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Kinetic Study For The Microbial Production Of Cellulase Enzyme By Optimizing The Substrate Cellulosic Lemongrass Waste Generated After Essential Oil Extraction

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Abstract

Cellulosic waste from industrial and agricultural processes presents an opportunity for producing valuable biomolecules like cellulases. Microorganisms such as fungi, bacteria, and protozoans produce cellulases, and they thrive when cultivated on cellulose-rich materials. In current study the Cellulase enzyme are produced using waste cellulosic materials of lemongrass. The lemongrass yields 1 – 2% v/w essential oil, and almost 98-99% cellulosic waste is generated theoretically and practically. Aspergillus niger was cultured for cellulase production. Different substrate quantities (4%, 6%, 8%, and 10% w/v in Mineral Salt Media) were tested, with pretreatment involving 0.1% v/v sulfuric acid. The kinetic study revealed increasing enzyme activity up to 48 hours of incubation. Reaction kinetics indicated first-order kinetics for substrate utilization and product formation, with a maximum yield coefficient of 11.56 g/g observed at 8% w/v lemongrass substrate concentration. This approach offers a promising means of utilizing cellulosic waste to produce valuable products like cellulase, potentially reducing costs compared to plant-derived sources such as essential oils, while also providing an eco-friendly method for waste management and resource utilization.

Keywords: *Eco-friendly, cellulosic matters, enzyme activity, Aspergillus niger, yield coefficient*

1. INTRODUCTION

Lemongrass *(Cymbopogon citratus)* belongs to the Poaceae family and is classified within the *Cymbopogon* genus. It has over 120 species and is mostly farmed in tropical and subtropical locations worldwide. It is extensively used in the cosmetics, pharmaceutical, agricultural, and food flavor sectors. *Cymbopogon citrates* is a perennial grass that exhibits reduced robustness compared to other lemongrass types. Typically, it is grown as a ratoon crop. The crop is harvested 4 to 6 months after planting, with further harvests occurring at intervals of 2 to 3 months. The object is severed at a height of 20 cm above ground level. The remaining portion of the crop on land is a pressing concern in regards to air pollution that requires an urgent resolution. The extraction of essential oil from *Cymbopogon citratus* (lemongrass) varies significantly depending on the environment, genetic variety, and care of the culture. However, on a dry basis, the essential oil content is only 1-2% [1, 2]. Due to the limited extraction techniques, the highest yield of essential oil is only 1-2% v/w. Therefore, a large amount of lemongrass is necessary to fulfill the market demand for essential oil. In the second phase of essential oil extraction, about 98-99% of the waste produced is cellulosic waste, which constitutes a significant quantity [3]. Anselme Payen was

the first individual to isolate this remarkable chemical from green plants. Cellulosic materials have always played a significant role as a source of nourishment for farmers to enrich their soil. Additionally used as animal feed. As mandated, they incinerated cellulosic waste to generate heat and prepared meals with firewood.

Cellulose is the substrate of cellulase, which is the most abundant polysaccharide on Earth. Researchers often use this plant material as a primary source for producing microbial metabolites [4]. Microbial production of cellulase enzyme may be derived from cellulosic waste. Cellulases can be produced through solid-state fermentation (SSF) using various substrates, including sugarcane bagasse, wheat bran, wheat straw, rice bran, rice straw, corncobs, banana waste, wheat flour, corn flour, mustard oil cake, sesame oil cake, cotton oil cake, cassava flour, steamed rice, sago hulls, sago humps, and apple pomace [5]. *Trichoderma reesei* RUT C30 has been reported to utilize wheat bran for cellulase production under SSF conditions. Enzymes are produced using straightforward and economical SSF procedures. The biochemistry of the substrate plays a crucial role in influencing cellulase production. Various agricultural residues, including rice straw and husk, wheat straw, sorghum hulls, soybean hulls, groundnut husk, maize stalks and cobs, sawdust, corn stover, sugarcane bagasse, prickly palm cactus husk, and yellow mombin fruit, serve as potential sources for cellulase synthesis [6,7]. The affordability and abundance of these substrates have heightened interest in microbial systems capable of utilizing lignocellulosic biomass for cellulase production. Both aerobic and anaerobic bacteria, as well as white and brown rot fungi and actinomycetes, contribute to cellulase synthesis. In submerged fermentation, the cellulolytic enzyme production of *Nocardiopsis sp.* KNU has been assessed using biomass sources such as water hyacinth, paddy straw, maize straw, soybean husk, and sugarcane bagasse. Various bacteria exhibited notable production of cellulase enzymes at various incubation durations. Various strains and surfaces exhibit distinct incubation periods [8]. Lemongrass may serve as a substrate for the synthesis of Cellulases. The Table 1, indicate the % composition of different component present in lemongrass. The percentage of cellulose is very high in lemongrass and is not affected during the extraction process, as the only essential oil along with few bioactive components is extracted during extraction process.

S. no.	Components of lemongrass	% Dry Weight Composition	Extracted along with essential oil
1	Cellulose	25-35%	No
2	Hemicellulose	20-30%	No
3	Lignin	5-15%	No
4	Extractives	0.2-0.5%	Yes
5	Proteins and Ash	5-10%	No

Table 1: Components of lemongrass oil

Cellulase, designated as an E.C. 3.2.1.4 enzyme, is responsible for hydrolyzing 1,4 β-D glycosidic bonds in cellulose. This enzyme group includes endoglucanase, exoglucanase, cellobiohydrolases, and β-glucosidase, which work together to degrade cellulose into glucose through hydrolysis. Exoglucanases, specifically 1,4-β-D-glucancellobiohydrolase, break down disaccharide units from either end of crystalline cellulose. Endoglucanases, also referred to as 1,4-β-D-glucan-4-glucanohydrolases, are capable of efficiently breaking down amorphous cellulose. These enzymes can also hydrolyze substituted celluloses like CMC and HEC by breaking them down internally. β-Glucosidases play a crucial role in converting cellobiose and other soluble oligosaccharides into glucose [9].

Cellulose is widely utilized across various industries. Recent surveys of the enzyme market highlight the increasing demand for cellulase in sectors such as healthcare, pharmaceuticals, food and beverages, animal feed, textiles, pulp and paper, biofuel production, chemical processing, detergents, and waste management. The use of it in the production of coffee, wine, and fruit juice is associated with the food and beverage industry. Biostoning is particularly beneficial for wet processing of textiles, specifically for denim fabric. This process utilizes biological agents to improve the softness of textile fibers, remove excess dye from fabrics, and improve water absorbance. Additionally, biostoning effectively reduces the formation of pills, creates a cleaner surface structure with

minimal fuzz, and enhances the overall appearance, texture, and color of the fabric, all without the need for chemical coating. Here are a few significant industrial applications for this enzyme [10].

Trichoderma reesei fungal cellulases are the most common textile enzyme [11]. Furthermore, actinomycetes belonging to the genera *Streptomyces* and *Thermobifida*, along with bacteria from *Pseudomonas* and *Sphingomonas*, produce enzymes for textile dye decolorization and degradation. The textile industry's best cellulase uses are biostoning and biopolishing. In other industries, it manufactures laundry detergents as well as cleaning and washing chemicals. Cellulase shows potential as an effective antibacterial treatment for *Pseudomonas* biofilms. Cellulases have remarkable potential in addressing healthcare challenges by effectively combating antibiotic-resistant microorganisms. Ecologically efficient cellulase enzyme producers include fungi, bacteria, and actinomycetes. Cellulases are popular in the paper, coffee, and textile industries because they can biotransform chemicals. It is a prominent biocatalyst that have enabled bio-industries to thrive. Cellulase is produced by several bacteria and fungi. Filamentous fungi are selected for commercial enzyme synthesis due to their higher enzyme production compared to yeast and bacteria. Cellulase production is a common trait across almost all Aspergillus fungus, which gives them a potential advantage in the enzyme industry. *Aspergillus* and *Trichoderma* are acknowledged for their ability to manufacture cellulase [12]. It is a group of enzymes that facilitate the breakdown of cellulose by hydrolysis. Cellulase production from lemongrass waste offers a sustainable solution for enzymatic hydrolysis of cellulose. Lemongrass, rich in lignocellulosic content, serves as an economical substrate for cellulase production. The development of fungus on natural substrates typically occurs at a sluggish pace. However, this constraint may be addressed by using appropriate mechanical and chemical pre-treatment methods on the raw substrate. Pre-treatment causes structural modifications in cellulosic substrates, potentially affecting the physicochemical characteristics of the substrate. The pretreatment step enhances the efficiency of using natural substrates in the manufacturing process [13].

In the past, enzymes that are important for industry have been obtained by a process called submerged fermentation (SmF). This approach is favored due to its ease of management and the ability to regulate environmental conditions like temperature and pH. However, using the solid-state fermentation (SSF) method has the capacity to increase productivity and reduce the costs linked to enzyme synthesis [14]. Filamentous fungi are commonly utilized in solid-state fermentation (SSF) due to their ability to grow on solid substrates with minimal moisture. Research has demonstrated that agro-industrial byproducts, including wheat straw, wheat bran, and rice straw, serve as effective materials for cellulase enzyme production [15]. SSF offers several advantages, including increased efficiency, a simple process, little financial commitment, low energy use, decreased water waste, enhanced product retrieval, the lack of foam accumulation, and applicability to poor countries [16].

Numerous studies have explored cellulase production by filamentous fungi using both submerged fermentation (SmF) and solid-state fermentation (SSF). However, there have been limited studies comparing the cellulase production under these two conditions. The effectiveness of microbial growth for cellulase production using agro-industrial waste as a raw material has been observed in both SmF and SSF. Moreover, studies have analyzed the cellulase production of *Aspergillus niger* in both SmF and SSF systems [17]. The potential uses of cellulases and the effective utilization of cellulosic waste suggest that lemongrass waste could be a viable resource for cellulase enzyme production. This approach may help reduce the costs associated with both lemongrass-derived essential oils and cellulase while simultaneously promoting waste utilization. In current study the kinetic study was performed for the production of Cellulase utilizing lemongrass waste. The substrate concentration was adjusted to maximize microbial growth and product yield. Additionally, the reaction rate and order were determined for the process.

2. MATERIALS AND METHODS

The study was conducted by using chemicals of Himedia, Merk and glasswares of Borosil. The lemongrass variety “Cymbopogon flexuous” was procured from the organic farms of Bareilly, Uttar Pradesh and was kept at room temperature throughout the period during which the experiments were conducted as mentioned in Fig. 1.

Fig. 1: Fresh lemongrass cut from fields

Prior to the essential oil extraction procedure, lemongrass was subjected to convective shade drying. The lemongrass, comprising both the leaves and stems, was cut into tiny pieces using a cutter to maximize the drying surface area. The most effective drying process led to increased oil extraction and improved preservation of qualitative characteristics. The lemongrass was dried in the shade at various time intervals to determine the highest possible amount of essential oil obtained after a certain period. Fig. 2 demonstrates the use of the hydrodistillation technique to extract essential oil from organic lemongrass. An analysis was done to determine if lemongrass waste obtained after hydro-distillation contains any vital components or whether it is completely useless. Lemongrass waste generated from essential oil extraction was utilized as a substrate for microbial cellulase production.

Fig. 2: Pre-distilled lemongrass vs post-distilled Lemongrass

2.1. Isolation of Aspergillus niger from soil

The *Aspergillus niger* was earlier obtained in our laboratory from a soil sample collected from the mango orchard at AUUP Lucknow Campus, Lucknow. The strain was grown on a slant medium composed of 20% potato infusion, 2% dextrose, and 2% agar (PDA) in distilled water, maintaining a pH of 5.6 ± 0.2. The temperature required for incubation was consistently maintained at 28 °C for 96 h [18].

2.2. Preparation of inoculums

A uniform suspension for microbial inoculation was prepared by growing a pure culture of Aspergillus niger on potato dextrose (PD) medium. The inoculum medium contained 200 mg/L of potato infusion, 20 mg/L of dextrose, and had a pH of 5.6 ± 0.2. Incubation was carried out at 28 ± 1 °C for 72 hours. The 10% created inoculums were put into production medium under sterile conditions to facilitate microbial growth and production creation. The addition of inoculum reduces the duration of the lag phase and enhances the yield of the product.

2.3. Production media preparation for Cellulase

2.3.1. Pre-treatment of substrate

The substrate Lemongrass cellulosic waste was pre-treated with 100 ml; 0.1% sulphuric acid v/v prepared in distilled water.

2.3.2. Production Media composition:

Mineral Salt Media (MSM) was used as an enrichment medium. The 20g, 30g, 40g, and 50g pre-treated substrate lemongrass was added to the 500-500 ml MSM in 4 separate 1000 ml Erlenmeyer flask, the sample was then sterilized using an autoclave at 121 °C and 15 psi for 15 minutes. Once the flask had cooled down, it was infected with a 10% concentration of a previously produced inoculum that had been incubated for 72 h. The production flasks were placed in a shaker incubator and mixture was incubated at 28°C with a shaking speed of 100 rpm for 72 hours, with samples collected at 24-hour intervals.

2.4. Quantitative estimation of protein, glucose, and enzyme activity:

The kinetics research on cellulase synthesis was conducted at 24-hour intervals. Protein measurement was performed using Lowry's technique, glucose estimation was done using the DNS method and CMCase activity in the enzyme was evaluated using the DNS method [19].

3. RESULTS AND DISCUSSION:

The results of solvent extraction obtained concluded that post hydro-distillation lemongrass does not have any citral content and is totally a waste product. The lemongrass waste was further utilized for the Cellulase production.

3.1. Kinetics study of Cellulase enzyme production by Solid State Fermentation (SSF):

A kinetic analysis was performed to examine substrate utilization and product formation during the microbial production of cellulase, using cellulosic waste as the substrate. Additionally, a comparative kinetic study was carried out to evaluate cellulase synthesis from lemongrass-derived cellulosic waste at varying concentrations (4%, 6%, 8%, and 10% w/v in MSM).

3.2. Kinetics study for protein content:

The kinetic study observes that the protein concentration was gradually increasing during the process up to 72 h during the Cellulase production. The maximum protein concentration was observed at 4% substrate concentration during Cellulase production. The protein content data analysis was done graphically as shown in Fig. 3.

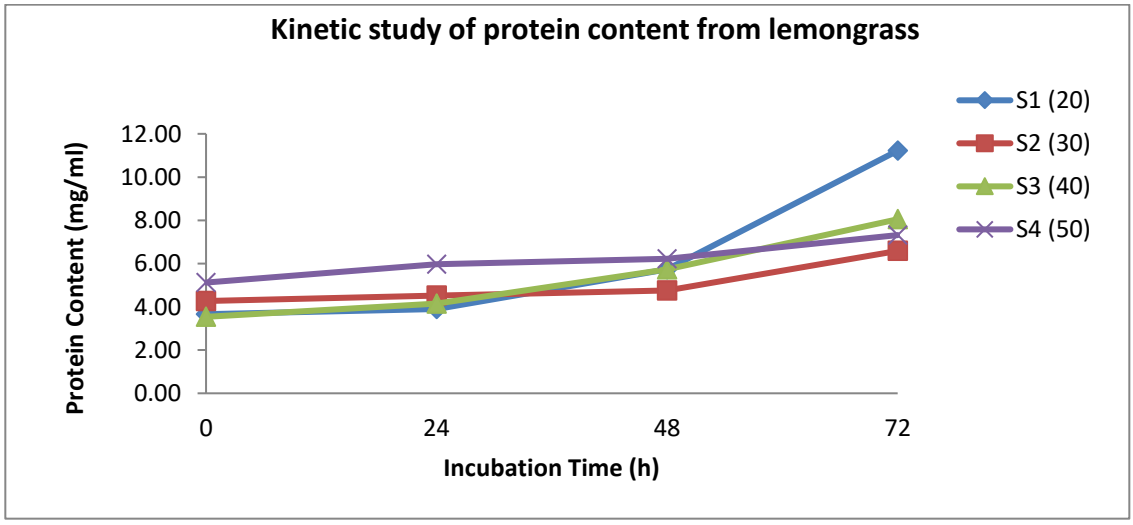

Fig. 3: Comparative kinetic study of protein concentration at different concentration of substrate

3.3. Kinetics study for substrate utilization:

The glucose content was gradually decreasing in all the 4 batches of different substrate, during the kinetic study of the Cellulase production upto 72 h. The substrate utilization data was analyzed graphically as shown in Fig. 4. All the studies of different substrate concentration were showing almost similar pattern.[20]

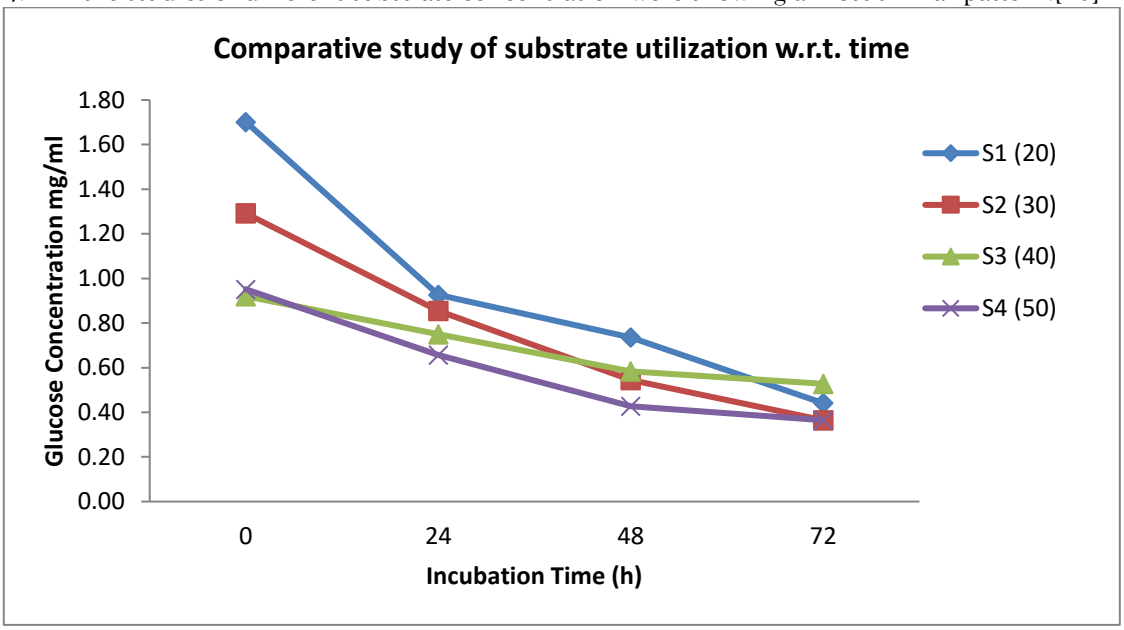

Fig. 4: Comparative kinetic study of glucose concentration at different concentration of substrate

3.4. Kinetics study for enzyme activity and specific enzyme activity:

The enzyme activity was maximum at 48 h incubation in all the 4 batches of different substrate concentration during the synthesis of Cellulase by the *Aspergillus niger* and then decreases till 72 h because of the formation of other by products. The maximum enzyme activity observed was 134.2U/mg/ml in the batch of the substrate concentration 8%, as shown in Fig. 5

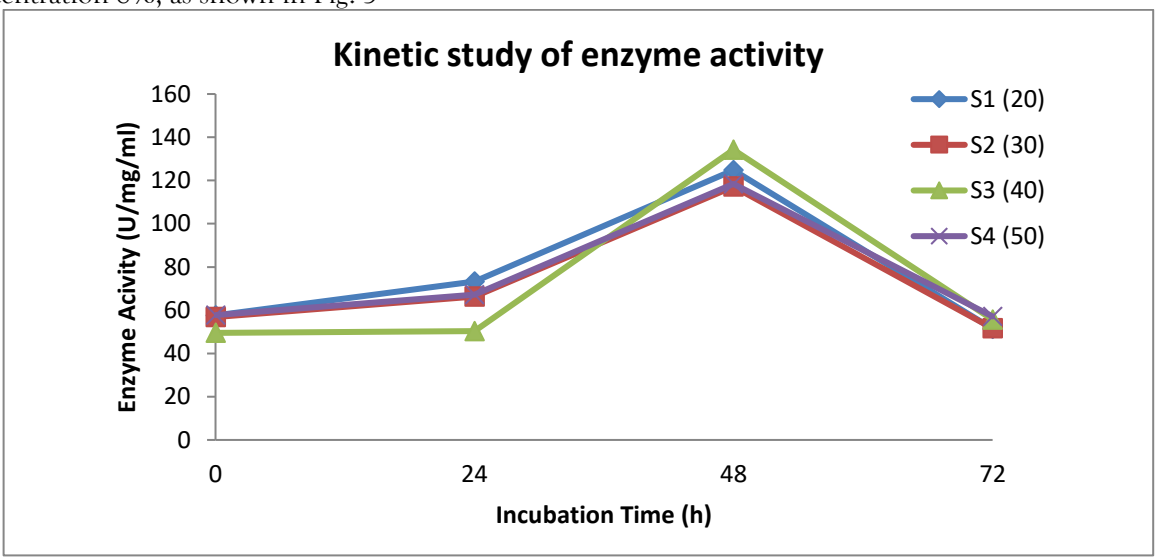

Fig. 5: Comparative kinetic study of enzyme activity for different concentration of substrate

The graphical data shown in Fig. 6, infer that specific enzyme activity increases in almost all the different concentration of lemongrass waste upto 48 h and then decreases for 72 h. The specific enzyme activity was observed high almost at 48 h but maximum was 24.64 U/ml at 6% substrate concentration.[21]

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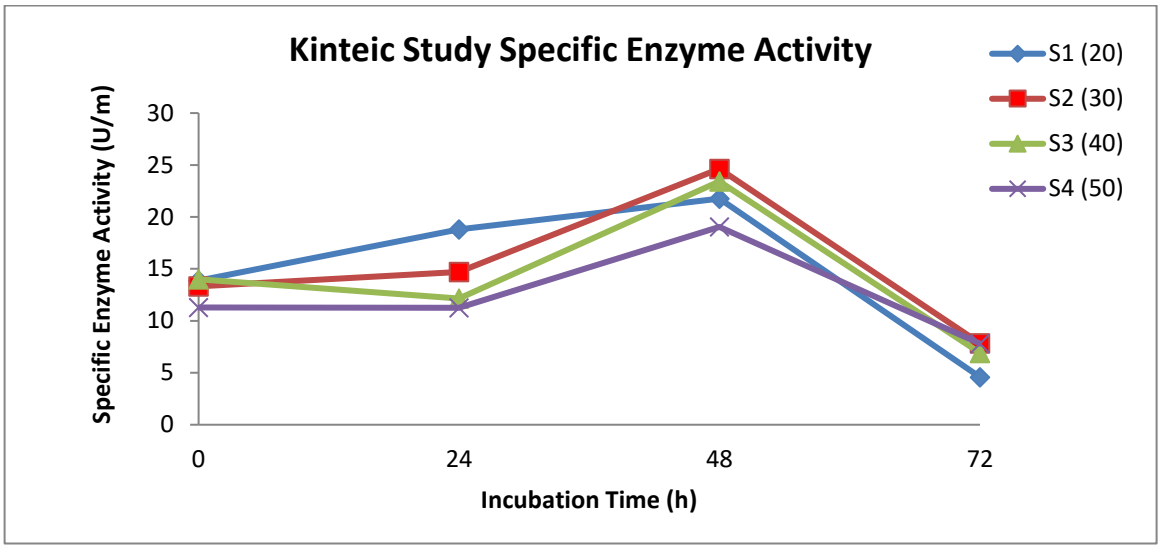

Fig. 6: Comparative kinetic study of specific enzyme activity at different concentration of substrate

3.5. Yield coefficient of product formation with respect to substrate utilization:

The maximum yield coefficient was observed 11.56 g/g for the substrate concentration of 8% in MSM. The yield coefficients for Cellulase enzyme produced with respect to substrate concentration are mentioned in Table 2.

S. no.	Sample	Yield coefficient ($Y_{P/S}$)
1	S1	6.00
2	S2	2.49
3	S3	11.56
4	S4	3.73

Table 2: Yield coefficient of Cellulase enzyme in samples of different substrate concentration

3.6. Rate of Reaction for substrate utilization and product formation:

The rate of product formation was increasing while the rate of substrate utilization was decreasing for all quantities of lemongrass with time up to 72 h and the best fit is observed for 40 gm (8%) of lemongrass as shown in the Fig. 7.

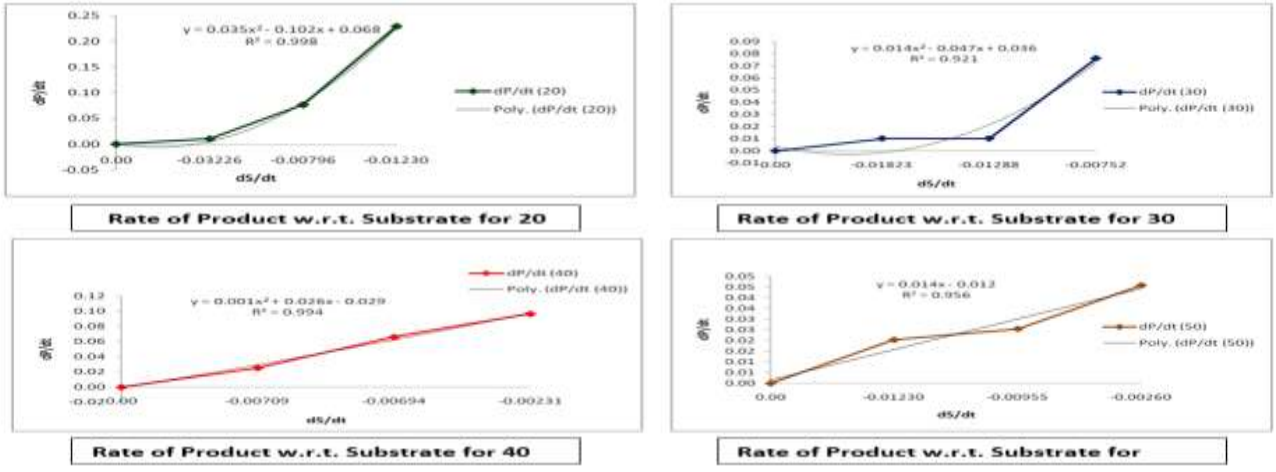

Fig. 7: Comparison of rate of product formation with respect to substrate utilization for all different quantity of lemongrass

3.7. Order of reaction

The order of reaction analyzed for the process and as the plot curve justifies that the order of reaction is 1st order for all quantities of lemongrass as shown in Fig. 8.

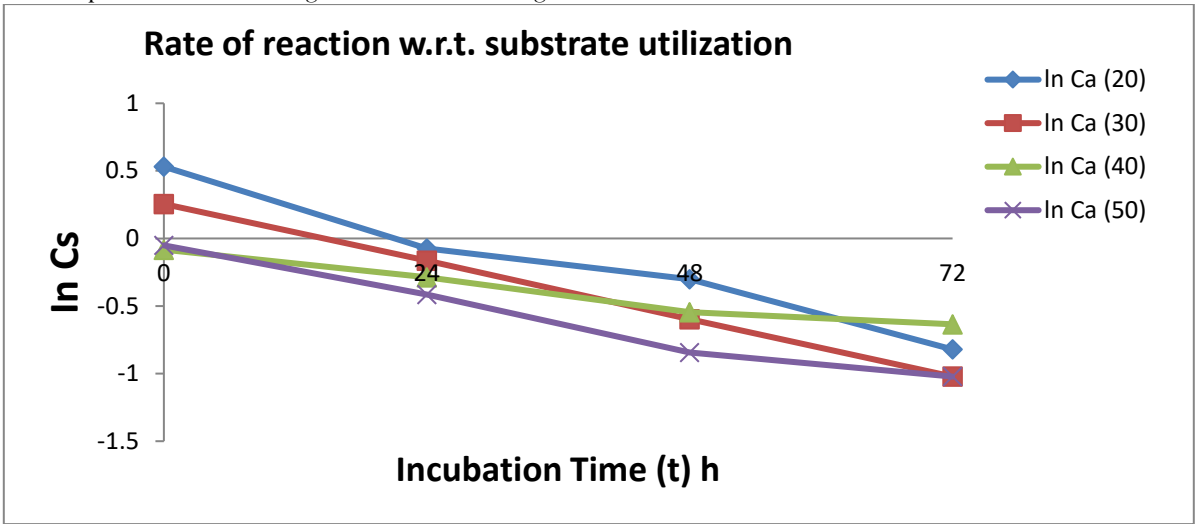

Fig. 8: Rate of reaction of 1st order for substrate utilization

The high cost of lemongrass essential oil is mostly attributed to its poor oil output, which usually falls within the range of 0.9% to 1% of the total dry weight of the lemongrass plant. The objective of this research was to reduce the expensive production cost of lemongrass essential oil by investigating the possibility of using the leftover waste material from the oil extraction process. The objective was to mitigate or diminish the total expense by concurrently generating commercially useful cellulase enzymes from this waste. In order to do this, the waste material generated from lemongrass was used as a substrate for the cultivation of cellulase enzymes via the utilization of the fungus *Aspergillus niger*. The research conclusively showed that *Aspergillus niger* was capable of efficiently synthesizing cellulase when grown using lemongrass waste as a substrate. The cellulase enzyme activity reached a maximum of 134.2 U/mg/ml when an 8% concentration of lemongrass waste substrate was used in the batch procedure. Prior research has shown the synthesis of cellulase by *Aspergillus niger* on several substrates, including wheat straw and sawdust. The highest recorded CMCase activity was 338.02 U/ml/min and 249.10 U/ml/min, respectively [22]. Previous studies have documented the highest CMCase activity for wheat straw and sawdust as 0.024 U/ml/min and 0.018 U/ml/min, respectively [23]. The cellulase enzyme activity found in this work, using lemongrass waste as a substrate, has shown significant promise in comparison to the outcomes obtained from other waste materials. This underscores the potential of lemongrass waste as a feasible substrate for cellulase synthesis.

Kinetic research was conducted on the cellulase enzyme derived from lemongrass waste. The study focused on analyzing the protein content, glucose concentration, and enzyme activity of the generated cellulase enzyme. Different concentration of substrate was taken (4%w/v, 6%w/v, 8%w/v, and 10%w/v) to study the activity of enzyme. Kinetics study of cellulase production from lemongrass concluded that protein content increases for all quantity of substrate with time up to 72 h while the glucose concentration decreases for all quantity of substrate with time up to 72 h as the enzyme production occurs by the fungus *Aspergillus niger* and the specific enzyme activity increases for all quantity of lemongrass with time up to 48 hrs and then decreases till 72 h. The optimum incubation time was 48 h for the Cellulase production in the case of lemongrass waste as a substrate, whereas previous research reports are showing 72 h incubation time for the substrate Wheat straw and Saw dusk [24]. The maximum substrate utilization of lemongrass observed was 58%w/w and that ranges from 37%w/w to 58%w/w for all the studies concentration of substrate taken (4%w/v, 6%w/v, 8%w/v and 10%w/v), whereas the substrate Wheat straw and Saw dusk was showing maximum substrate utilization upto 11%w/w and 23 %w/w respectively [25,26]. Rate of product formation with respect to substrate utilization increases for all

quantities of lemongrass with time up to 72 hrs and the best fit is observed for 8%w/v of lemongrass as well the maximum yield was also achieved at 8%w/v substrate concentration of lemongrass waste. The substrate utilization was showing the first order reaction for the process. The results of lemongrass waste for the Cellulase production were more promising as compared to previously reported cellulosic waste. [27,28]

4. CONCLUSION:

A significant amount of cellulosic waste is produced by the agricultural and industrial sectors. It is necessary to use the produced cellulosic wastes as a substrate to manufacture different biomolecules. The use of trash is necessary for two reasons. Firstly, it helps in minimizing and reusing waste to protect the environment. Secondly, it adds value to the production process and reduces the cost of the final product. The waste from lemongrass was used for the synthesis of the cellulose enzyme and showed encouraging outcomes. The production of 1–2% v/w essential oil from lemongrass resulted in a waste generation of 98–99% w/w. The concentration of the essential oil derived from lemongrass was insufficient, resulting in a high cost. Utilizing lemongrass waste may increase the value of the product and eventually lower the cost of the essential oil. Due to its economic significance, it is necessary to generate the cellulose enzyme at a cheap cost by using inexpensive substrates or waste materials as substrates. Utilizing lemongrass waste for the production of cellulose may effectively mitigate environmental waste and decrease the expenses associated with essential oil and cellulose production. The efficient concentration of Lemongrass waste was utilized to produce Cellulase. The current study can be further optimized for achieving maximum Cellulase productivity at pilot plant so that it can be processed at industrial scale for getting actual cost benefits for the products. This study can benefit aroma industry by reducing the cost of essential oil and can also benefit Cellulase enzyme industries. The reduce cost of Cellulase can benefit the various others industries where the Cellulase are applicable.

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