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Development of protocol for biofuel production from cellulose

Manjari singh*
Department of Biotechnology, SR Institute of Management & Technology, Dr. APJ Abdul Kalam Technical University, Lucknow

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ABSTRACT

Worldwide, there is an increasing need for ethanol production as a result of rapid industrialisation and population expansion. Due to their primary importance as food and feed, conventional crops like maize and sugar cane cannot produce enough biofuels to meet the world's demand. Ligno-cellulosic materials, including pseudo stem debris from bananas, have value and make desirable feedstock for the production of bioethanol. Utilising agricultural industrial waste has the advantages of being affordable, renewable, and plentiful. Bioethanol from banana pseudo stem waste has the potential to be a noteworthy technological advancement thanks to an efficient method. The real effort involves employing acid pre-treatment, hydrolysis, and fermentation by Aspergillus Niger to bio convert cellulose from banana pseudo stem waste, collected from an agro-industry, into ethanol. Pre-treating the Banana Pseudo stem waste fibres with diluted sulfuric acid and then heating the resulting combination to a high temperature to disrupt the cellulose's crystalline structure in order to make it easier for diluted acids to hydrolyse the cellulosic component. The cellulose content is hydrolysed into reducing sugars using a dilute acid hydrolysis process that requires 72 hours of incubation at a high temperature. The hydrolysed waste is then fermented with Aspergillus Niger under ideal incubation conditions to create ethanol.

Keywords: Biofuel production; Cellulose; Banana pseudo stem; Aspergillus niger; Fermentation; Bioethanol

Address for correspondence:

Manjari singh,
Department of Biotechnology, SR Institute of Management & Technology, Dr. APJ Abdul Kalam Technical University, Lucknow
E-mail: singhmanjari143@gmail.com
Phone No.: +917518453931

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INTRODUCTION

Bioethanol, also known as renewable ethanol or agroethanol, is a type of biofuel produced from various organic materials, primarily plant biomass. It is considered a sustainable alternative to fossil fuels due to its renewable nature and lower carbon emissions. Bioethanol can be used as a substitute for gasoline in vehicles, either in pure form or blended with gasoline. The production of bioethanol involves the fermentation of sugars derived from biomass sources such as corn, sugarcane, wheat, cellulose, and lignocellulosic waste materials. These raw materials undergo a series of biochemical and chemical processes to convert the sugars into ethanol. The main cost element in bioethanol production is the feedstock, which determines the economic viability of the process.

The increasing global demand for ethanol production is driven by several factors, including rapid industrialization, population growth, and the need to reduce dependence on fossil fuels. Conventional crops like corn and sugarcane have limitations in meeting the growing demand for biofuels due to their primary value as food and feed sources. Therefore, the utilization of lignocellulosic biomasses, including agricultural waste materials like banana pseudo stem waste, has emerged as an attractive alternative for bioethanol production. The use of agro-industrial waste as a feedstock for bioethanol production offers several advantages. It is cost-effective, abundant, and contributes to the preservation of the environment by utilizing waste materials that would otherwise undergo natural degradation. Banana pseudo stem waste, for instance, represents a significant biomass resource with low acquisition costs. Its conversion into ethanol can not only address waste management concerns but also contribute to the production of a valuable biofuel.

The bioconversion of cellulose from banana pseudo stem waste into ethanol involves various steps, including acid pre-treatment, hydrolysis, and fermentation by microorganisms such as Aspergillus Niger. These processes break down the complex structure of cellulose into fermentable sugars, which are then converted into ethanol through fermentation. Research studies have shown promising results, indicating the potential to obtain a significant volume of ethanol from each ton of dry biomass treated. Ethanol purification is a critical operation in bioethanol production, especially when it is intended for blending with gasoline. Anhydrous ethanol, with low water content, is required for complete miscibility with gasoline. Various separation techniques, including adsorptive distillation, are employed to remove water from ethanol. The development

of bio-based adsorbents from banana waste as an alternative to molecular sieves for ethanol dehydration is being explored to optimize energy efficiency and reduce costs [1].

Derived bioethanol: Bioethanol is produced from a wide variety of raw materials including corn starch, sugar cane and lignocellulosic waste materials like waste biomass from banana. The feedstock is the primary cost component in the production of bioethanol. Worldwide Demand for ethanol production is increasing continuously due to rapid industrialization and growth in population. Conventional crop like sugarcane not able to meet the global demand of biofuel production due to primary value of food, the use of lignocellulosic biomasses has been considered an appealing option for replacing non-renewable energy sources with a more sustainable alternative.

The Argo industrial wastes stand out within these biomasses, due to their availability and low acquisition cost. Making use of this biomass for the production of alcohol for fuel could be very attractive alternative by not only contributing to the preservation of the environment through removing this waste from the land.

The advantage of utilizing agro industrial waste is abundant, renewable and cost effective. The utilization of Banana Pseudo stem waste for bioethanol production holds promise as an economically viable technology. This involves the bioconversion of cellulose from Banana Pseudo stem waste obtained from an Agro-industry into ethanol through acid pre-treatment, hydrolysis, and fermentation using Aspergillus Niger. According to Souza et al. (2014), considering the saccharification yield and fermentation efficiency, it is possible to obtain 187L of ethanol from each ton of dry biomass treated.

Fruit wastes, including banana, are not only the cheapest but also easily available sources for bioethanol production. In a study by Janani et al. (2019), a comparison of ethanol efficiency produced from different fruit wastes was conducted, and the concentration of ethanol in banana was found to be 5.40% (Andrew et al., 2014). Talkad et al. (2016) obtained results for Saccharomyces cerevisiae - EMS & UV Mutated, revealing purity levels of 73.5% and 53.9% respectively, while acid treatment showed a purity level of 53.9%. Ethanol purification is a critical operation for various purposes in bioethanol production.

For blending with gasoline ethanol to be used must be anhydrated since hydrated ethanol with more than 2% (v/v) water is not completely miscible with gasoline. Distillation can concentrate the ethanol up to azeotropic point but further dehydration technique adds costs to Bioethanol production. Among all separation techniques for ethanol dehydration, adsorptive distillation stands out as the most effective method. Molecular sieves used for dehydration purpose which require high temperature for regeneration so we should find some alternative adsorbent for energy optimization. There is an incentive to find new cost effective bio based material sources for this application Deposition of the bio based adsorbents is also environmental friendly as these are biodegradable reported by Benson and George. Ability of starch and cellulose in selectively adsorption of water is a result of interaction in the form of hydrogen bonding between free hydroxyl groups (-OH) units and the water molecules given by K. Beery, and M. Ladisch. In this work, new bio based adsorbents that are able to separate the azeotropic water –ethanol mixture are developed from banana waste. The water content in the feed solution varied and final ethanol the concentrations obtained some alternative adsorbent for energy optimization [2].

There is a strong motivation to discover new, cost-effective bio-based materials for this application. It has been reported by Benson and George (2017) that the use of biodegradable bio-based adsorbents is environmentally friendly. The ability of starch and cellulose to selectively adsorb water is a result of the interaction between the hydroxyl groups (-OH) and water molecules, as explained by K. Beery and M. Ladisch (1996). In this study, novel bio-based adsorbents derived from banana waste have been developed to effectively separate the azeotropic water-ethanol mixture. The water content in the feed solution varied, resulting in different concentrations of ethanol obtained.

The increasing global demand for ethanol production is driven by rapid industrialization and population growth. However, conventional crops like corn and sugarcane are unable to meet this demand due to their primary value as food and feed. Therefore, the utilization of lignocellulosic substances such as Banana Pseudo stem waste becomes an attractive feedstock for bioethanol production. Utilizing agro-industrial waste offers several advantages, including cost-effectiveness, renewability, and abundance. The conversion of Banana Pseudo stem waste into bioethanol presents a promising and economically viable technology. This involves the bioconversion of cellulose from Banana Pseudo stem waste obtained from an Agroindustry into ethanol using acid pre-treatment, hydrolysis, and fermentation by Aspergillus Niger.

The process begins with the pre-treatment of Banana Pseudo stem waste fibers, where they are treated with dilute sulfuric acid and subjected to high temperatures. This process aims to break down the crystalline structure of cellulose, facilitating the subsequent hydrolysis of the cellulosic component by dilute acids. The hydrolysis process converts the cellulose content into reducing sugars through a 72-hour incubation period at high temperatures. Finally, the fermented hydrolysed waste is subjected to fermentation using Aspergillus Niger under appropriate incubation conditions, resulting in the production of ethanol. This comprehensive process allows for the efficient conversion of Banana Pseudo stem waste into valuable bioethanol [3].

Status of fossil fuel in the world: Fossil fuels are formed through natural processes involving the anaerobic decomposition of buried deceased organisms, storing energy derived from ancient photosynthesis. These fuels, such as petroleum, coal, and natural gas, are the result of organisms that lived millions of years ago, with some dating back as far as 650 million years. Fossil fuels have high carbon content and have traditionally been the primary energy sources.

In today's energy markets, there is a significant surge in energy demand driven by strong economic growth, particularly in developing countries like China and India. However, the capacity to meet this escalating demand for fossil energy is hindered by limited production capacity and inadequate infrastructure, including pipelines, refining facilities, and terminals (CERA, A global sense of energy insecurity). Many nations are increasingly concerned about their energy security and are moving towards nationalizing energy production and distribution. Consequently, substantial investments in production capacity and infrastructure are necessary to ensure access to essential energy resources. India, for instance, consumes more than 250 million tonnes of fossil fuels annually, with diesel alone accounting for approximately 40 million tonnes. This highlights the significant reliance on fossil fuels within India's energy consumption patterns [4].

Need for biofuel: The escalating global cost of fossil fuels has prompted major economies worldwide to explore renewable and more affordable alternatives to meet their energy needs. Energy efficiency plays a vital role in the energy landscape as it enables a reduction in the reliance on primary energy resources and leads to significant savings. Opportunities for enhancing energy efficiency exist throughout the entire energy value chain, offering substantial potential for improvements.

The 2013 WEC report titled "World Energy Perspective: Energy Efficiency Technologies" provides quantitative indicators for different phases of the value chain and specific industries. However, achieving energy efficiency is not solely dependent on utilizing efficient technologies; it also requires consideration of economic factors. Energy efficiency technologies will be widely adopted when they are economically viable, demonstrates long-term benefits, and encounter no significant implementation barriers. This comprehensive approach ensures that energy efficiency solutions not only deliver environmental advantages but also make economic sense [5].

What is biofuel?: Biofuels are a renewable form of energy that harnesses the energy stored in biomass, which can be derived from various sources such as plants, animals, microorganisms, and organic wastes. They serve as alternative substitutes to alleviate the scarcity of fossil fuels. When compared to fossil fuels, biofuels offer several advantages. They can be in the form of liquids or gases, depending on the specific type of biofuel, the feedstock used, and the production technology employed. One prominent example of a biofuel is bioethanol, which is derived from agricultural sources rather than petrochemicals. Bioethanol is a transparent, colourless liquid that has a boiling point of 78 °C. It finds widespread use in industries, medicine, and as a motor fuel. Additionally, bioethanol is utilized in antifreeze compounds, rocket fuels, pharmaceuticals, printing, cosmetics, and other applications. By tapping into the potential of biofuels like bioethanol, we can reduce our reliance on non-renewable resources and explore more sustainable energy alternatives.

Renewable alternatives to fossil fuels, such as biofuels, have gained prominence due to their comparable quality to diesel and petrol. Moreover, biofuels exhibit lower levels of pollution compared to their fossil fuel counterparts. This has led to increased global efforts to produce ethanol from starch and sugar-producing crops, driven by environmental concerns and the desire to reduce dependence on imported fossil fuels. Among biofuels, bioethanol and biodiesel are the most widely produced types.

Government policies play a significant role in driving the use of biofuels for transportation. Concurrently, biodiesel production in a particular country expanded eleven-fold, increasing from less than 1 billion to nearly 11 billion liters. Collectively, these fuels accounted for 1.8% of the world's transport fuel by energy value. In Europe, there has been a consistent upward trend in biofuel utilization in road transport over the past decade, rising from 0.1% in 1997 to 2.6% in 2007. These statistics highlight the growing significance of biofuels in addressing energy needs while considering environmental sustainability [6].

How biofuels can be utilized as source of energy: Biofuels can penetrate the primary transport markets through two approaches: blending into the existing fuel supply or direct usage with minimal engine modification. The introduction of ethanol-blended petrol commenced in January 2003, with the current implementation at a 5% blend level. However, there exists a shortfall of 225 million litres of ethanol to meet the demand of oil companies, estimated at 435 million litres (Anonymous, 2006). To bridge this gap, the exploration of alternative ethanol sources becomes imperative.

Both bioethanol and biodiesel can be blended with conventional petrol and diesel, respectively. Currently, blends of up to 5% biofuel are authorized for use in vehicles across the EU and Asian countries. These biofuels are expected to be the primary focus of production in the EU and Asian regions until 2010. In line with this, India has implemented reforms by incorporating 10 to 15% ethanol blending in its gasoline usage. Among the top ethanol-producing nations, Brazil leads with the highest production volume of ethanol (15,099 million liters per year), followed by the U.S. (13,381 million liters per year), China (3,649 million liters per year), and India (1,749 million liters per year). Sugar cane is the predominant source of ethanol in Brazil, while corn serves as the primary feedstock for ethanol production in the U.S. (Peterson, 2006). These figures highlight the significant contributions of various countries to global ethanol production and the diverse sources used in its manufacturing process [7].

Status of biofuel production the India: India holds the fifth position globally, following China, Japan, Russia, and the U.S., in terms of fossil fuel consumption. As a significant crude oil importer, India expends approximately Rs. 1,200 billion in foreign exchange each year to fulfil 75% of its oil requirements (Anand, 2006). This reliance on imports has negatively impacted the country's balance of payments, especially with the unprecedented surge in crude oil prices.

To address this challenge, many crude oil importing nations, including India, have implemented policies mandating the addition of 5 to 10% ethanol in petrol and diesel. As a result, there has been a significant increase in ethanol

production in recent years. These government mandates play a crucial role in meeting international agreements and national targets for reducing greenhouse gas (GHG) emissions. They also contribute to the growing demand for ethanol-based biofuels. By incorporating ethanol into gasoline, countries aim to enhance energy security, reduce dependence on fossil fuels, and mitigate the environmental impact of transportation. These mandates serve as proactive measures toward achieving sustainable and greener energy solutions while promoting the transition to a low-carbon economy.

Substrates used for biofuel: Bioenergy encompasses a wide range of energy fuels derived from various biological sources and converted through different technologies to generate heat, power, liquid biofuels, and gaseous biofuels. In developing countries, the term "traditional biomass" primarily refers to fuel wood, charcoal, and agricultural residues utilized for household cooking, lighting, and space heating. There exists a diverse array of potential biofuels, but the globally recognized ones are bio-ethanol and biodiesel. Bio-ethanol can be produced from several crops such as sugarcane, corn (maize), wheat, and sugar beet. On the other hand, biodiesel is derived from straight vegetable oils, both edible and non-edible, as well as recycled waste vegetable oils and animal fat.

Leading countries in the production of biofuels for transportation include the USA, Brazil, and the European Union. In the United States, ethanol production primarily utilizes corn, while in Brazil; it relies heavily on sugarcane ethanol. In the European Union, biodiesel production mainly revolves around rapeseed. These biofuels play a significant role in diversifying the energy mix, reducing greenhouse gas emissions, and promoting sustainable energy sources. With advancements in bioenergy technologies and increasing global focus on renewable fuels, bio-ethanol and biodiesel hold promise as key components of a more sustainable and environmentally friendly energy future [8, 9].

The industrial processes involved in pulp and paper production, tobacco processing, pig iron manufacturing, and other sectors generate byproducts such as bark, wood chips, black liquor, and agricultural residues. These byproducts have the potential to be converted into bioenergy. By utilizing renewable non-fossil carbon sources like organic wastes and biomass derived from various organic matter (such as plants, grasses, fruit wastes, and algae), a continuous and sustainable energy supply can be ensured. The cost-effectiveness of ethanol production through fermentation is heavily influenced by the raw material expenses, which constitute more than half of the production costs. Therefore, securing a reliable and affordable supply of raw materials is crucial to achieve lower production costs. The utilization of agro-industrial and food processing wastes for the production of value-added products has gained significant attention. This approach not only contributes to energy generation but also reduces environmental pollution.

Among the various biofuels available in the market, the most common one is fatty acid methyl ester/s (FAME) or biodiesel. Biodiesel can be produced from vegetable oil crops like rapeseed, palm, or soybean, as well as from the conversion of waste materials such as cooking oil. This diversification of feedstock sources for biofuel production enhances sustainability and reduces dependence on traditional fossil fuels. By effectively harnessing these bioenergy resources and optimizing the utilization of waste materials, we can achieve a more environmentally friendly and economically viable energy landscape [10].

These biofuels are specifically targeted for different market segments. Bioethanol is produced through microbial fermentation of various starches and sugars derived from crops such as sugar beet and wheat. Other substrates used include Cassava Starch, fruit wastes, corn flour, Jackfruit Seeds, sweet sorghum, sweet potato, and more. These bioethanol varieties are primarily aimed at the gasoline market as blending components. In addition to bioethanol, there is also a focus on producing purified and liquefied biogas to cater to the natural gas market. The majority of ethanol currently produced is classified as first-generation biofuel, which is derived from the fermentation of sugar or starch obtained from food crops such as wheat, corn, sugar beet, sugar cane, etc. However, it's worth noting that an increase in ethanol production from these sources can potentially contribute to a rise in food prices, as it leads to higher demand for corn and wheat.

To address these concerns and promote sustainability, second-generation biofuels have emerged. These biofuels are derived from starch-rich crops and lignocellulosic crops, which offer promising alternatives. Utilizing low-cost feedstock for fuel-ethanol production is a key objective in order to reduce the final product cost. Waste materials generated by the food industries are being explored as viable substrates, provided that they are available in large quantities and their transformation into ethanol is demonstrated to be feasible. By diversifying the feedstock sources and exploring waste materials, the biofuel industry aims to achieve both economic and environmental sustainability in the production of bioethanol and other biofuels [11].

Fruit processing waste as source of biofuel: By employing mechanical drying techniques, various agricultural wastes such as mango peel, mango kernel, citrus peel, pineapple peel, and tomato processing wastes can be preserved throughout the year. Previous studies have reported ethanol production concentrations of 4.02% (w/v) in citrus peel waste, 3.5% (w/v) in grapefruit peel, and 4.2% (w/v) in pineapple peel (Ban-koffi and Han, 1990; Nishio et al., 1980; Wilkins et al., 2007).

For industrial alcohol production, the yeast Saccharomyces cerevisiae and the facultative bacterium Zymomonas mobilis are considered favorable candidates. Z. mobilis offers advantages over S. cerevisiae in terms of ethanol productivity and tolerance. However, yeast remains the preferred choice for commercial ethanol production due to its ability to ferment glucose into ethanol as the predominant product. Yeast is known for its high ethanol tolerance, rapid fermentation rates, and resilience to temperature and substrate concentration variations (Linden

and Hahn-Hagerdal, 1989).

Hang and colleagues have reported a solid-state fermentation process that yields approximately 42 liters of ethanol per metric ton of wet pomace. This represents an energy recovery of approximately 20% of the total energy content in the pomace. Gupta and associates conducted a study on the solid-state fermentation of apple pomace using various organisms such as Saccharomyces cerevisiae, S. diastaticus, Pichia fermenting, Candida utilis, and C. tropicalis. Among these organisms, S. diastaticus demonstrated the highest yield, reaching a maximum of 2.8% with a fermentation efficiency of 43.8%. S. cerevisiae followed with a net yield of 2.6% and a fermentation efficiency of 40.6%. The fermentation efficiency of these strains can be enhanced by supplementing the substrate with nitrogen, phosphorus, and trace elements. Nitrogen supplementation was found to have a greater impact on alcohol production compared to phosphate or trace element supplementation, likely due to the specific requirements of each strain. During mango processing, significant quantities of solid and liquid wastes are generated. These include stones, stalks, trimmings, and fibrous materials, which are obtained during the raw material preparation phase.

The mango processing industry contributes approximately 40 to 50% of the total fruit waste, with 5 to 10% being pulp waste and 15 to 20% kernel waste. Liquid waste refers to the waste material generated during fruit washing, packaging, blanching, cooling, and cleaning of plant and machinery. Finding a suitable utilization for this mango waste presents both a necessity and a challenge. For instance, if a factory processes five tons of Totapuri mangoes per hour, it would generate approximately six tons of peel waste per day during an 8-hour work period. In India alone, an estimated 0.4 to 0.6 million tons of mango peel waste is generated annually (Anonymous, 2004). Currently, this waste is either used as cattle feed or discarded in open areas, contributing to environmental pollution [12, 13].

MATERIALS AND METHODS

Collection of sample

Fruits or Cellulosic waste was collected from local market from Lucknow

Iodine test

1. Add a few drops of iodine
3. Appearance of deep blue color.
4. Starch in the solution.
5. Test solution.

Benedict's test

- To 2 mL of Benedict's reagent
- Add five drops of the test solution to the mixture.
- Place the mixture in a water bath and heat it for duration of five minutes.
- Cool the solution.
- Formation of red, yellow or green
- Colour/precipitate.
- The sugar estimation method employed is the dinitrosalicylic acid method, which is a simple, sensitive, and easily adaptable technique for handling a large number of samples simultaneously.

Medium and chemicals: All chemicals used as were of analytical grade purchased from E-Merck, Sigma. MP biomedical, (USA). Media were purchased from Hi-media (Hi-media, India) as laboratory use quality and chemicals like sodium alginate, calcium chloride, Starch, Glucose, ammonium sulphate [14] (**Tab. 1, 2.**).

One ml of individual solutions was mixed in 492 ml of DW to bring the vol. up to 500 ml. Dried ingredients were mixed prior to addition of water. Then 500 ml of DW were added. Mineral solution and basal solution was mixed so that the final vol. was one litre [15] (**Tab.3.**).

Biochemical estimations

Sugars

Reducing sugar estimation: Sample preparation: 2 ml of culture filtrate was neutralized with 0.1 N NaOH solution, using phenolphthalein an indicator. Then 2 ml of lead acetate (45%) was added. The sample was allowed to sit at ambient temperature for duration of 10 minutes. Then 2.2 ml of 23% potassium oxalate was added and volume was made up to 50 ml with distilled water. It was filtered through Whatman filter paper for further used sugar tests.

Preparation of DNS reagent: 1 g. of 3, 5 Di-Nitro

Tab. 1. Growth medium used for amylolytic micro-organism (MP-7 media).

Component	g/liter
Monopotassium phosphate	4
Disodium phosphate	6
Ammonium sulphate	2
Starch	10

Tab. 2. Minerals.

Mineral	**Solution**
FeSO4	0.2 g + 200 ml DW
MgSO4	4 g + 200 ml DW
CaCl2	0.2 g + 200 ml DW
Boric Acid	0.002 g + 200 ml DW
MnSO4	0.002 g + 200 ml DW
ZnSO4	1.14 + 200 ml DW

Tab. 3. Potato dextrose broth (PD Broth).

Component	g/Litre
Peptone	10
Malt extracts	3

Salicylic Acid was mixed with 20 ml of 2N NaOH. 30 gram of Na K Tartrate was added and volume was made up to 100 ml. 0.4 mL substrate was taken in a fresh tube and 0.1 ml of enzyme total volume was make up to 0.5 ml and 0.5 ml of distilled water was added. Then 1 ml of 3, 5 Di-Nitro salicylic acid was mixed in the solution and kept in boiling water bath for 10 min. After 10 min, the samples were taken out and cooled under running tape water. 10 ml of DW was added and reading was taken at 546 nm [16].

Estimation of sugar by Folin-Wu method

Principle: In the presence of glucose, the alkaline copper reagent undergoes a reduction reaction, converting cupric ions to cuprous ions. Alternatively, cupric sulfate can be transformed into cuprous oxide. Additionally, glucose reduces phosphomolybdic acid to phosphomolybdous acid, resulting in a blue color when the optical density is measured at 420 nm.

Reagents required

To prepare the alkaline copper-reagent, begin by dissolving 40 grams of anhydrous sodium carbonate (Na_2CO_3) in approximately 400 mL of water. Transfer this solution to a 1-liter flask. Next, dissolve 7.5 grams of tartaric acid in the sodium carbonate solution. In a separate container, dissolve 4.5 grams of CuSO4 in 100 mL of water. Combine the two solutions and thoroughly mix them together. Finally, add enough water to bring the total volume up to 1 liter [17].

Phosphomolybdic acid reagent: Add 5 gms of sodium tungstate to 35 gm of 5 Di-Nitro salicylic acid was mixed in the solution and kept in boiling water bath for 10 min. After 10 min, the samples were taken out and cooled under running tape water. 10 ml of DW was added and reading was taken at 546 nm. 5 Di-Nitro salicylic acid was mixed in the solution and kept in boiling water bath for 10 min. After 10 min, the samples were taken out and cooled under running tape water. 10 ml of DW was added and reading was taken at 546 nm. 5 Di-Nitro salicylic acid was mixed in the solution and kept in boiling water bath for 10 min. After 10 min, the samples were taken out and cooled under running tape water. 10 ml of DW was added and reading was taken at 546 nm [18].

PROCEDURE

Pipette out a standard glucose sodium solution within the range of 0 to 1 mL. Make up the volume to 2 mL by adding distilled water to the pipetted solution. Add 2 mL of alkaline Cu-reagent to all the test tubes containing the glucose sodium solution. Mix the contents of the test tubes thoroughly, Place the test tubes in a boiling water bath and keep them there for 8 minutes. Wait for 10 minutes and mix the contents of the test tubes. Make up the volume in each test tube to 25 mL with distilled water; Take the optical density reading at 420 nm using a spectrophotometer [19].

Protein estimation

Protein concentration was estimated by Lowry et al. (1951) method using with Bovine serum albumin (BSA) as a standard with minor modifications. This method is most commonly method for determination of protein in cell free extraction because if its high sensitivity and efficiency of estimating quantities as low as 20 µg of proteins can be measured. The –CONH-(Peptide bond react with copper sulphate in the alkaline medium to give blue colour complex). Furthermore, it is worth mentioning that the presence of tyrosine and tryptophan residues in proteins can lead to the reduction of the hosphomolybdate and phosphotungstate components present in the Folin-Ciocalteu reagent, resulting in the formation of blue-colored products.

Composition of protein quantification reagent

Alkaline Na Alkaline Na_2CO_3 reagent: Dissolve 2.0 g Na_2CO_3 in 0.1 NaOH and volume make up to 100 ml with 0.1 N NaOH.

Copper sulphate reagent: 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate solution.

Alkaline sodium copper sulphate reagent: 1ml of reagent 2 was mixed with 50ml of Reagent 1. It is important to note that the mixture described in the procedure is unstable and should be freshly prepared before each use. Folin's reagent: Dilute the reagent appropriately so that its, is 1 N in respect of its acid content.

0.1 N NaOH.

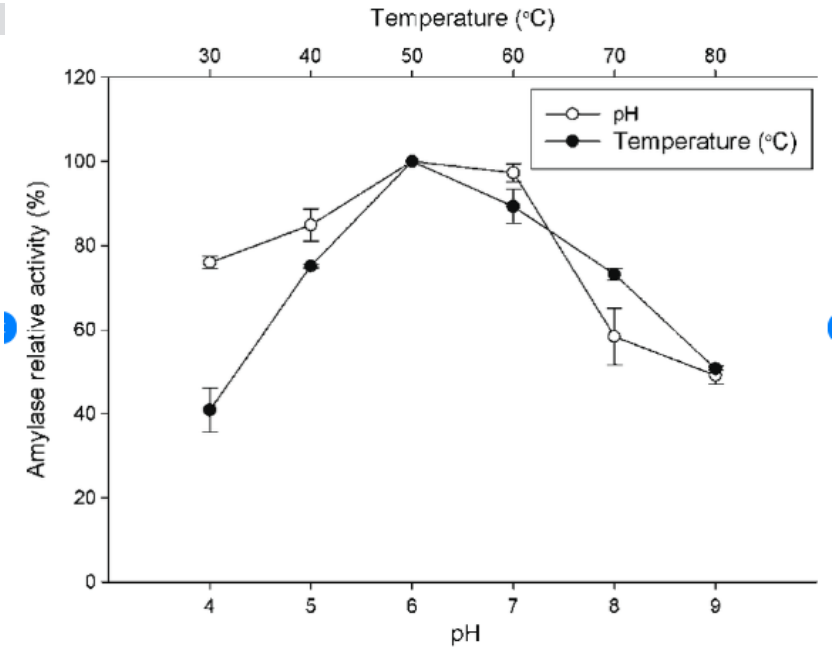
Bovine serum albumin (BSA) 100mg/ml solution in distilled water Precipitated sample was dissolved in 50mM /5pH acetate buffer the sample were taken in different concentrations (50µl, 100µl, 200µl) and then makeup water label at 200µl and then freshly prepared alkaline copper sulphate reagent. Was added properly and after freshly prepared alkaline copper sulphate reagent. Was added properly and after10 min 0.5ml folin (FCR) reagent. The contents instantaneously allowed developing for 30 min. To determine the absorbance at 660 nm, the instrument should be set using a reagent blank. The reagent blank consists of 1 mL of 0.1 N NaOH instead of the sample aliquot. Another set of tubes taken suitable aliquot of BSA solution (in a range of 0-100µg) standards and reading were recorded. The standard curve determination the amount of protein in the sample [20-24].

Enzyme assays by analytical methods

Amylase activity: Amylase activity was assayed as per method of Wood and Bhat (1988) using soluble starch as standard. To 0.4 mL of 1% soluble starch in 0.05M acetate buffer (pH 5.0) taken in a test tube, 0.2 mL of culture filtrate was added and incubated at 35°C for 60 minutes. The absorbance was recorded at 550 nm against the blank (of 0.05M acetate citrate buffer). One unit of amylase activity was expressed as 1 µ mole of glucose released per mL enzyme per minute (**Fig. 1.**).

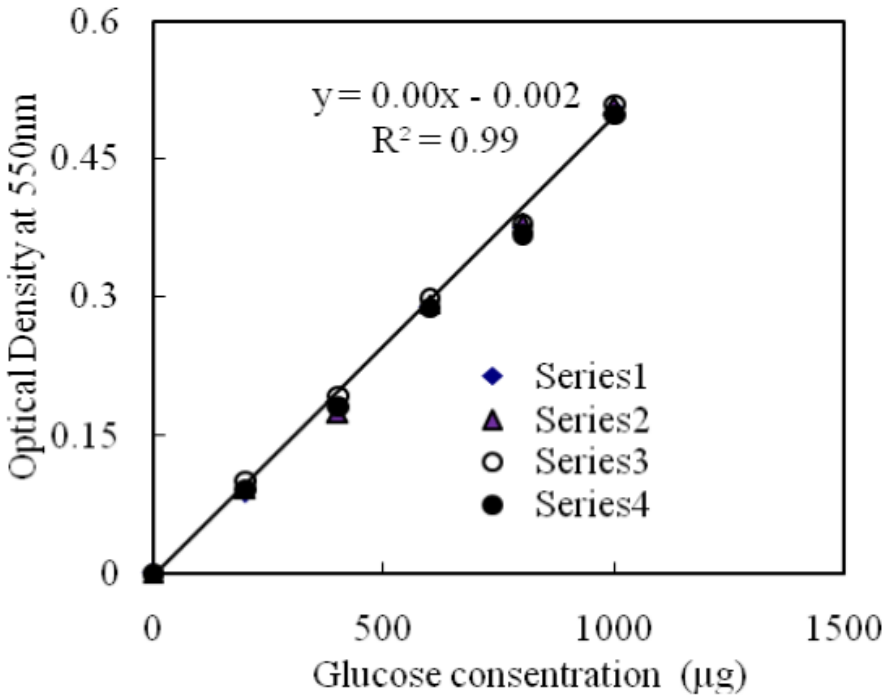
Glycosidase activity: The activity of glycosidase was assessed using a modified version of the Bergham and Pettersson method. In this experiment, 0.5 mL of the enzyme solution was mixed with a 0.5% starch solution in a 0.05M acetate buffer at pH 5.0. The mixture was then incubated at 30 °C for 60 minutes. To halt the reaction, the mixture was heated in a boiling water bath for 10 minutes. The enzyme's activity was determined by measuring the concentration of glucose released into the medium over time (**Fig. 2.**). In this study, one unit of the enzyme was defined as the quantity required liberating one µmole of

Fig. 1. Amylase activity.



Effect of pH and temperature on purified α-amylase activity. The enzyme activities are represented relative to the maximal values

Fig. 2. Glycosidase activity.



reducing sugar under the specified assay conditions.

Ethanol assay by dichromate colorimetric method: The majority of chemical oxidation methods rely on the utilization of potassium dichromate to completely oxidize ethanol in the presence of sulfuric acid, resulting in the formation of acetic acid. This particular reaction is widely employed due to the ready availability of high-purity potassium dichromate and the solution's stability in air over an extended period. The theoretical stoichiometry of the reaction is depicted below.

2Cr2O7--+ 3C2H5OH + 16H+ -----> 4Cr+++ + 3CH3COOH + 11H2O

Due to the significant spectral overlap between the yellowish dichromate (Cr2O7, Cr(VI)) and the intensely green reduced chromic product (Cr+++, Cr(III)), Beer's law cannot be applied for accurate measurement. Instead, the solution of interest needs to undergo analysis at various wavelengths to determine the separate concentrations of dichromate and chromic ions, while also ensuring that the overall quantity of chromium is accounted for atoms remains conserved according to material balance. Alternative determination methods relying on the reaction mentioned above are commonly employed. In these methods, any reactant or product involved in Reaction (1) can be analyzed using a separate reaction. For instance, the excess dichromate present in the solution can be titrated with other oxidizing reagents like ferrous ammonium sulfate.

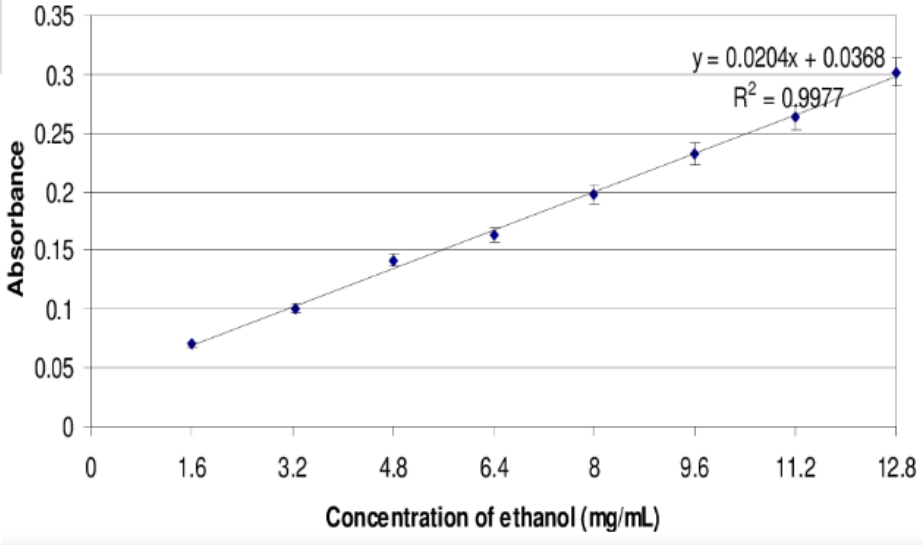


Fig. 3. Ethanol assay by dichromate colorimetric method.

Cr2O7--+ 6Fe++ + 14H+ -----> 2Cr+++ + 6Fe+++ + 7H_2O
To improve the visual identification of the endpoint during titration, organic indicators like sodium diphenylamine sulfonate and 1, 10-phenanthroline can be employed. Another effective approach involves using iodometric titration. By maintaining an appropriate concentration of sulfuric acid in the solution, the oxidation of ethanol is directed towards acetic acid rather than acetaldehyde [25-28] (**Fig. 3.**).

RESULT AND DISCUSSION

The utilization of celluloses derived from *T. reesei* fungi in the biofuels sector has proven to be effective in breaking down vegetable feedstocks into substrates suitable for various processes. *C. molischiana* is another microorganism showing promise for bioethanol production, as it has the ability to convert glucose, fructose, and sucrose-containing substrates into ethanol. The process of glucose production through *T. reesei* RUT-C30 fermentation is intricate and influenced by several factors, such as pre-incubation time, initial pH, and initial cellulosic concentration. In a preliminary study, a fermentation experiment was conducted over a span of 5 days, with the measurement of reducing sugar and glucose levels at 24-hour intervals. Optimal conditions for pre-culture incubation time, initial pH, and initial cellulosic concentration were determined to be 2 days, pH 7, and 20 g/L, respectively, in terms of maximizing reducing sugar and glucose production from cellulose. Following 2 days of incubation under these optimized conditions, the maximum quantities of reducing sugar and glucose obtained were 4.1 g/L and 2.6 g/L, respectively. Hence, celluloses sourced from *T. reesei* fungi have been successfully employed in the biofuels industry to degrade vegetable feedstocks for the production of versatile substrates. Additionally, *C. molischiana* stands out as a promising microorganism for bioethanol production, specifically capable of converting glucose, fructose, and sucrose-containing substrates into ethanol [29].

Glucose production through *T. reesei* RUT-C30 fermentation is a complex process influenced by various factors, including pre-incubation time, initial pH, and initial cellulosic concentration. In an initial study, a fermentation experiment was conducted over duration of 5 days, with the measurement of reducing sugar and glucose levels monitored at 24-hour intervals. The optimal conditions for pre-culture incubation time, initial pH, and initial cellulosic concentration were determined to be 2 days, pH 7, and 20 g/L, respectively, in terms of maximizing the production of reducing sugar and glucose from cellulose. Under these optimized conditions, the maximum quantities of reducing sugar and glucose reached were 4.1 g/L and 2.6 g/L, respectively, after 2 days of incubation. *T. reesei* RUT-C30 is known for its high production of cellulolytic enzymes, including cellulases and xylanases. To achieve complete hydrolysis of cellulose into glucose, a combination of exoglucanases (cellobiohydrolases), endoglucanases, and β-glucosidases is required. The extracellular cellulolytic system of In T. reesei, cellobiohydrolases or exoglucanases make up around 60 to 80% of the composition, followed by endoglucanases at 20 to 36%, and β-glucosidases at approximately 1%. These enzymes work synergistically to convert cellulose into glucose.

The impact of temperature on cellulose hydrolysis and the production of reducing sugar and glucose was examined by increasing the temperature from 30°C to 50°C at various time intervals (24, 36, 48, and 60 hours). The investigation focused on the change in glucose levels over time when the temperature was raised to 50°C. Under constant temperature conditions of 30°C, *T. reesei* RUT-C30 exhibits an abundant production of cellulolytic enzymes, including celluloses and xylanases. The complete hydrolysis of cellulose to glucose necessitates the presence of exoglucanases (also known as cellobiohydrolases), endoglucanases, and β-glucosidases. The extracellular cellulolytic system of *T. reesei* consists of approximately 60 to 80% cellobiohydrolases or exoglucanases, 20 to 36% endoglucanases, and 1% β-glucosidases, which work synergistically to convert cellulose into glucose. It is important to note that the optimal temperature for this cellulose system is approximately 50°C. Thus, the investigation aimed to assess the changes in cellulose

hydrolysis and the production of reducing sugar and glucose when the temperature was elevated to 50°C.

To investigate the impact of temperature on cellulose hydrolysis and the production of reducing sugar and glucose, the temperature was increased from 30°C to 50°C at specific time intervals (24, 36, 48, and 60 hours). The following optimized conditions were employed for the conversion of cellulose into reducing sugar and glucose: To initiate the cultivation process of *T. reesei*, a pre-culture is prepared and allowed to grow for 2 days. The pre-culture is then used to inoculate the cultivation medium with a 10% (v/v) inoculum. The initial pH of the medium is set to 7, and the initial cellulose concentration is maintained at 20 g/L. Additionally, the temperature is gradually increased to the desired level as part of the cultivation process. 50°C at 36 hours. After 72 hours of *T. reesei* cultivation, the temperature was then lowered back to 30°C, and *C. molischiana* was introduced. The maximum ethanol concentration achieved was 3.0 g/L at 120 hours, resulting in a yield of 0.15 g of ethanol per gram of cellulose. Notably, the ethanol yield of 0.15 g per gram of cellulose based on glucose was significantly higher than the typical yield of 0.48 g of ethanol per gram of glucose observed in Saccharomyces cerevisiae. Molischiana has the capability to utilize not only glucose but also cellodextrins. The yeast produces β-glycosidase, which aids in the breakdown of cellobiose into glucose. Furthermore, *C. molischiana* can ferment cellodextrins with a degree of polymerization ranging from 2 to 6, leading to ethanol production. In summary, the investigation demonstrated that increasing the temperature to 50°C during cellulose hydrolysis affected the production of reducing sugar and glucose. Additionally, the use of *C. molischiana* in the subsequent fermentation process resulted in a higher ethanol yield compared to conventional yeast strains, owing to its ability to utilize both glucose and cellodextrins as substrates [30-35].

CONCLUSION

Numerous studies have focused on enhancing the efficiency of fermentation in dilute-acid hydrolyzate by addressing the issue of hydrolyzate toxicity. Various strategies have been proposed to overcome this challenge, and two promising approaches are the use of high cell concentrations in the cultivation media and the formation of cell aggregates for improved protection against harsh environmental conditions. In our current research, we combined these concepts to develop an encapsulated yeast cell system, aiming to enhance the in situ capacity of cells and their tolerance against inhibitors found in toxic mediums like wood hydrolysates. By encapsulating the yeast cells, we provided them with a natural defense mechanism in the form of aggregation, which offers superior protection. The effectiveness of this encapsulated yeast cell system was demonstrated by successfully fermenting both a toxic synthetic medium and undetoxified hydrolyzate in both batch and continuous cultivation setups. Remarkably, the encapsulated system exhibited significantly higher rates of volumetric glucose consumption and ethanol production compared to free-cell cultivation. These improvements indicate the potential of encapsulated yeast cells for fermenting inhibitory media.

Overall, our study highlights the advantages of combining high cell concentrations and encapsulation as a strategy for efficient fermentation of dilute-acid hydrolyzate. This approach not only reduces fermentation time but also enhances the cells' tolerance against inhibitors, thereby paving the way for more effective utilization of renewable resources in biofuel production and other biotechnological applications. The utilization of encapsulated cells in cultivation demonstrates notable improvements in the fermentation process compared to free-cell systems. Specifically, when using encapsulated cells, there is a decrease in glycerol and acetate yields, while the ethanol yield is enhanced. This emphasizes the increased capability of encapsulated cells for on-site detoxification, accompanied by a greater proportion of viable cells. The advantages of encapsulated cells are further demonstrated in continuous cultivation using undetoxified lignocellulosic hydrolyzate. In this scenario, the dilution rates achievable with encapsulated cells are several times higher compared to free-cell cultivation. This indicates that encapsulated cells possess an increased ability to tolerate and detoxify the inhibitory compounds present in the hydrolyzate.

Furthermore, when evaluating the volumetric ethanol productivity, the encapsulated cell system performs comparably to the system operating in a synthetic medium without any inhibitors. This suggests that encapsulated cells are capable of achieving efficient ethanol production even in the presence of inhibitory substances. Overall, the utilization of encapsulated cells in the fermentation of lignocellulosic hydrolyzate offers several benefits, including reduced production of unwanted by-products such as glycerol and acetate, increased ethanol yield, higher dilution rates in continuous cultivation, and comparable ethanol productivity to non-inhibitory synthetic media. These findings highlight the potential of encapsulated cell systems as a valuable approach for optimizing biofuel production from renewable resources.

The distinctive advantages observed in encapsulated cells are likely attributed to their specific growth pattern, forming tightly packed cell communities with physiological and morphological adaptations. Notably, the membrane of the capsules exhibits high selectivity towards the components present in different mediums, such as wood hydrolyzate, orange peel hydrolyzate, or limonene-contained medium. This selective membrane enables successful fermentation of these mediums, even in the presence of 1.5% (v/v) limonene. In the case of wood hydrolyzate, orange peel hydrolyzate, or limonene-contained medium, the selective membrane of the capsules effectively allows fermentation to occur while maintaining the desired concentration of limonene. This demonstrates the capability of the encapsulated cell system to handle inhibitory compounds and create a favorable environment for fermentation. In summary, the encapsulated cell system's advantageous features can be attributed to the packed-cell community growth pattern, along with physiological and morphological modifications.

The selective membrane of the capsules enables successful fermentation of different mediums, including those containing inhibitory substances like limonene. These findings highlight the potential of encapsulated cells as a versatile and robust approach for fermenting complex mediums and overcoming inhibitory challenges in biofuel production and other biotechnological applications.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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