are also responsible for product removal at uniform rate from cell wall. The similar effect can be observed in both the enzymes production. For CMCase production rotation has 3rd highest SI impact 54 % and for FPase 2nd highest SI impact was observed 61%.

Sarkar *et al.,* 2012 *reported the effect of rotation on cellulase production. At rotational speed 120 rpm and temperature 37ºC CMCase and FPase activity was increased in cellulase production. From ANOVA analysis (Table 8) rotation has 5 % contribution for CMCase production while*

it is 10% for FPase production. The same contributed by Fig 11.

FPase assay	Av.OD (550 nm)	CMCase assay	Av. OD(550 nm)
Trial 1	0.256	Trial 1	0.284
Trial 2	0.262	Trial 2	0.363
Trial 3	0.485	Trial 3	0.454
Trial 4	0.557	Trial 4	0.597
Trial 5	0.639	Trial 5	0.683
Trial 6	0.586	Trial 6	0.543
Trial 7	0.957	Trial 7	0.896
Trial 8	0.262	Trial 8	0.766
Trial 9	0.331	Trial 9	0.526
Trial 10	0.392	Trial 10	0.492
Trial 11	0.285	Trial 11	0.405
Trial 12	0.355	Trial 12	0.334
Trial 13	0.238	Trial 13	0.389
Trial 14	0.296	Trial 14	0.326
Trial 15	0.312	Trial 15	0.349
Trial 16	0.454	Trial 16	0.264
Trial 17	0.426	Trial 17	0.218
Trial 18	0.434	Trial 18	0.246

Tabl 6: Main effects determination by level difference (Average effects of factors and interaction)

Column/factors	L2-L1	L2-L1
Temperature (°C)	1.435	0.217
pH	1.408	0.353
Tween 80 (%)	0.609	-0.321
Water hyacinth (%) w/v	0.714	0.319
Nitrogen source	0.247	-0.411
Incubation time (days)	-0.248	-0.988
Inoculum (%)	-0.515	-0.723
Rotation (rpm)	-0.246	-0.026

Conclusion and Future Scope

In our work increasing 1.5% WHresulted in increase in FPase 76% as comparably to **Despande *et al.*, 2009.***while CMCase activity as 50% higher while FPase was 87 % higher. This is because of increase mass transport when media were supplied with Tween 80-1.5%, peptone 1%, rotation-100 rpm; inoculums level -7.5%, incubation time-7days and WH-7.5% in SSF mode. Various chemical pre-treatment were done such as HCl, NaOH, and Lactic acid along with steam explosion. Out of all the experiments performed, NaOH (8%) gave the best result for pre-treatment. While as other results were not significant. Trichoderma reesei selected with WHfor cellulase production. Optimization the effective conditions for production of CMCase temp-40 °C, pH-5, tween80 3%, WH7.5%, nitrogen source 1% as peptone, incubation time 7days, inoculum 5% at rotation 100 rpm for FPase production only two condition was difference inoculum was higher (10%) and rotation speed was 150 rpm. Production of cellulase for CMCase was 52% more increase activity observed after media optimization and similar for FPase was 84% increase activity observed after media optimization.*

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Recent trend in Bio-fortified wheat production-A review

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Abstract

The daily dietary recommendation is different for both men and women; women's require 8 mg/day zinc while men requires 11 mg/day. Daily meal does not have these micro-nutrition in the diet therefore, it should be supplied from outside source. Therefore, Biofortified wheat is an best option where, More than 2 million people are suffering from malnutrition (FAO, 2016). There are various products of cereals such as Flour from cereals is used for making bread & other products for breakfast cereals, as pasta, snack foods, dry mixes, cakes, pastries, and tortillas. If more bio-fortified cereals can be grown it could enhance the nutritional value of the food as well as sales. Some of the major players in the Indian breakfast cereals market are Kellogg Co., Nestle, PepsiCo, Bagrry's India Ltd and Marico. Kellogg's frootloops, Kellogg's rasin bran, Quaker, Wheaties, Nestle fitness and private brands using such products. As per WHO recommendations now Food Safety and Standards Authority of India (FSSAI) has make it policy for wide food fortification program and now. Common staple foods such as wheat flour, rice, edible oil, and milk have been selected as food vehicle for different micronutrient fortifications.

Keywords: Bio-fortified wheat, FSSAI, bran, malnutrition, breakfast, Folic acid, Thiamine

1.1 Introduction:

Bread wheat (Triticum aestivum L.) is the second most important food crop worldwide after rice. There are various reports of bio-fortified wheat that has been reported in past few year which are rich in zinc (Cardoso et al., 2018; Velu et al., 2015; Zia et al., 2020).

Wheat (Triticum aestivum) is one of the most important food crops globally which provides one-third of the total protein requirements of the populations (Shewry, 2009). It has a protein content ranging from 7 to 15% and plays a vital role in the human diet (Ciclitira & Ellis, 1987). Wheat proteins are generally rich in glutamic acid (Glu) and prolines (Pro), the functional amino acids in extensible dough formation. Protein content along with the amino acid composition determine the nutritional quality of wheat grains (Joye, 2019). The amino acid composition of wheat is not balanced and lacks the adequate amounts of EAA like Lys, Thr, and Met (Jiang, Tian, Zhi, & Zhang, 2008). Processing of wheat into different products results in further depletion of its amino acid contents. With the modern popularization concept of a healthy diet, people are becoming more concerned about health and nutrition (Garg et al., 2018). Recently, colored wheat has gained lots of attentions due to its health beneficial properties (Gupta, Meghwal, & Prabhakar, 2021; Saini).

Different colors of anthocyanin bio-fortified wheat viz., purple, blue and black depend upon the types and position of anthocyanins in wheat layers (Garg et al.,2016). The purple color is due to localization of anthocyanins in the pericarp layer, blue in the aleurone layer, while, black is combination of the two.

Recent publications have characterized anthocyanins and other nutrients from colored wheat, studied origin and genetics of grain color, developed high yielding regionally adapted germplasm, studied the effect of genotype and environment on anthocyanin accumulation, developed different products, studied the effect of cooking on the stability of anthocyanins and their antioxidant activity and effect of abiotic stresses (Lachman, Martinek, Kotikov □a, Ors □ak, & ˇSulc, 2017; Mbarki et al., 2018; Paznocht et al., 2018).

Several studies have reported its grass, sprouts, whole plant and seeds of colored wheat for their anti-inflammatory activity, antimicrobial activity, and other human health benefits including positive effects on neurodegenerative disorders, high-fat diet-induced

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derangements like body weight gain, blood cholesterol and glucose and diabetes (Gupta et al., 2021; Mbarki et al., 2018; Paznocht et al., 2018; Saini et al., 2020; Sharma et al., 2018, 2020, 2021; Sytar, Bo□sko, ˇZivˇc□ak, Brestic, & Smetanska, 2018). Due to the growing trend towards nutrition-rich foods, nutritional profiling of these designer crops along with their suitability for the production of high-quality end products has become utmost important. Presently, little is known about the nutritional background of colored wheat grains particularly their amino acid profile (Tian, Chen, & Wei, 2018). Hence, the current study was designed to evaluate the profile of amino acids in flours and chapatti samples of black, blue and purple colored anthocyanin biofortified wheat developed at National Agri-Food Biotechnology Institute (NABI), Punjab, India (Garg et al., 2016). The effect of cooking upon the amino acid content of colored wheat chapatti samples in comparison to white wheat chapattis was also evaluated as anthocyanins are reported to reduce protein degradation in food products upon heating (Oancea, Aprodu, Rapeanu, Bahrim, & Stanciuc, 2017). This would be the first study reporting the effect of cooking on amino acid content of anthocyanin rich colored wheat chapattis. Such information could be useful in assessing the quality of diets to meet nutritional requirements of people, particularly of South Asian countries who mainly consume wheat in the form of chapattis.

Zinc deficiency has resulted in various consequences in Asian countries specially India and Pakistan. Therefore, to cop up with major mineral deficiency such as "Iron" and "Zinc" by the plant breeders and agronomist. But real question is that if Bio-fortified wheat has been successfully completed mission of coping with malnutrition. Assessment of release of "Zn" has been done on what variety Triticum aestivum L. var. Zincol-2016 (Zia et al., 2020). Mostly, fortification done via fertilizer added in excess zinc (Cardoso et al., 2018) but also there is report of genetic modification technique by which Zinc bio-fortification has been done. In this regard X-ray analysis & QTL mapping has been prove to be very useful tool to detect trace of metal present in wheat flour(Cardoso et al., 2018)(Crespo-Herrera, Govindan, Stangoulis, Hao, & Singh, 2017). WHO started fortified wheat since 2011 and has permitted to add many other components such as Vit A,
(https://documents.wfp.org/stellent/groups/public/documents/manual_guide_proced/wfp251105.pdf)

Table 1: Micronutrient rate and chemical form (source WHO public manual available at www.wfo.org)

Vitamin A 250 n.s	1.0 mg/kg	Dry vitamin A palmitate
Vitamin B1	4.4 mg/kg	Thiamine mononitrate
Vitamin B2	2.6 mg/kg	Riboflavin
Vitamin B3	35.0 mg/kg	Nicotinamide
Folic acid 1.0	mg/kg	Folic acid
Vitamin B 12	0.008 mg/kg	Cyancobalamin
Iron	15 mg/kg	NaFeEDTA
Zinc 30 mg/kg	Zinc oxide.	

As per latest report fortified wheat now has been used by Big firms like ITC, HUL & Cargill to fortify wheat flour. (https://economictimes.indiatimes.com). Market leaders like the ITC, General Mills, Hindustan Unilever, Patanjali, and Cargill has started fortification of their flagship brands of wheat flour (atta) such as Aashirwaad, Pillsbury, Annapurna, Patanjali and Nature Fresh respectively," the FSSAI. These fortification of wheat is done in aim to cop-up with malnutrition and undernourished people which is more than 50% in Africa and around 15% in India and on around 50% in women's. According to FSSI report folic acid amalgamation is essential to absorb "Fe" in the body and also cost not more than one rupees per kg while they can save more than Rs 25 on prevention by medicine.

Average levels of nutrients to consider adding to fortified wheat flour based on extraction, fortification compound and estimated *per capita* flour availability.

Nutrient	Flour Extraction rate	Compound	Level of nutrient to be added in parts per million (ppm) by estimated average per capita wheat flour availability (g/day)[a]			
			<75[b] g/day	75-149 g/day	150-300 g/day	>300 g/day
Iron	Low	NaFeEDTA	40	40	20	15
		Ferrous Sulfate	60	60	30	20
		Ferrous Fumarate	60	60	30	20
		Electrolytic Iron	NR[c]	NR[c]	60	40
	High	NaFeEDTA	40	40	20	15
Folic acid	Low or high	Folic Acid	5	2.6	1.3	1
Vitamin B_{12}	Low or high	Cyanocobalamin	0.04	0.02	0.01	0.008
Vitamin A	Low or high	Vitamin A Palmitate	5.9	3	1.5	1
Zinc[d]	Low	Zinc Oxide	95	55	40	30
	High	Zinc Oxide	100	100	80	70

a. These estimated levels consider only wheat flour as main fortification vehicle in a public health program. If other mass-fortification programs with other food vehicles are implemented effectively, these suggested fortification levels may need to be adjusted downwards as needed.
b. Estimated per capita consumption of <75 g/day does not allow for addition of sufficient level of fortificant to cover micronutrients needs for women of childbearing age. Fortification of additional food vehicles and other interventions should be considered.
c. NR = Not Recommended because very high levels of electrolytic iron needed could negatively affect sensory

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properties of fortified flour.
d. These amounts of zinc fortification assume 5 mg zinc intake and no additional phytate intake from other dietary sources.

* This is an extract from the relevant guidance document (1). Additional guidance information can be found in this document.

Antioxidant status in Bio-fortified wheat

According to latest work done order of antioxidant in biofortified wheat have been worked out which was in order of black > blue > purple > white (Kumari et al., 2020). Antioxidant is mainly present in wheat which is transferred via chapatti and it does not get degraded.

NUTRITIONAL SECURITY VERSES FOOD SECURITY IN SOCIETY

As *per WHO program bio-fortification wheat can give a nutritional security to Indian citizen by undermining impact of increasing malnutrition in children and pregnant women's. Earlier food hunger was an issue so Indian Government has launched a program for Food Security to reduce hunger index. As per Global perspective India's current hunger Index is around 110 (2021) and therefore, has launched several program such as Midday Meal Scheme (MMS), Integrated Child Development Services scheme (ICDSS) and the Public Distribution System (PDS) under National Food Security Act, in 2013 with mission to provide for food and nutritional security to poor people at affordable prices.*

Now with more data, nutritional security has been defined more accurately such as bio fortified cereal grains specially wheat, rice and other crops like maize(Aziz et al., 2019; Cormick et al., 2020; Murphy & Westmark, 2020; Wang, Kong, Liu, Fan, & Zhang, 2020). According to a report PM Kalyan Yojana around 1.74 Crore was spent to provide Covid-19, effected poor people to overcome the economic impact. Extended effort done by Government in form of providing concept of multi nutria-farm where fortified cereal grain is permitted to grow with increased content of protein and micronutrients, namely vitamins and minerals to provide sufficient nutrition to malnourished children's (Alvi & Gupta, 2020).

Anthocyanins are water soluble pigments that belong to class flavonoids in which white wheat contains more phenolic acid as antioxidants. Comparatively therefore, total phenolic acid and antioxidant content (134>122>120>13 in total anthocyanin test TAC) enhanced in order of color such as black>blue>purple>white (S. Sharma et al., 2018).

Major thiamine storage compartment of wheat or rice, the aleurone, is removed during refinement. For this reason, refined wheat flour and polished rice are common foods fortified with thiamin. The Food and Drug Administration of the United States (FDA) mandates that refined wheat flour be enriched with 2.9 mg thiamine per pound flour (FDA, 2018), and 66 total countries have fortification programs in place for wheat flour, maize flour, and/or rice (GFDx, 2019).

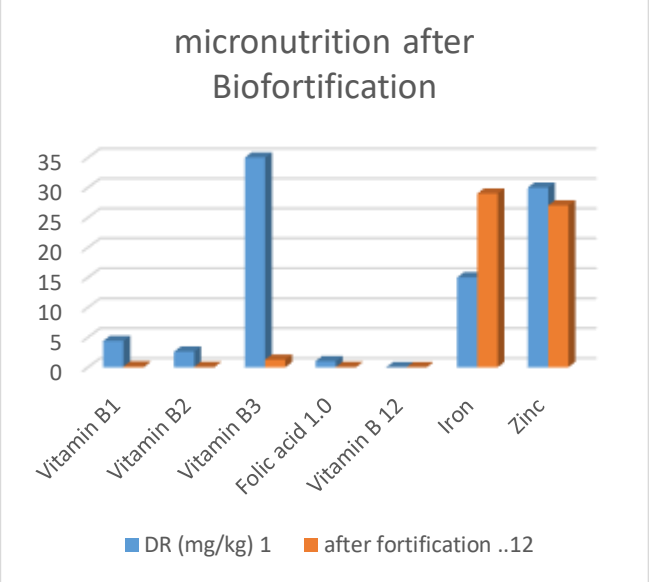

Fig 1. Source cited Comparative vitamins Daily requirements verses available in wheat (Garg et al., 2021)

	micronutrition	DR (mg/kg)	after fortification
1	**Vitamin A**	1	..12
2	**Vitamin B1**	4.4	0.12
3	**Vitamin B2**	2.6	0.04
4	**Vitamin B3**	35	1.25
5	**Folic acid 1.0**	1	0.04
6	**Vitamin B 12**	0.008	0.004
7	**Iron**	15	29
8	**Zinc**	30	27

Grain Zn content in wheat improved from 28.96 to 36.61 mg·kg-1 and that in flour increased from 10.51 to 14.82 mg·kg-1 after Zn fortification (Wang et al., 2020).

Zinc wheat

Grain Zn content in wheat was 31.84 mg·kg−1 globally but varied across continents, for example, 25.10 mg·kg−1 in Europe, 29.00 mg·kg−1 in Africa, 33.63 mg·kg−1 in Asia, and 33.91 mg·kg−1 in North America. Grain Zn content in wheat improved from 28.96 to 36.61 mg·kg−1 and that in

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flour increased from 10.51 to 14.82 mg·kg−1 after Zn fortification.

Furthermore, Zn content varied in the different processed components of wheat; that is, Zn content was 12.58 mg·kg−1 in flour, 70.49 mg·kg−1 in shorts, and 86.45 mg·kg−1 in bran. Zinc content was also different in wheat-derived foods, such as 13.65 mg·kg−1 in baked food, 10.65 mg·kg−1 in fried food, and 8.03 mg·kg−1 in cooking food. Therefore, the suitable Zn fortification, appropriate processing, and food type of wheat are important to meet people's Zn requirement through wheat.(Wang et al., 2020).

1. WHO. Recommendations on wheat and maize flour fortification: meeting report - interim consensus statement. Geneva, World Health Organization; 2009 (http://www.who.int/nutrition/publications/micronutrients/wheat_maize_fortification/en/).

Table 2.

Vitamin	Physiologic roles	Deficiency
Thiamin (B_1)	Co-enzyme functions in metabolism of carbohydrates and branched-chain amino acids	Beri-beri, polyneuritis, and Wernicke-Korsakoff syndrome
Riboflavin (B_2)	Co-enzyme functions in numerous oxidation and reduction reactions	Growth, cheilosis, angular stomatitis, and dermatitis
Niacin (nicotinic acid and nicotinamide)	Co-substrate/co-enzyme for hydrogen transfer with numerous dehydrogenases	Pellagra with diarrhoea, dermatitis, and dementia
Vitamin B_6 (pyridoxine, pyridoxamine, and pyridoxal)	Co-enzyme functions in metabolism of amino acids, glycogen, and sphingoid bases	Naso-lateral seborrhoea, glossitis, and peripheral neuropathy (epileptiform convulsions in infants)
Pantothenic acid	Constituent of co-enzyme A and phosphopantetheine involved in fatty acid metabolism	Fatigue, sleep disturbances, impaired coordination, and nausea
Biotin	Co-enzyme functions in bicarbonate-dependent carboxylations	Fatigue, depression, nausea, dermatitis, and muscular pains

2. FAO (Food and Agriculture Organization), IFAD (International Fund for Agricultural Development), WFP (World Food Program). The State of Food Insecurity in the World 2015. Meeting the 2015 International Hunger Targets: Taking Stock of Uneven Progress. Rome: FAO; 2015.

One of the best wheat rich in anthocyanin is purple wheat now started to be grown in various part of India and other countries. In Mohali Punjab by scientist (Beleggia et al., 2021; Francavilla & Joye, 2020; Hirawan, Diehl-Jones, & Beta, 2011; Paznocht et al., 2018; S. Sharma et al., 2018).

There was production of double bio fortification product rich in both Zinc and iron and was reported to be high as compared to wheat (S. Sharma et al., 2018).

A new variety of Bio-fortified bread wheat variety developed and named as HI 1633 (Pusa Vani), have been developed by the Indian Agricultural Research Institute, Regional Station, ICAR- Indore.

Bio-fortified bread wheat variety with yield 47 q/h-1 and can be grown late and also has been characterized for best quality of bread and biscuit while micronutrient reported was around 41 ppm of both iron and zinc(Sai Prasad et al., 2021).

In removing malnutrition

Bio-fortification is the process of enriching the staple food crops with nutrients and a way towards more nourishing future. ZINC deficiency generally caused 48.1 per cent among under five years children in Uttar Pradesh (Tewari, Rani, Singh, Singh, & Singh, 2017). In combating malnutrition such bio-fortification is highly required achieve the goal of nutritional security in Uttar Pradesh as well as India. (Tewari et al., 2017). **Bio-fortified coloured wheat are known for their anthocyanin content** *(N. Sharma et al., 2022). Black and blue wheat flour and chapatti samples had higher total amino acid content, and nutrition index in comparison other flour and chapatti samples, while EAA content were similar among all flour and chapatti samples. After chapatti cooking, average percent reduction in amino acid content in black, blue, purple and white wheat were 11.41, 12.4, 19.0, and 23.8%, respectively. White chapatti samples exhibited >20% losses in the contents of 14 amino acids, while black chapatti showed >20% losses in case of only (N. Sharma et al., 2022). The higher retention of amino acids in coloured wheat might be due to the masking and protective effects of anthocyanin's on proteins and amino acids from heating and oxidative damages.*

Conclusion

As per vision 2030 main aim is to provide nutrition rich food to poor lactating mothers and undernourished children's (Jose, Gulati, & Khurana, 2020). In Indian scenario trend is changing where people are now more aware of growing food organically. Large farmers in Punjab growing fortified wheat such as purple wheat.

Affordability may be an issue because price are high of these products as per reports 63−76% of the rural poor could not afford a recommended diet in 2011 (Raghunathan, Headey, & Herforth, 2021). Some authors believe that Millet can be one of the good alternative to that area where it's difficult to grow cereals such as draught affected (Brahmachari, Sarkar, Santra, & Maitra, 2019). Backyard poultry farming is also suggested as one of the best alternative in rural families with improved income and nutrition (M. Kumar, Dahiya, & Ratwan, 2021).

The fortification done via two methods 1) Agronomic method 2) Genetic engineering Bio fortification methods.
Breeding method or GE methods can increase the availability and efficiency of micronutrients like Iron and Zinc.

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