Studies on microbial interaction for the production of biofuel through fatty acid platform

Thesis

Submitted By

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of his ***Master of Bioprocess Engineering*** studies**.** All information in this document have been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are fully authentic to this work.

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CERTIFICATE

This is to certify that the thesis entitled "***Studies on microbial interaction for the production of biofuel through fatty acid platform***" submitted by **Ms. Sumona Pathak** (Roll No: M4BPE19009), in the partial fulfillment for the requirements for the award of the degree of ***Master of Engineering*** with specialization in ***Bioprocess Engineering, Jadavpur University, Kolkata*** during the academic session 2017-2019 is a bonafide record of the project work carried by her under the supervision of ***Prof. (Dr) Ranjana Chowdhury*** and no part of this work has been presented earlier for any other degree or diploma in any institute or university. The approval does not necessarily endorse or accept every statement made, opinion expressed or conclusion drawn as recorded in the thesis. It only signifies the acceptance of the thesis for the purpose for which it is submitted.

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Abstract

The present study was conducted to determine biofuel production through volatile fatty acid platform using mixed consortia collected from natural sources. The acid production potential of the isolated acidogenic mixed consortium was accessed by monitoring the fall of system pH which was found to be 4.5- 5 that indicates the consortium to be acidogenic. Primarily, an optimized dilute acid pretreatment method was designed using the parameters acid concentration, loading and autoclave time and the response was considered to be the total amount of reducing sugar. The optimum pretreatment condition obtained from the study of acid pretreatment was 1% dilute sulphuric acid at 0.1 % loading when autoclaved for 30 minutes. Growth kinetic study of the mixed consortia had been carried out individually for both glucose and hydrolysate in the Reinfored Clostridial Broth (RCM) to determine the monod kinetic parameters i.e. maximum specific growth rate (μ_{max}) and substrate saturation constant (K_s). A summative type of model has been found and specific growth rates of mixed strain using glucose-hydrolysate combination was plotted against concentration of reducing sugar in hydrolysate and glucose as independent variables. Mathematical modeling of specific growth rate was performed and the surface plots and 3-D plots were generated using experimental and predicted values of concentration of biomass and pH. The survival and growth of a solventogenic bacterium on growth medium that was amended with the acidic effluent of the acidogenic consortium has shown positive growth.

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1. Introduction

In recent years, the world energy demand is continuously increasing, which forces overexploitation of fossil fuel reserves. A large fraction of the world's total energy demands is supported by non-renewable fossil resources such as coal, oil and natural gas. The burning of fossil fuels contributes to the increase in atmospheric CO2 level and poses a threat to the environment, as it is directly related to global warming. Alternate sources of clean and renewable energy need to be found so as to meet the ever increasing demand for energy and to counter greenhouse effects. Emissions of carbon dioxide can be limited with alternative sources of energy such as biofuels. [1] Biofuel (biodiesel, bio-alcohol and biogas) can be a suitable alternative to fossil fuel, as it is derived from biological processes. Biofuel can be produced from different kinds of feed-stocks such as food crops, agriculture waste, municipal waste, industrial waste, waste cooking oils and animal fats by using different thermochemical or biochemical processes.[2-5]

Thermochemical methods subject biomass to pyrolysis or gasification while biochemical processes convert the biomass first into sugar and then the sugar is applied to microbial fermentation.[6-8]

Biofuels are derived from biological material mainly from plants, microorganisms, animals and wastes. All biofuels possess the same basic and renewable origin. Biofuels are based by the "present-day" photosynthetic conversion of solar energy to chemical energy which sets them apart from fossil fuels that are based on ancient photosynthesis. [9] The line between renewable biofuels and non-renewable fossil fuels is sometimes vague and only complete life-cycle analyses in the future will reveal which feed-stocks are truly renewable to be used in biofuel production.

1.1 Classification of Biofuel

Depending on the origin and production technology of biofuels, they are generally called as the first, second and third generation biofuels while the fourth generation biofuels make use of novel synthetic biology tools and are just emerging at the basic research level. Biofuels have become the largest renewable fuel produced and consumed in the world due to increasing demands to replace the fossil fuels with renewable ones, thus reducing greenhouse gas emissions and ease climate change. On the other hand, there are serious concerns about competition of land for food and fuel production, particularly due to the fact that the current technology for biofuel production depends on feedstock derived from the edible fraction of food plants (corn, rapeseed, sugar beet and others).

1.1.1 1G. First Generation Biofuels

This is the first class of biofuels, the production technologies use feedstocks suitable for human/animal consumption as food material (e.g. animal fats, flax, soy, rapeseed, mahua, mustard, hemp, sunflower, jatropha, palm oil, field pennycress, and pongamia pinnata) for biofuel production. This causes concern regarding the conflict between 'food and fuel', in context of the prominent global food crisis 1G biofuel production, which is achieved mostly from simple/complex carbohydrates, directly leads to 'food vs. fuel' conflict. Food versus fuel is the dilemma regarding the risk of diverting crops or farmland for biofuel production adding to the detriment of the food supply. First generation biofuels (produced primarily from edible parts of food crops) are limited in their ability to achieve targets for fossil oil-product substitution, climate change mitigation and economic growth, due to the prevalence of the aforementioned conflicting issue of food vs. fuel. The cumulative impacts of these factors have increased the

interest in developing biofuel platforms produced from non-food biomass, which is the next class of biofuels. [10-14]

1.1.2 2G. Second Generation Biofuels

The concerns on global food crisis stimulated the development of the second generation biofuels produced from the ligno-cellulosic feedstocks. These second generation biofuels could avoid many of the concerns facing first generation biofuels and potentially offer greater cost reduction potential in the longer term. Lignocellulosic biomass consists of the residual, non-edible parts of food crops (e.g., stems, leaves and husks) as well as other non-food crops (e.g., switch grass, jatropha, cereals that bear little grain, fuelwood and industrial waste such as wood residues, skins and pulp from fruit pressing. Feedstocks from lignocellulosic materials primarily used for 2G biofuel production include cereal straw, bagasse, forest residues, sewage and municipal wastes. [15] Presently, the 2G biofuels are being seen as the energy vectors that can substitute the usage of fossil-fuels in near term and hence, are rendered most important among all generations.

1.1.3 3G. Third Generation Biofuels

In this class of biofuel technology, biofuel is produced by algae (or other aquatic species), is spotlighted as the third-generation biofuel. [13] Algae can be cultured (i.e., bio-manufactured) using sea or wastewater in either indoor photo-bioreactor systems or in open outdoor water reservoirs/ponds. These are biodegradable, and are relatively harmless to the environment if spilled.

1.1.4 4G. Fourth Generation Biofuels

The 4G biofuel technologies are aimed at not only producing sustainable energy but also a way of capturing and storing CO2. [16] Biomass materials, which have absorbed CO2 while growing, are converted into fuel using the same processes as second generation biofuels. This process differs from second and third generation production as at all stages of production the carbon dioxide is captured using processes such as oxy-fuel combustion. The carbon dioxide can then be geo-sequestered by storing it in old oil and gas fields or saline aquifers. This carbon capture makes fourth generation biofuel production carbon negative rather than simply carbon neutral, as it is 'locks' away more carbon than it produces. This system not only captures and stores carbon dioxide from the atmosphere but it also reduces CO2 emissions by replacing fossil fuels.

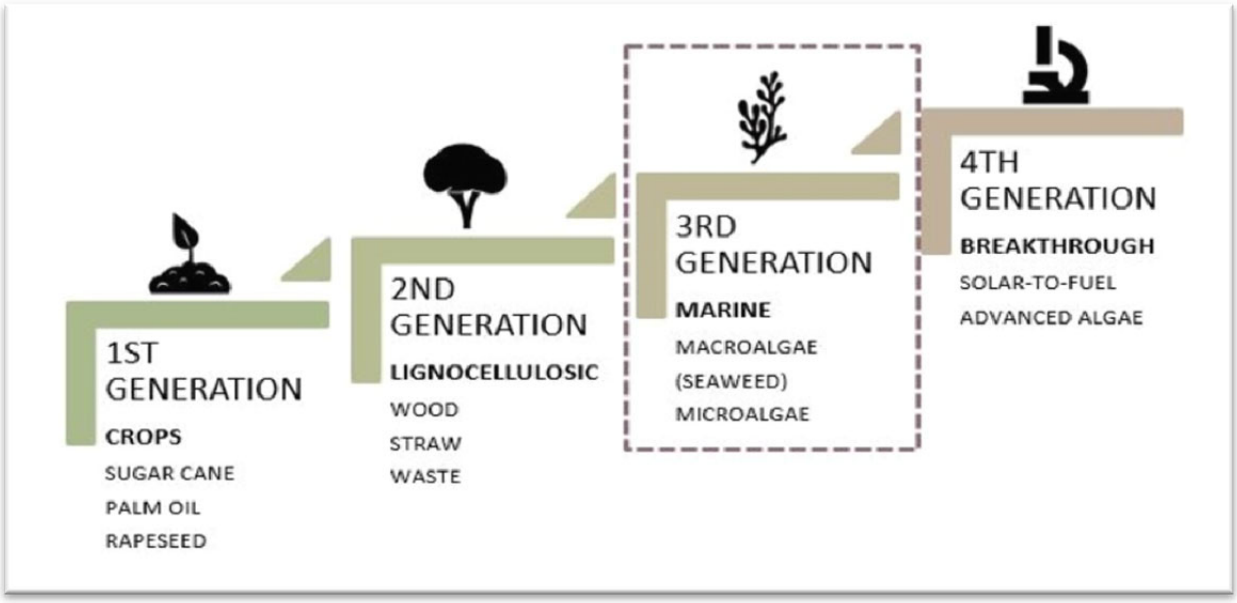

Figure1: Generation of biofuels [source: www.google.com]

1.2 Lignocellulose: The feedstock for 2G Biofuels

The inedible parts of plants are feeding the next generation of biofuels. The woody material that gives plants their rigidity and structure comprises three main types of carbon-based polymer — cellulose, hemicellulose and lignin — collectively called *lignocellulosic biomass*. When taken apart, these polymers yield chemical components that can be used to make biofuels. Cellulose, after all, is a polymer of glucose.[14] And if this sugar can be extracted, it can be fermented to make ethanol or the longer-chain alcohol butanol. Hemicelluloses are polymers of various sizes that incorporate a range of different sugars, whereas lignin has a polymer backbone made from phenolic groups, which are ring-shaped, carbon-based structures. Other useful chemicals such as furans — molecules with a circular structure consisting of four carbon atoms and an oxygen atom — can be pulled out of lignocellulosic biomass and could serve as alternative, high-energy-density fuels. Most of the current efforts in second-generation biofuel production focus on ethanol, with furans and butanol at earlier stages of development.

The structural complexity of lignin makes it recalcitrant to digestion by bacterial enzymes. The presence of lignin hinders the microbial enzymatic reaction due to its complex structure. Many physico-chemical, structural and compositional factors hinders the hydrolysis of polysaccharide components in lignocellulosic biomass to sugars and other organic compounds that can later be converted into fuels. Thus, pretreatment for removal of lignin is absolutely essential for biochemical production of fuels from complex feedstocks.

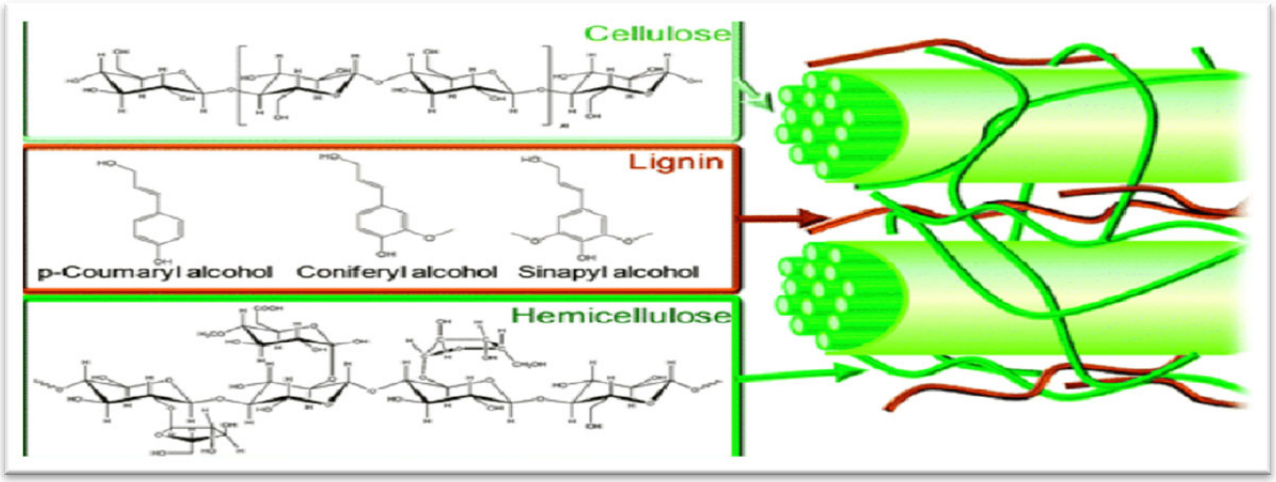

Figure 2: Structure of lignocellulosic biomass [source: www.google.com]

1.2.1 Pretreatment of lignocellulosic wastes

Lignocelluloses are mainly comprised of cellulose, hemicellulose and lignin. The presence of lignin limits the fullest usage of cellulose and hemicellulose. To convert these energy rich molecules into simpler forms, it is necessary to remove the lignin from lignocellulosic materials. A number of pretreatment methods such as concentrated acid hydrolysis, dilute acid hydrolysis, alkali treatment, sodium sulphite treatment, sodium chlorite treatment, steam explosion, ammonia fiber explosion, lime treatment and organic solvent treatment have been used frequently to remove lignin and improve the saccharification of the cell wall carbohydrates. Dilute acid hydrolysis is a fast and convenient method. The acid hydrolysis pretreatment removes the hemicellulosic portion which mainly contains pentoses like xylose and arabinose which in turn adds up the quantity of fermentable sugars available for fermentation process and some fraction of lignin but rest of the lignin remains intact to the cellulosic substrate.

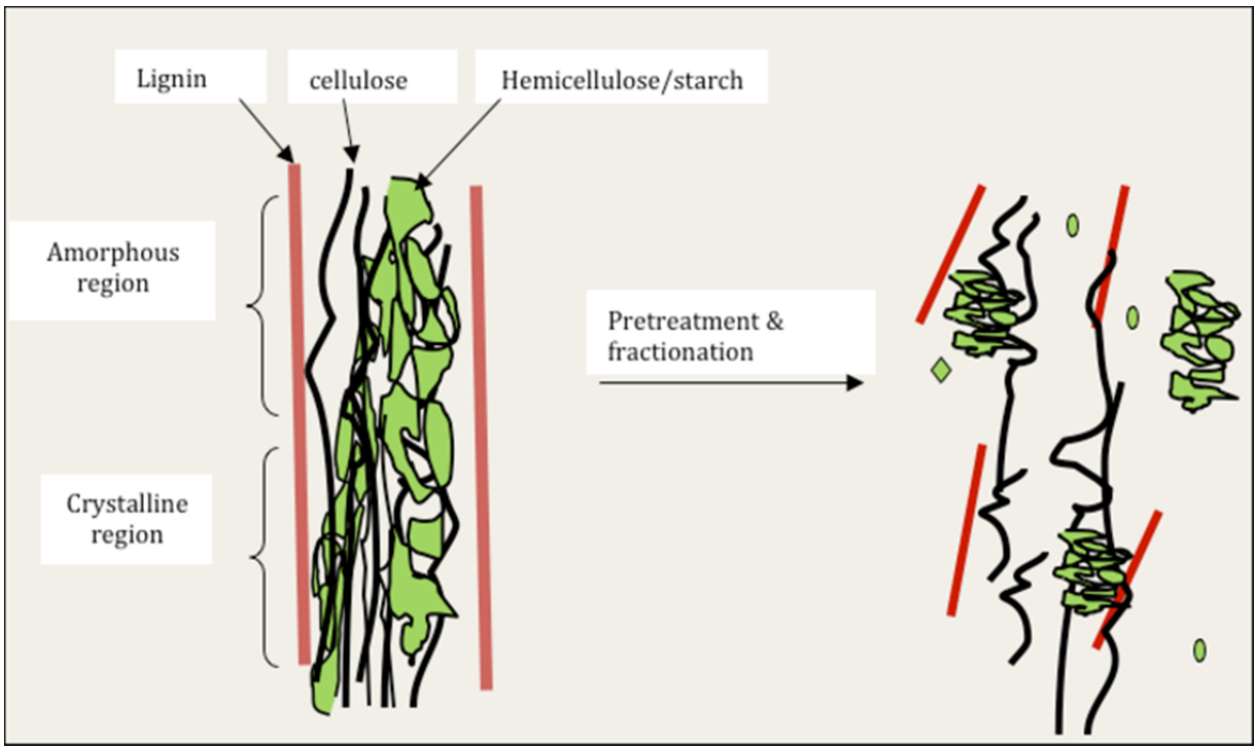

Figure 3: The change in structure of feedstock after pretreatment procedure [www.google.com]

1.3 Platforms for Biofuel Production

Various technologies to convert biomass to usable fuels have been developed. The typical four platforms are (i) sugar, (ii) syngas, (iii) Volatile Fatty acid and (iv) biogas platform.

The sugar platform uses hexose and pentose sugars that are extracted or converted from plant body. The syngas platform involves a thermochemical chemical conversion process using pyrolysis or gasification of plant mass to syngas followed by biological conversion of the syngas to biofuels. The volatile fatty acid platform is used to produce higher bioalcohols from long-chain fatty acids. The biogas platform is used to produce methane gas from municipal solid wastes (MSWs) or other organic wastes through an anaerobic digestion (AD) process that exploits rapid acidogenesis and slow methanogenesis. [17] The anaerobic digestion is the biological conversion in the absence of oxygen of degradable organic compounds into methane

and carbon dioxide. [18] Anaerobic Digestion (AD) includes four steps, the first step of anaerobic digestion is the breakdown (hydrolysis) of organic compounds into soluble mono- and oligomers (monosugars, amino acids and fatty acids) by hydrolytic enzymes that is followed by acidogenesis, which includes both hydrolysis and fermentation into volatile fatty acids (VFAs), H2 and CO2 by acidogenic bacteria. The final steps are the acetogenesis (production of acetic acid, and H2 by proton-reducing acetogens) and the methanogenesis steps (production of CH4 and CO2 by acetoclastic methanogens and CH4 by CO2 reducing methanogens). However, the volatile fatty acid platform is mentioned as an individual platform, it is actually originated from the bi Sugar Platform

1.3.1 The sugar platform for converting lignocellulose to ethanol via sugars consist of four steps that are (a) pretreatment to change structural features to make cellulose accessible to enzymes, (b) formation of fermentable sugars that is known as saccharification, (c) fermentation of the sugars to ethanol, and (d) separation and purification of the ethanol.

1.3.2 Syngas Platform

Synthesis gas which is also known as syngas, it comprises of a mixture of carbon monoxide, hydrogen, and carbon dioxide and it can be produced sustainably with the syngas platform through a thermochemical step with biomass feedstock. The produced syngas is converted to ethanol and acetic acid using carboxydotrophic bacteria.

1.3.3 Development of Volatile Fatty acid Platform

Anaerobic conversions are among the oldest biological process technologies utilized by mankind. AD involves a transformation of organic matter by a mixed culture bacterial ecosystem without oxygen. It is a natural process that produces a gas principally composed of methane and

carbon dioxide. In this process, Acidogenesis is the production step of Volatile Fatty Acids (VFAs) platform and Methanogenesis step is eliminated as that will lead to biogas production.

The first step of AD is the hydrolysis of plant matter. This step breaks down biopolymers and other organic material to smaller molecules. The second step is the conversion by acetogenic bacteria of products of the first step to organic acids, carbon dioxide, and hydrogen. Acetogenic bacteria produce acetic acid however other organic acids are also produced. The principal organic acids produced are acetic acid (CH3COOH), propionic acid (CH3CH2COOH) and butyric acid (CH3CH2CH2COOH). Ethanol (CH3CH2OH) and other products are also produced in smaller fractions. [17]

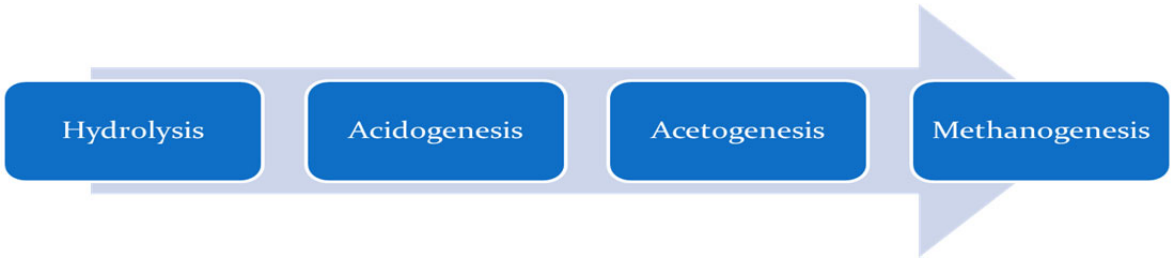

Figure 4: Steps involved in Anaerobic Digestion

Biological processes need a sterilization process to culture a specific microorganism. But the mixed VFAs fermentation process does not require a sterilization process and instead it uses a mixed microbial community better known as mixed consortia that is being produced from hydrolysis and acidogeneis steps. [19-20]The mixed culture can provide energy savings and it is an economical process. Lignocellulosic biomass is composed of lignin, hemicellulose, and cellulose and it is used as raw material for biofuel production. This type of biomass is recalcitrant in nature and microbes are not able to utilize it. Therefore mild pretreatment is done to make it usable by the microorganisms.

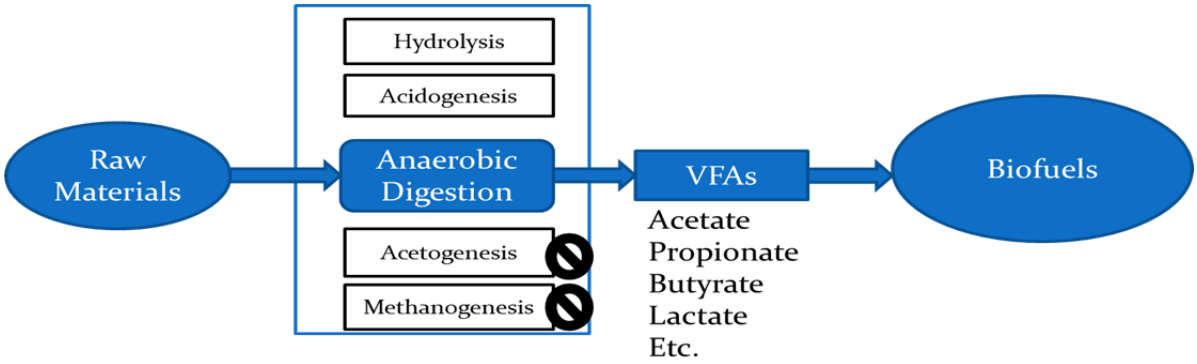

Figure 5: Concept of VFA platform

1.4 Carboxylate Platform

The carboxylate platform can depolymerize biomass (e.g., cellulose) into monomers (e.g., glucose) and then ferments these monomers into carboxylate salts without added enzymes. The carboxylate platform ferments glucose to carboxylic acids (e.g., acetic acid, butyric acid). Most of the carboxylic acids are present as their carboxylate salts (e.g., acetate) as the pH of the fermentation is almost neutral hence the term carboxylate platform. In its simplest and most robust form, the carboxylate platform uses a mixed consortium of microorganisms (e.g., Clostridia, Bacilli) that transform biomass into carboxylates namely short-chain fatty acids (SCFAs, e.g., acetic, propanoic acids) and medium-chain fatty acids (MCFAs, e.g., butanoic, pentanoic, hexanoic, heptanoic, octanoic acids). The mixed-culture fermentation is an example of consolidated bioprocessing, in which the fermenting organisms produce both hydrolytic enzymes (e.g., cellulase) and fermentation products (SCFAs and MCFAs). Throughout the world, anaerobic digesters are used to break down a wide variety of biomass components (e.g., sewage sludge, manure, food scraps) into biogas, a mixture of methane and carbon dioxide. These digesters are robust, low-tech devices that can be operated by someone with minimal training. Because methane has a low commercial value, it is preferable to inhibit methane production and operate the digester as a stuck fermenter that accumulates the

intermediate SCFAs and MCFAs and their salts[21]. Due to the wide variety of microorganisms, each has its own specialized niche, a wide variety of biomass components — not only just cellulose — can be transformed into SCFAs and MCFAs. The sugar platform employs a single microorganism and sterile operating conditions to transform sugar intermediates into final products and the desired organism dominates the culture. While the carboxylate platform does not require sterility, the inoculum sources include soil, rumen fluid, compost, and other natural materials. The carboxylates are nearly at the low energy state; therefore the biological transformations are driven by thermodynamics rather than the challenging task of maintaining monoculture fermentations.

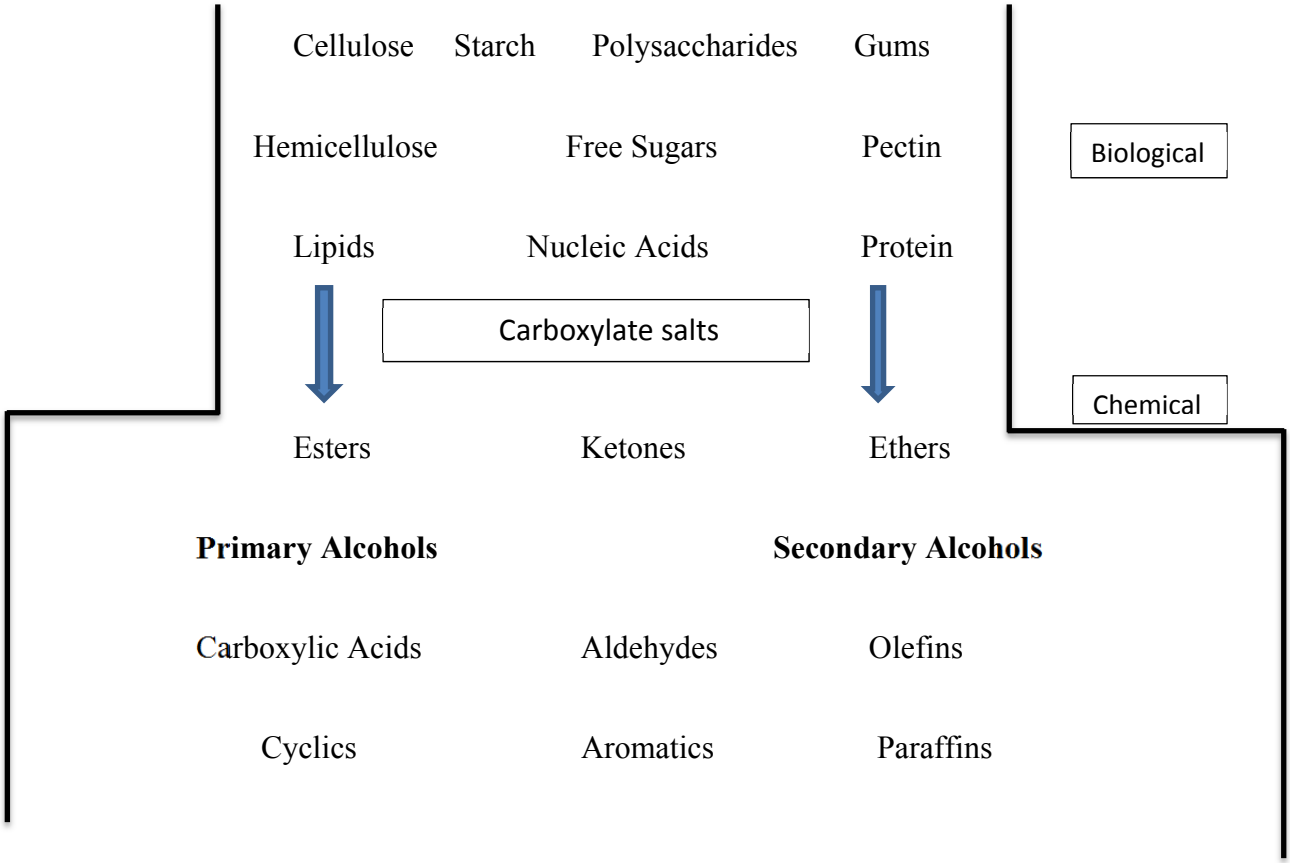

Figure 6: The carboxylate platform converts a wide array of biomass components to carboxylate salts via fermentation.[21]

The scale quantifies the Gibbs energy efficiency, which is defined as:

$$\eta G \equiv 1 - (\Delta G°r / \Delta G°combustion)$$

According to this definition, when 1 mole of glucose is converted to 2 moles of lactic acid, the Gibbs energy efficiency is 95.7%. The remaining Gibbs energy (4.3%) is available to be converted to adenosine triphosphate (ATP), an energy molecule that powers cellular metabolism.

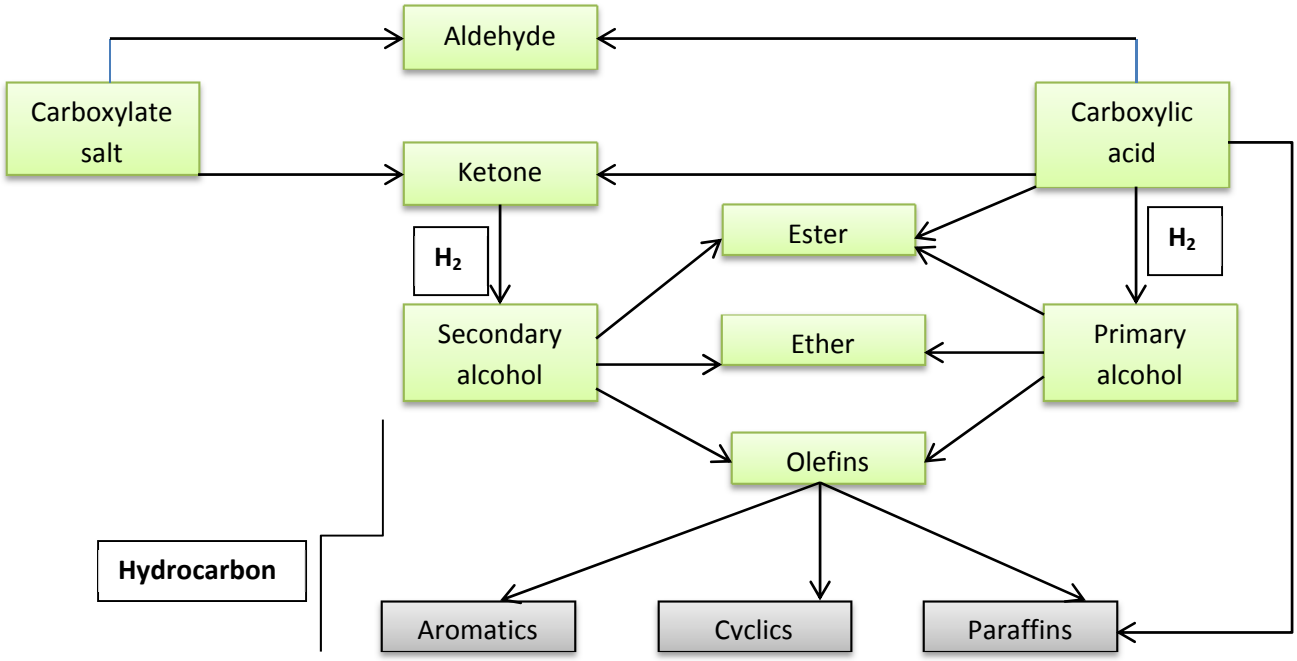

Figure 7: Subsequent chemical processes can transform these carboxylate salts into chemicals and fuels.[21]

1.4.1 Secondary alcohols: Two carboxylic acids are catalytically joined to form a ketone with the loss of one molecule of carbon dioxide. The ketones are hydrogenated to secondary alcohols, which are dehydrated to olefins that are hydrotreated to form saturated hydrocarbons.

1.4.2 Primary alcohols: Carboxylic acids are converted to primary alcohols. Although several routes are possible, here we show a process commercialized by Celanese (6) in which carboxylic

acids are catalytically hydrogenated to primary alcohols without the loss of carbon dioxide. The primary alcohols are then dehydrated, oligomerized, and hydrotreated. The reaction conditions during oligomerization (temperature, pressure, residence time, catalyst) determine the mix of products, that include paraffins, olefins, aromatics, and cyclics.
In the mixed culture, the hydrogen partial pressure determines whether NAD is in its oxidized form (NAD+) or its reduced form (NADH). The following classes of microorganisms are typically found in the consortium:

- *lactic acid* formers convert glucose to lactic acid
- *ethanologens* ferment glucose to ethanol and carbon dioxide (other routes to ethanol include the decarboxylation of lactic acid and the reduction of acetic acid)
- *acidogens* directly ferment glucose to acids such as acetic, propanoic, and butanoic acids (acetic acid can be made from ethanol, which occurs more readily at low hydrogen partial pressure)
- *acetogens* convert carbon dioxide and hydrogen into acetic acid
- *chain elongators* convert carboxylic acids to longer-chain carboxylic acids in the presence of reductants (e.g., ethanol, higher alcohols, lactic acid, and hydrogen) or when there is a reducing environment provided by an electrical voltage.

Biological processes need a sterilization process to culture a specific microorganism. But the mixed VFAs fermentation process does not require a sterilization process and instead it uses a mixed microbial community better known as mixed consortia that is being produced from hydrolysis and acidogeneis steps. The mixed culture can provide energy savings and it is an economical process. Lignocellulosic biomass is composed of lignin, hemicellulose, and cellulose and it is used as raw material for biofuel production. This type of biomass is recalcitrant in nature

and microbes are not able to utilize it. Therefore mild pretreatment is done to make it usable by the microorganisms.

Therefore the carboxylate platform ferments a wide variety of biomass feedstocks into mixed carboxylic acids (Short-chain fatty acids and Medium chain fatty acids) by using a mixed culture of microorganisms. Certain chemical reactions occur that transform these intermediates into industrial chemicals and hydrocarbon biofuels. [21] The carboxylate platform allows for the biological conversion of carboxylates to valuable products, such as lipids and hydrocarbons. The convenient feature of carboxylate platform include flexible feedstocks, higher yields, scalability, non-sterile operating conditions and the use of an active and stable natural mixed consortium. Moreover the carboxylate platform can be practiced in an appropriate manner that can integrate well with existing infrastructure.

2. Literature Review

Sl No.	Paper name	Author name	Review
1	Producing biofuels via the carboxylate platform	Holtzapple et al. (2015)	The carboxylate platform ferments a wide variety of biomass feedstocks into mixed carboxylic acids (Short-chain fatty acids and Medium chain fatty acids) by using a mixed culture of microorganisms. Certain chemical reactions occur that transform these intermediates into industrial chemicals and hydrocarbon biofuels. The carboxylate platform allows for the biological conversion of carboxylates to valuable products, such as lipids and hydrocarbons. The convenient feature of carboxylate platform include flexible feedstocks, higher yields, scalability, non-sterile operating conditions and the use of an active and stable natural mixed consortium. Moreover the carboxylate platform can be practiced in an appropriate manner that can integrate well with existing infrastructure. [21]
2	Volatile Fatty Acid Platform: Concept and Application	Kim et al.	VFA platform can use organic biomass mixtures with high water content under stable control methods helps to enable high productivity and conversion to valuable products, biofuels and biochemicals. By conducting anaerobic digestion using multiple substrates will be able to increase the VFA production. A substrate with high volatile solid indicates high degradation degree and can be used as preliminary data to track the biodegradability capability. Combined pretreatments (biological and physical) as chemical-free

			pretreatment seems to be attractive. The pretreatment processes have been extensively studied with several combination strategies, optimization process, improvement on the methodology and procedures that includes using of new equipment, new microbial strains and novel approaches, the feasibility and economic impact are still a major drawback in this area. Therefore the pretreatment studies should consider the aim of the pretreatment and the concluding research must highlight the achievement of the pretreatment objectives including whether the pretreatment process is cost-effective or has potential or is not feasible for further improvement. [17]
3	Analysis of cell growth dynamics of *Pediococcus acidilactici* in the presence of inulin in an optimized microenvironment	Koruri et al. (2015)	In this study it deals with the optimization of cell growth rate of the candidate probiotic *Pediococcus acidilactici* in the presence of the specific prebiotic inulin. Three independent variables which are concentration of inulin, concentration of glucose and pH have been selected for optimization study using response surface methodology. Theoretical analysis suggests that the maximum cell growth rate occurs at pH 7, 20 g/dm3 concentration of inulin and 20 g/dm3 concentration of glucose. Validation of these values has been done through a set of programmed experiments. Studies on cell dynamics in the presence of different concentrations of inulin have also been carried out to identify any limitation on the initial inulin concentration and the results clearly indicate that cell growth increases with the increase in inulin concentration. On the other hand, there is a critical value of the prebiotic concentration (20 g/dm3

			inulin) beyond which the cell growth is inhibited. A summative type growth model has been proposed to explain the growth behaviour of *P. acidilactici* in the presence of the dual substrate, i.e. glucose and inulin. While growth on glucose follows Monod model, Haldane-type substrate-inhibited growth model holds good for growth on inulin. All the intrinsic kinetic parameters for model equations have been determined experimentally. [22]
4	Downstream extraction process development for recovery of organic acids from a fermentation broth	Bekatorou et al. (2016)	Organic acids (OAs) recovery from an acidogenic fermentation broth, as that is the main problem regarding the use of OAs for production of ester-based new generation biofuels or other applications. Alcohols performed better in extracting OAs from aqueous media as compared to other solvents, reaching in some cases recoveries of about 100%. Cost-effective OAs extraction from fermentation broths can hence be achieved using alcohols as solvents at ambient temperatures. 3-methyl-1-butanol led to the highest OAs recovery in a repeated extractions process from all the tested alcohols. However, 1-butanol is a better option due to its availability, lower market prices and possibility to produce as full bio-product form renewable resources.[23]
5	Continuous acidogenesis of sucrose, raffinose and vinasse using mineral kissiris as promoter	Lappa et al. (2015)	Kissiris promotes the acidogenesis of sucrose, raffinose and vinasse. Initially, the effect of pH (4–8) and fermentation temperature (18–52°C) plays an important role on the accumulation of low molecular weight organic acids (OAs) during sucrose acidogenesis. The promoting effect of kissiris was confirmed when compared to free cells that resulted in

			80% increase of OAs production. The optimum conditions that were used (pH 8; 37°C) during acidogenesis of sucrose/raffinose mixtures. A continuous system was also operated for about more than 2 months. When sucrose and sucrose/raffinose mixtures were used, lactic acid type fermentation overruled while when vinasse was used, butyric acid type fermentation occurred. Total OAs concentrations were about more than 14 g/L and ethanol concentrations were 0.5–1 mL/L. Culture adaptation in vinasse was very necessary to avoid underprivileged results. Hence the proposed process is promising for new generation ester-based biofuel production from industrial wastes. [24]
6	Acidogenesis of cellulosic hydrolysates for new generation biofuels	Kandylis et al. (2016)	The acidogenesis of lignocellulosics and liquid effluent have been discussed extensively with potential goal for the production of a new generation biofuel. This type of new biofuel can be produced through esterification of volatile fatty acids with ethanol (produced simultaneously during the acidogenesis) or/and with another alcohol. And this will overcome the major problems that are being faced during bioethanol production and it concerns the high energy demand of the bioethanol production plant particularly it was found that the main volatile fatty acids formed are formic, acetic, propionic, butyric, lactic and valeric. Their formation depends on various factors such as NADH/NAD+ proportion, pH, organic load and chemical composition of the waste that is being treated. These conditions affect the survival of microorganisms and the

			formation of predominant acetic, butyric and lactic acid. The use of ɣ-alumina promotes the formation of volatile fatty acids simultaneously with bioethanol production and both could be used for esterification. [25]
7	Favouring butyrate production for a new generation biofuel by acidogenic glucose fermentation using cells immobilized on c-alumina	Syngiridis et al. (2014)	The continuous acidogenic fermentation of glucose at high initial pH of about 8.9 was substantially advantaged by cells immobilized on ɣ-alumina that leads to higher VFA yields and favours butyric acid formation. On the other hand, batch fermentation at pH 6.5 favoured the ethanol-type fermentation. Hence it can be concluded that the adjustment of pH and the type of operation mode during acidogenic fermentation of glucose can alter the profile of the produced VFAs and ethanol concentration, that is of great importance for generation of new biofuel technologies based on conversion of various waste streams. [26]
8	Current status and strategies for second generation biofuel production using microbial systems	Bhatia et al. (2017)	To protect the environment and meet increasing energy demand, renewable sources of energy need to be adopted The food against fuel issue resembles that first generation biofuels appear unsustainable. Hence using lignocellulosic biomass for biofuel production clearly needs to be explored and promoted. Due to some technological barriers, the production of biofuel from lignocellulose (second generation biofuel) is currently not that cost effective. Although microbial fermentation is an ecofriendly way to convert lignocellulose into biofuel so it will take time to become a commercial reality. Biofuels of different generations can contribute interdependently to fulfill energy

			demand. It focuses on the pretreatment of biomass, the production of biofuel (biodiesel, bioalcohol, and biogas) using microbial systems and the various efforts that have been implemented to improve biofuel production. Naturally, existing microbes or engineered ones can be cocultured together to improve certain functions such as increase the substrate utilization range, improve the productivity and facilitate the recovery process. Co-culture technique may help to improve the overall economics of the production process so as to reduce the steps needed for biomass pretreatment and other metabolic engineering required to improve microbes. [27]
9	Production of medium-chain volatile fatty acids by mixed ruminal microorganisms is enhanced by ethanol in co-culture with *Clostridium kluyveri*	Weimer et al. (2015)	The aim of this study was to increase the production of caproate and valerate in short-term *in vitro* incubations. Mixed bacterial communities from the rumen ferment cellulosic biomass primarily to C2–C4 volatile fatty acids and that perform only limited chain extension to produce C5 (valeric) and C6 (caproic) acids. Coculture of mixed ruminal microbes with a rumen-derived strain of the bacterium *Clostridium kluyveri* converted cellulosic biomass plus ethanol to VFA mixtures that include valeric and caproic acids as the major fermentation products over a period of 48–72h run time. Concentrations of caproate reached about 6.1 gL^{-1} that is similar to or greater than those reported in most conventional carboxylate fermentations that employ substantially longer run times. [28]
10	Acidogenic fermentation of wheat straw after chemical and	Tsafrakidou et al. (2018)	Acidogenic fermentations were performed on substrates consisting

	microbial pretreatment for biofuel applications		of non-treated, chemically delignified and microbially delignified wheat straw. *T. viride* hydrolysis of the chemically delignified straw slightly improved the organic acids (OAs)yields. On the other hand, *P. chrysosporium* delignification led to acidogenesis results comparable to those of chemical delignification. In both the cases, acidogenesis was further promoted by the use of culture immobilized on γ-alumina. So, low cost microbial pretreatment combined with simple cell immobilization techniques may offer advantages for the utilization of lignocellulosics by anaerobic acidogenesis. Hence the proposed process is a cost-effective, environmentally friendly approach for 2nd generation, ester-based, biofuels similar to biodiesel. [29]
11	Upgrading syngas fermentation effluent using Clostridium kluyveri in a continuous fermentation	Gildemyn et al. (2017)	Ethanol is used to elongate short-chain carboxylic acids to medium-chain carboxylic acids (MCCAs). Clostridium kluyveri can be used as a biocatalyst to upgrade syngas fermentation effluent into MCCAs at pH> 5.5. Besides changing the operating conditions in regards to substrate loading rates and composition, the effect of in-line product extraction, pH, and the use of real syngas fermentation effluent on production rates were tested as well. Increasing the organic loading rates resulted in proportionally higher production rates of n-caproic acid, that were up to 40 mM day−1 (4.64 g L−1 day−1) at carbon conversion efficiencies of 90% or higher. A lower ethanol/acetic acid ratio (3:1 instead of 10:1) enabled faster and more efficient n-caproic acid production. Therefore, the use of real effluent from syngas fermentation, without added yeast extract, but with

			added defined growth factors, maintained similar production rates. [30]
12	New contributions for industrial n-butanol fermentation: An optimized Clostridium strain and the use of xylooligosaccharides as a fermentation additive	Grassi et al. (2018)	In this work, they developed a second-generation fermentation process using cellulosic sugar as the substrate. It showed a viable technical process for ABE fermentation. Using a n-butanol tolerant *C. saccharoperbutylacetonicum* strain, a lignocellulosic hydrolysate and a simple and low-cost additive, xylooligosaccharides or cellulose pulp/enzyme, it has been observed a substantial increase in the n-butanol yield and productivity. It showed a result of n-butanol titer of 12.5 g/L and a productivity of 0.43 g/L.h were obtained, a respective gain of 17% and 58% relative to the initial process. Hence it can lead to a new technological advance that can contribute to the return of the production of renewable n-butanol. [31]
13	Sugar recovery from rice straw by dilute acid pretreatment	Kim et al. (2012)	At first rice straw was pretreated with dilute sulfuric acid in order to decrease the amorphous portion and enhance enzyme accessibility. Dilute acid pretreatment process was optimized by the use of a statistical method and the relationships between each factor were thoroughly investigated. After then Saccharification of pretreated rice straw was performed and then it followed by fermentation of glucose, the hydrolysate of the saccharification process. The optimal conditions that dilute acid pretreatment process followed: temperature 110°C, reaction time of 14.02 minutes and acid concentration 1.2%. After dilute acid pretreatment, the solid weight was decreased by about 20% and 73.14% of the theoretical maximum content

			of xylose was solubilized. Glucose was recovered at a rate of about 90% at 24h after rice straw was treated with dilute acid. Qualitative analysis such as SEM, XRD, and FT-IR were conducted after the pretreatment process and the results supported the pretreatment process well. [32]
14	Mixed consortia in bioprocesses: role of microbial interactions	Ghosh et al. (2016)	The utilization of mixed culture has become a current research trend of applied microbiology, bioprocess engineering and biotechnology. In comparison to monocultures, the mixed cultures can jointly perform complex processes efficiently, yielding the desired product at an expanded rate. The interactions between the microbial partners in these mixed cultures are expected to have a significant impact on the combined performance of the microorganisms and the bioprocess as a whole. Pervasiveness of positive interactions (commensalism or mutualism) among microbial members of a mixed culture or consortia can significantly enhance the product outcome of the bioprocess, ensuring their industrial application and long-term stability. While on the other hand negative interaction (parasitism, predation or ammensalism) leads to elimination of microbial members from the consortia causing the destruction of community structure as well as disruption of cumulative performance. Hence, a prior knowledge on the typeof interaction between the microorganisms is also essential for the optimization of the performance of the designed consortia. [33]
15	Mesophilic and thermophilic conditions select for unique but highly parallel microbial	Hollister et al. (2012)	The carboxylate platform is a flexible, cost-effective means of converting lignocellulosic materials

	communities to perform carboxylate platform biomass conversion		into chemicals and liquid fuels. The metagenomes of two actively fermenting platform communities incubated under contrasting temperature conditions (mesophilic 40°C; thermophilic 55°C) but utilizing the same inoculum and lignocellulosic feedstock. The thermophilic community harbored genes affiliated with *Clostridia*, *Bacilli*, and a *Thermoanaerobacterium sp*, whereas the mesophilic community metagenome was composed of genes affiliated with other *Clostridia* and *Bacilli*, *Bacteriodia*, *ɣ-Proteobacteria,* and *Actinobacteria*. Both the communities were able to metabolize cellulosic materials and shared many core functions but significant differences were detected with respect to the abundances of multiple Pfams, COGs, and enzyme families. The mesophilic metagenome was enriched in genes related to the degradation of arabinose and other hemicellulose-derived oligosaccharides, and the production of valerate and caproate. While, the thermophilic community was enriched in genes related to the uptake of cellobiose and the transfer of genetic material. Functions assigned to taxonomic bins has indicated that multiple community members at either temperature had the potential to degrade cellulose, cellobiose, or xylose and produce acetate, ethanol, and propionate. This study suggests that both metabolic flexibility and functional redundancy contribute to the platform's ability to process lignocellulosic substrates and are likely to provide a degree of stability to the platform's fermentation processes we well. [34]

3. AIMS AND OBJECTIVES

1. Selection of a potential acidogenic bacterial consortium isolated from natural source to be used in VFA platform.
2. Selection and pretreatment of an Indian agro-waste for the extraction of fermentable sugar for production of VFAs.
3. Studies on the trends of biomass growth and pH by the selected acidogenic consortium on glucose and hydrolysate of pretreated agro-waste.
4. Determination of growth kinetics of the acidogenic consortium on glucose and agro-waste hydrolysate.
5. Development of mathematical model equations for prediction of the biomass growth and pH in the presence of mixed carbon substrates (glucose + hydrolysate) in batch systems.
6. Validation of the mathematical model by comparison with the experimental data obtained from the mixed substrate system.
7. Investigation of the survival and growth of a solventogenic bacterium on growth medium amended with the acidic effluent of the acidogenic consortium.

4. Materials and Methods

4.1 Material

Pretreatment of Rice Straw

Feedstock: Rice Straw

Chemicals: Sulphuric acid, Distilled water

Analytical Method (DNS): 3,5-dinitro salicylic acid, Sodium hydroxide (Merck), Sodium-potassium tartrate (Merck), de-ionized water, amber colored screw-cap bottle

Apparatus: Screw capped bottle, micropipette (Tarsons), 1 ml Micro-tips (Tarsons), test-tube holder, beaker, conical flask, measuring cylinder and scissor.

Instruments: Digital weighing machine (Sartorius), Autoclave (Gurpreet Engineering Works), Hot air Oven, Double Beam UV-Vis Spectrophotometer (Perkin Elmer), Heater (Bhattacharya & Co) and mixer grinder (Philips).

Microorganism/s used:

1. *Clostridium acetobutylicum* MTCC 11274

   Clostridium acetobutylicum (strain no. 11274) was purchased from the culture collection centre (MTCC) in freeze dried form. The bacterium was revived to viable form using Reinforced Clostridial Medium (RCM) and Glucose as the carbon source.

2. Acidogenic consortium isolated from natural source

A mixed culture of bacteria has been isolated from the mud sample collected from the agricultural field of Hooghly district. The culture has been enriched in RCM using glucose as the C-substrate.

Growth medium for C.acetobutylicum (Reinforced Clostridial Agar medium):

Table 1: Reinforced Clostridial Medium (RCM) Composition

Sl No.	**Description**	**Amount (on the basis of 1000ml)**
1	Peptone (Merck)	10 gm/l
2	Beef Extract (Merck)	10 gm/l
3	Yeast Extract (Merck)	3 gm/l
4	Glucose (Merck)	5 gm/l
5	NaCl (Merck)	5 gm/l
6	L-cysteine hcl (Merck)	0.5 g/l
7	Sodium acetate (Merck)	3 gm/l
8	Starch (Merck)	1gm/l
9	Distilled water	1000 ml

Revival of cultures: Dried form of microorganisms, petri dishes, paraffin oil, paraffin film, glass elbow (spreader), and inoculating loop.

Batch set up: Erlenmeyer flasks (E-Flasks), micropipette (Tarsons), 1 ml Micro-tips (Tarsons), non-absorbent cotton plug, parafilm tape.

Instruments: Digital weighing machine (Sartorius), Autoclave (Gurpreet Engineering Works), Hot air Oven, B. O bench, Luxmeter (Lutron, Taiwan), Argon generator, NMR.

Gram staining method: Microscopic glass slides, Crystal violet (Loba Chemie), Gram's iodine, Ethyl alcohol, Saffranin (Loba Chemie), Spirit lamp, fitted with camera (Olympus).

4.2 Experimental Methods:

4.2.1 Preparation of DNS Reagent Working Procedure

1) Colour reagent solution (solution 1) was prepared first in a conical flask. 12 grams of Sodium Potassium Tartrate was dissolved in 8 ml of 2M NaOH (0.64 gram)by direct heating and constant stirring.
2) 0.1M (0.4564 gram) of 3,5 – dinitro salicylic acid (DNS) solution (solution 2) was dissolved in 20ml of deionized water by direct heating and constant stirring.
3) Solution 1 was added gently with solution 2 with constant stirring and heating. In this solution 40ml deionized water was added and the change in colour is noticed. This DNS reagent solution is allowed to become cool and kept in an amber coloured bottle (to prevent it from light damage) inside the refrigerator.

4.2.2 Confirmatory test of non-reducing sugar in the hydrolysate of rice straw

- 3ml of DNS reagent were added in each of them and 3ml of samples were added in respective test tubes in the 1:1 ratio.
- The three test tubes were kept in a beaker containing water and heated in a heating mantle at 900C for 10-15 minutes.
- The colour change was observed.
- The spectrophotometer was switched on and wavelength was selected as 540 nm. At first, the O.D of the blank was taken and made it zero.

- Then, O.D of all the tubes was taken. The cuvettes were washed each time after taking O.D.

4.2.3 Preparation of Glucose Calibration Standard Curve

- Six test tubes were taken and labeled as Blank and 1 to 5.
- 100 ml standard glucose stock solution (1%) was prepared.
- Dilutions of glucose standards with 0.1, 0.2, 0.4, 0.6, 0.8 % were made by transferring required amount of glucose from the standard glucose stock solution (1%) and the total volume by distilled water was adjusted.
- 3 ml of DNS reagent was added to all the tubes containing 3 ml of glucose solution of their respective concentrations (1:1 ratio) and kept in boiling water bath for 15 minutes.
- The spectrophotometer was switched on and wavelength was set at 540nm. At first, the O.D of the blank was adjusted to zero.
- Then, O.D of all the samples (no.1-5) was taken. The cuvettes were washed each time after taking O.D.

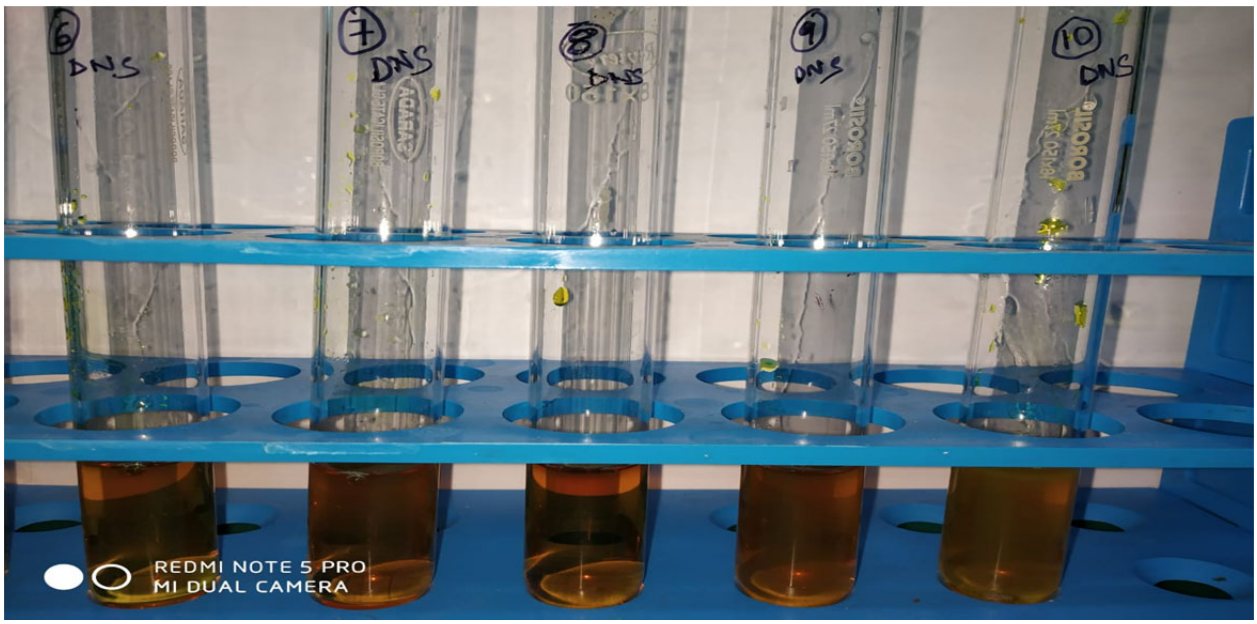

Figure 8: Glucose standards after DNS procedure

Table 2: Data for Glucose Calibration Curve

Sl. No.	Glucose %	Volume of glucose stock solution in ml	Volume of distilled water in ml
1	0.1	1	9
2	0.2	2	8
3	0.4	4	6
4	0.6	6	4
5	0.8	8	2

4.2.4 Pretreatment of Feedstock for biofuel production

Selection of feedstock

A large number of lignocellulosic residues like wheat straw, barely straw, corn stover, rice straw and switch grass can be used for butanol production. Rice straw is a major agricultural byproduct and one of the low cost and abundant lignocellulosic materials among agro residue that may properly utilized for biofuel production biologically because of its low lignin content.

Processing of Feedstock (mechanical size reduction, acid catalyzed pretreatment)

Size reduction often referred to as comminution of biomass play a crucial role in the preprocessing of biomass. Size reduction is an operation where the size distribution of biomass particles is adapted to a level determined by its final use. The purpose of biomass conversion into liquid biofuel, the average particle size is ideally in the micrometer range so as allowing enzymes to readily access.

The purpose of size reduction of biomass is to transform the material into a form that optimizes handling, storage, transportation and conversion (or direct combustion). Size reduction processes can be categorized as 1) single fracturing mechanisms such as cutting, shredding and/or shearing mechanisms as found in forage choppers, rotary veneer choppers, shredders, roller grinders, and crushers and 2) multiple-fracturing milling mechanisms such as knife mills, hammer mills, ball/rod mills, disk (attrition)mills, and ultrafine mills.

Milling is a high energy requirement process so it continuously increases the energy prices which in turn make it economically unfeasible. On the other hand the grinding is widely used for reducing the particle size which is a preliminary step to increase the specific surface area of the substrate and thus makes it more prone to enzymatic attack.

However, reducing only the particle size is insufficient in modifying the lignocellulosic structure. Thus, an amalgamation of other pre-treatment methods like acid hydrolysis, thermo mechanical extrusion and alkali pre-treatment becomes necessary.

4.2.5 Steps involved in processing of feedstock

Step 1

The feedstock is collected locally and stored

Step 2

The feedstock is then cut with scissors to 2 inches size and then oven dried at 85°C overnight

Step 3

Further size reduction using mixer grinder to obtain the particle size between 0.4mm to 2mm

Figure 9: Steps involved in preparation of feedstock

4.2.6 Acid hydrolysis of Rice Straw

The method of acid hydrolysis of rice straw by diluted H2SO4 was conducted at 121°C and 15 psi. Rice straw (5 g) was treated with 50 mL of H2SO4 solution (following a 1:10 ratio of biomass to acid solution) of various concentrations for different reaction times.

4.3 Sample Calculation

4.3.1 Determination of acid concentration

As for rice straw we have preferred dilute sulphuric acid of concentration of 0.5, 1.0, 1.5 w/w which we got from various literatures where the concentration of sulphuric acid is varied from 0.5-1.5w/w.

The density of H2SO4 is 1.82 g/cc which means 1.84 gram of H2SO4 in 1 cm3 of solution.

0.5 w/w of H2SO4 = 0.2747 ml H2SO4 in 99.5 ml of water.

Similarly 1 w/w of H2SO4 = 0.55 ml H2SO4 in 99ml of water.

1.5% w/w of H2SO4 = 0.824 ml H2SO4 in 98.5ml of water.

4.3.2 Determination of volume of acid solution to be added at different acid loading

VAcid = WRS * LA * (1/SA) * 100 where,

WRS = Weight of Rice Straw (RS)

LA = Acid to Rice Straw Ratio (Basis: 1g of acid solution)

SA = % strength of acid solution

Using the above equation the amount of acid solution is calculated for each 5g of rice straw used.

4.4 Preparation of nutrient media

- In order to perform microbial study, preparation of nutrient media is the most essential step for proper growth, product formation and maintenance of bacterial cultures. The composition of nutrient media was almost similar to that of growth media.
- The ingredients of the nutrient medium were weighed, according to the composition of the media prescribed in literatures, and dissolved in appropriate amount of distilled water. Then the media was autoclaved at 2.04 kg/cm2 for 15 minutes in order to make it sterile and free from any contamination. Then the media has been used for the preparation of seed culture and batch experiments.

4.5 Procedure for preparation of seed culture

- A 100 ml conical flask containing 100 ml sterile RCM broth is autoclaved.
- Glucose substrate is UV- sterilized and then added to the conical flask and shaken well to dissolve the glucose completely in the medium.
- A slant test tube culture of mixed consortia and chosen bacteria (*Clostridium acetobutylicum*) is taken.
- A loopful of the bacteria is carefully taken out from the test tube with the help of a sterile inoculating loop.
- The cotton plug of the conical flask is removed and the loopful of the bacterial inoculum is inserted into the flask. The sterile RCM broth is inoculated with the bacterial culture by dipping the loop containing the inoculum and transferred it to the media by gentle shaking.
- The loop is taken out from the flask and the cotton plug is replaced to the mouth of the flask and sealed tightly with parafilm strip.
- The flask is kept in the incubator at 37 ºc and incubated for 30 hrs.
- The total procedure was performed in the laminar air flow bench maintaining strictly sterile conditions. From the 30 h old broth culture several subcultures can be prepared as required for the experiment.

4.6 Gram staining procedure for microscopic observation of Bacterial Species

Gram positive and gram negative bacteria are differentiated based on their physical and chemical properties of their cell wall by the gram straining method which is basically a differential staining method. It is the basic foundation on which bacterial identification is

built and it divides the Eubacteria according to their sustainability into two fundamental groups.

Procedure:

1) Dye crystal violet is used to stain the smear. It is stained for 30 seconds and then water rinsed for about 20 seconds.
2) It is then treated with iodine solution for 1 minute and then water rinsed. Iodine acts as a mordant and increases the interaction between cells and the dye in order to stain the cell more strongly.
3) More decolourization of the smear is done by watching with 95% ethanol or acetone for about 10 to 30 seconds. In this step the differential aspect of gram stain is generated. Do the gram positive bacteria retain the crystal violet whereas the gram negative ones lose their crystal violet and become colourless.
4) Further the smear is counter stained using safranin which is one of the most common counter stain for about 30-60 seconds and then rinsed with water.
5) The excess water is removed by a blotting paper and then air dried.
6) Finally it is observed under microscope. The gram negative bacteria colour ranges from pink to red whereas the gram positive bacteria become dark purple.

4.7 Experimental procedure of batch study with varying conc. of glucose and hydrolysate as substrate

1) Each batch study was performed by varying glucose concentration in 50ml conicals, labelled for every two hour reading. 30ml of nutrient media was poured in the conical and flushed with argon gas through UV sterilized membrane filter.

2) The media was inoculated with 10% of inoculums (3ml for 30 ml suspension culture), with the help of a micropipette and sterile micro tip. Inoculums was taken from previously prepared seed culture had same concentration of substrate as that of batch experiments.
3) After inoculation, the head space volume of the conical flasks were sparged with argon gas for 2-3min. Sparging was immediately followed by air tight sealing of conical with cotton plug and paraffin film strips.
4) All the inoculated conical flasks was kept in incubator at 36-37oc and 3.5 klux light intensity for 24h incubation.
5) Above described steps was repeated for preparing three more batch set ups with varying glucose and hydrolysate concentrations of 0.5%, 1.0%, 2%, 3% and 5% in the media. Same amount of inoculums (10% of the total culture broth) from the corresponding seed culture were used to inoculate all of set ups.
6) After every two hour time interval, samples were collected from the conical flasks of respective hour and analysed by the conventional gravimetric method to determine the biomass concentration at particular time. The collected samples were centrifuged for 15mins at 10000 rpm in a table top centrifuge, in 1.5 ml pre weighed eppendorf.
7) After centrifugation, the supernatant was discarded and the pellet was allowed to dry for overnight in hot air oven. The eppendorf with dried pellet was weighed and subtracted by the weight of empty eppendorf. Therefore, the amount of biomass present in 1.5ml of sample and the biomass concentration in sample can be calculated.

8) On obtaining the biomass concentration at every second interval for corresponding glucose concentration, growth curves of cell concentration vs. Time was plotted. From these growth curves the growth kinetic parameters were calculated using Monod-model.

Figure 10: Batch study with varying conentration of glucose and hydrolysate as substrate

4.8 Experimental procedure of batch study with combinations of conc. of glucose and hydrolysate

1) Each batch study was performed by varying glucose and hydrolysate concentration 50ml conical were taken, labelled for every two hour reading. 30ml of nutrient media was poured in the conical and flushed with argon gas through UV sterilized membrane filter.
2) The media was inoculated with 10% of inoculums (3ml for 30 ml suspension culture), with the help of a micropipette and sterile micro tip. Inoculums was taken from previously prepared seed culture had same concentration of substrate as that of batch experiments.

3) After inoculation, the head space volume of the conical flasks were sparged with argon gas for 2-3min. Sparging was immediately followed by air tight sealing of conical with cotton plug and paraffin film strips.
4) All the inoculated conical flasks were kept in incubator at 36-37°c and 3.5 klux light intensity for 24h incubation.
5) Same procedure as that of glucose and hydrolysate substrate is followed.

4.9 Experimental procedure of batch study for the survival and growth of a solventogenic bacterium on growth medium amended with the acidic effluent of the acidogenic consortium.

1) It was performed by varying acidic effluent of the acidogenic consortium concentration. 50ml conical were taken, labelled for different sets. 30ml of nutrient media was poured in the conical and flushed with argon gas through UV sterilized membrane filter.
2) The media was inoculated with 3ml, 5ml, 10ml of inoculums for 30ml of suspension culture with the help of a micropipette and sterile micro tip. Inoculums was taken from previously prepared seed culture had same concentration of substrate as that of batch experiments.
3) 2% of *Clostridium acetobutylicum* was added from the seed culture.
4) After inoculation, the head space volume of the conical flasks were sparged with argon gas for 2-3min. Sparging was immediately followed by air tight sealing of conical with cotton plug and paraffin film strips.

5) All the inoculated conical flasks were kept in incubator at 36-37°c and 3.5 klux light intensity for 72h incubation.

6) The pH and OD was observed after that 72h of incubation.

4.10 Mathematical Modelling using MATLAB

The mathematical modelling was done using MATLAB 2017a using ODE45 which is based on Runge-Kutta method. Using specific growth rate datas of mixed strain using glucose-hydrolysate combination have been graphed against concentration of reducing sugar in hydrolysate and glucose as independent variables.

5. Theoretical Analysis

Evaluation of growth kinetic parameters (μ_m and K_s)

The specific growth rate (μ) refers to the steepness of a curve, and it is defined as the rate of increase of biomass of a cell population per unit of biomass concentration. It can be calculated in batch cultures, since during a defined period of time, the rate of increase in biomass per unit of biomass concentration is constant and measurable. This period of time occurs between the lag phase and stationary phases. During this period, the increase in the cell population fits a straight-line equation between ln x and t.

From the experimental data obtained by performing batch studies on mixed consortia the growth kinetic parameters were calculated. The growth of a bacteria growing in a batch study is given by,

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.. (1)$$

Integration of the above equation with limits of biomass concentration from X0 to X and that for time 0 to t the equation is converted to the form,

$\ln \frac{X}{Xo} = \mu t$ ……. (2)

Equation 2 is in the form y = mx, where m is the slope of the straight line and here equal to μ. So, if ln(X/X0) values of one bacteria are plotted against corresponding time values then the value of the slope of the curve will be equal to the value of specific growth rate (μ) of that bacteria.

Following this method four different μ values for two different substrate concentrations (1%, 2%, 3% and 5%) was calculated. Using these four μ values the growth kinetic parameters μm and Ks of the bacteria was evaluated. Linearized form of Monod's equation was used to calculate μm and Ks.

Linearized form of Monod's equation is given by,

$\frac{1}{\mu} = \frac{Ks}{\mu m} * \frac{1}{S} + \frac{1}{\mu m}$ …..... (3)

This equation is in the form y = mx + c, where the slope m of the curve gives the value of Ks/μm and the value of the intercept c is equal to 1/μm. From the value of the intercept μm is calculated and using this value Ks is calculated. 1/μ values are calculated and plotted against 1/S values and using equation 4 μm and Ks of the bacteria was evaluated.

This total procedure of batch study was performed for mixed consortia in Reinforced Clostridia Media (RCM) nutrient media individually for both the substrates glucose and rice straw hydrolysate and μm and Ks values for growth of the bacteria were calculated.

Coexistence of competing species of two substrates

Consider the specific growth rates of each microorganism described by the extended monod model for two substrates

$$\mu = \frac{\mu m1\ S1}{ks1 + S1 + a11S2} + \frac{\mu m2\ S2}{ks2 + S2 + a12S1}$$

Nomenclature

μ: Specific microbial growth rate (h^{-1})

T = Time (h)

S = Substrate concentration (g/l)

K_{S1} = Substrate saturation constant of mixed consortia in glucose medium (g/l)

K_{S2}= Substrate saturation constant of mixed consortia in hydrolysate medium (g/l)

μ_m = Maximum specific growth rate (h^{-1})

μ_{m1} = Maximum specific growth rate of glucose (h^{-1})

μ_{m2} = Maximum specific growth rate of hydrolysate (h^{-1})

S1 = Substrate concentration of glucose (g/l)

S2 = Substrate concentration of hydrolysate (g/l)

a12 = Mutual inhibitions constant (mg/l)

a11 = Reciprocal of a12 (mg/l)

6. Results and Discussion

6.1 Selection of Acidogenic Consortia from different natural sources

The microorganisms of the carboxylate platform ferment a wide variety of biomass feedstocks into mixed carboxylic acids and their salts, which in turn can be transformed into hydrocarbon fuels. Natural microorganisms that are metabolically potent to redirect renewable carbon sources into desired fuel products are contemplated as best choices to obtain high volumetric productivity and yield. There are many microorganisms (often environmental isolates) which possess native biochemical pathways that convert biomass into products that resemble biofuels.

Under the present study an attempt has been made for the isolation of acidogenic microbial consortium from some natural sites/sources. The selected natural sources from where sample have been obtained for isolation of microorganisms are:

- Agricultural field mud (Ag-M)
- Cow Dung (CD)
- Heat treated Cow Dung (CD-H)
- Rumen (Ru) fluid of calf
- Heat treated Rumen (Ru-H) fluid

Initially sample taken from all the selected natural sources grew in RCM medium in presence of glucose. pH of the medium fell from 6.8-7 to the range of 5-5.5 indicating production of acids.

All the samples were then inoculated in RCM containing RS hydrolysate as the carbon source and kept for 24 hours in incubator for the visible observation.

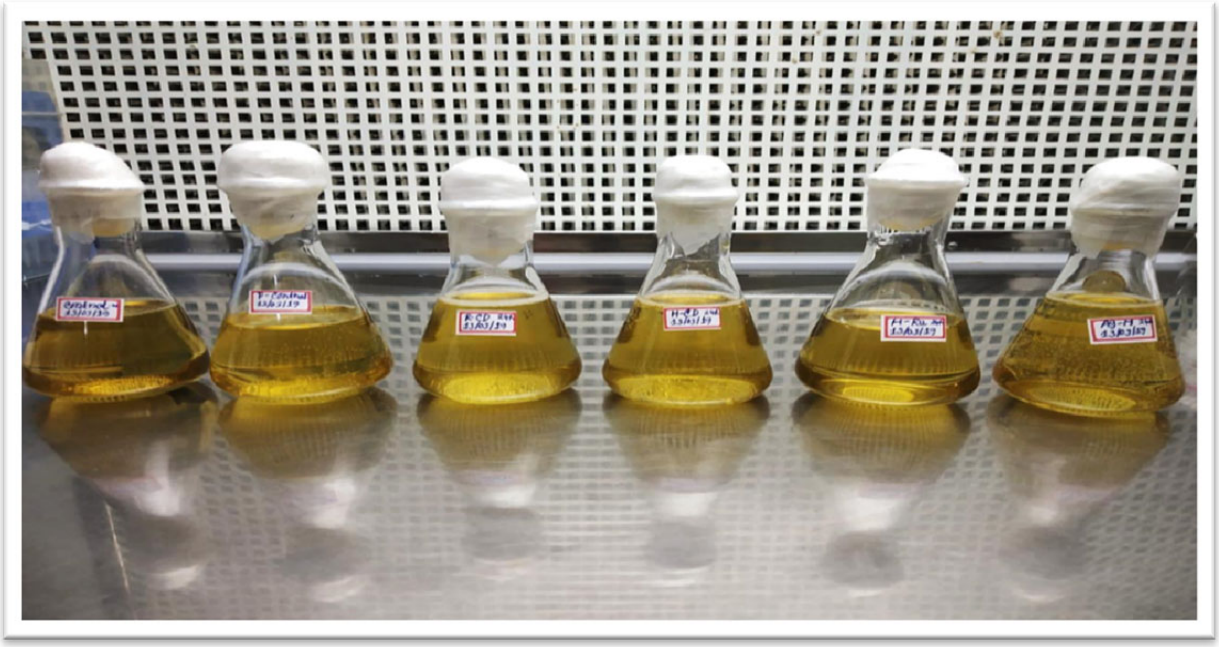

Figure 11: Different natural sources inoculated in RCM containing RS hydrolysate.

Figure 12: After 24hours it has been observed that only Ag-M grew well and adapted in RCM + Rice Straw Hydrolysate medium.

After 24hrs of growth it was observed that only the culture designated as Ag-M was able to grow using the RS hydrolysate. Hence, Ag-M was selected as the acidogenic consortium to be used in the VFA platform.

The acidic metabolites produced by Ag-M have been analysed and identified using NMR analysis. The NMR showed multiple peaks indicating production of mixed volatile fatty acids. Among the acids, acetic acid has been clearly identified as a product of the Ag-M.

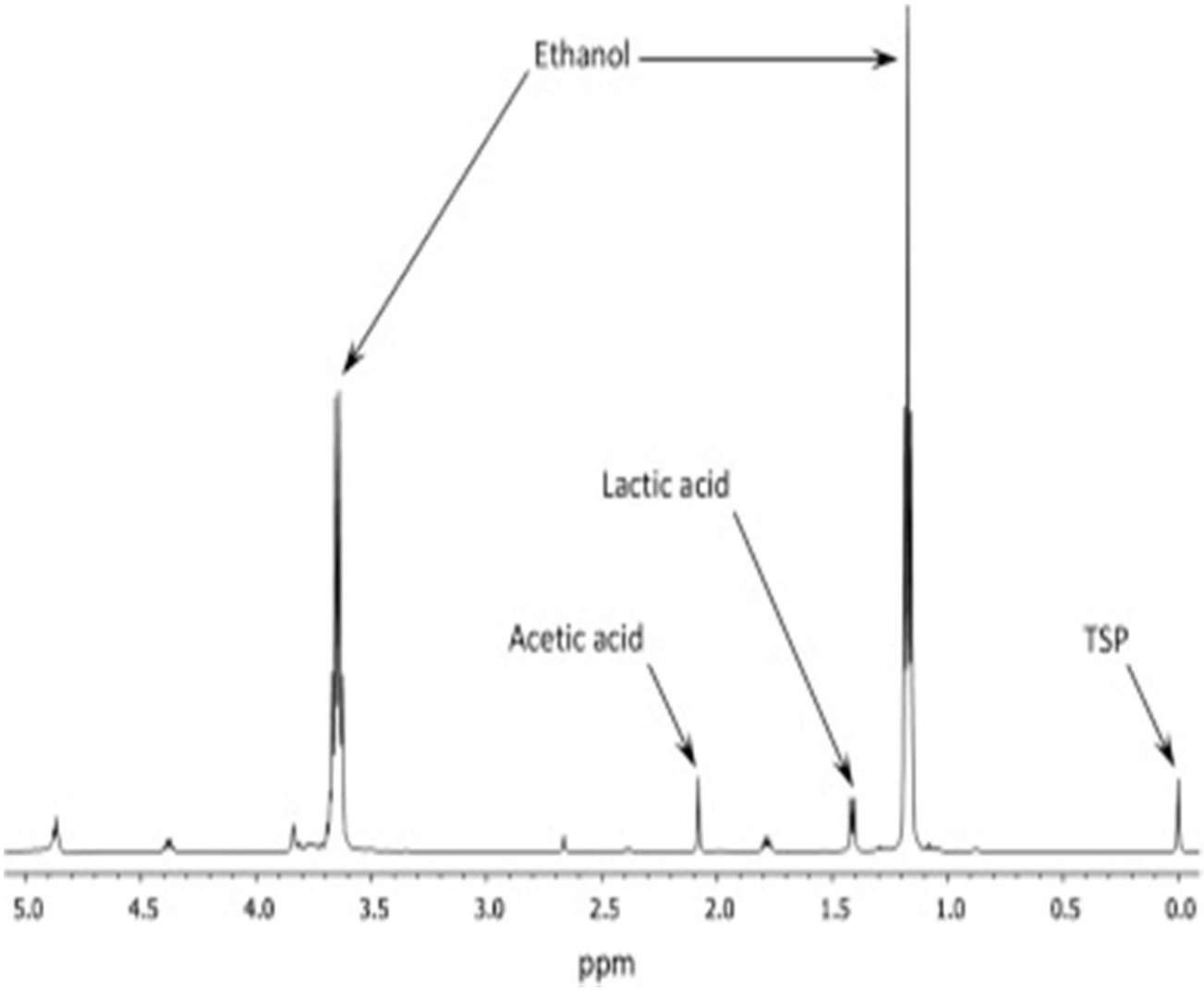

Figure 13: NMR standard curve of acetic acid [35]

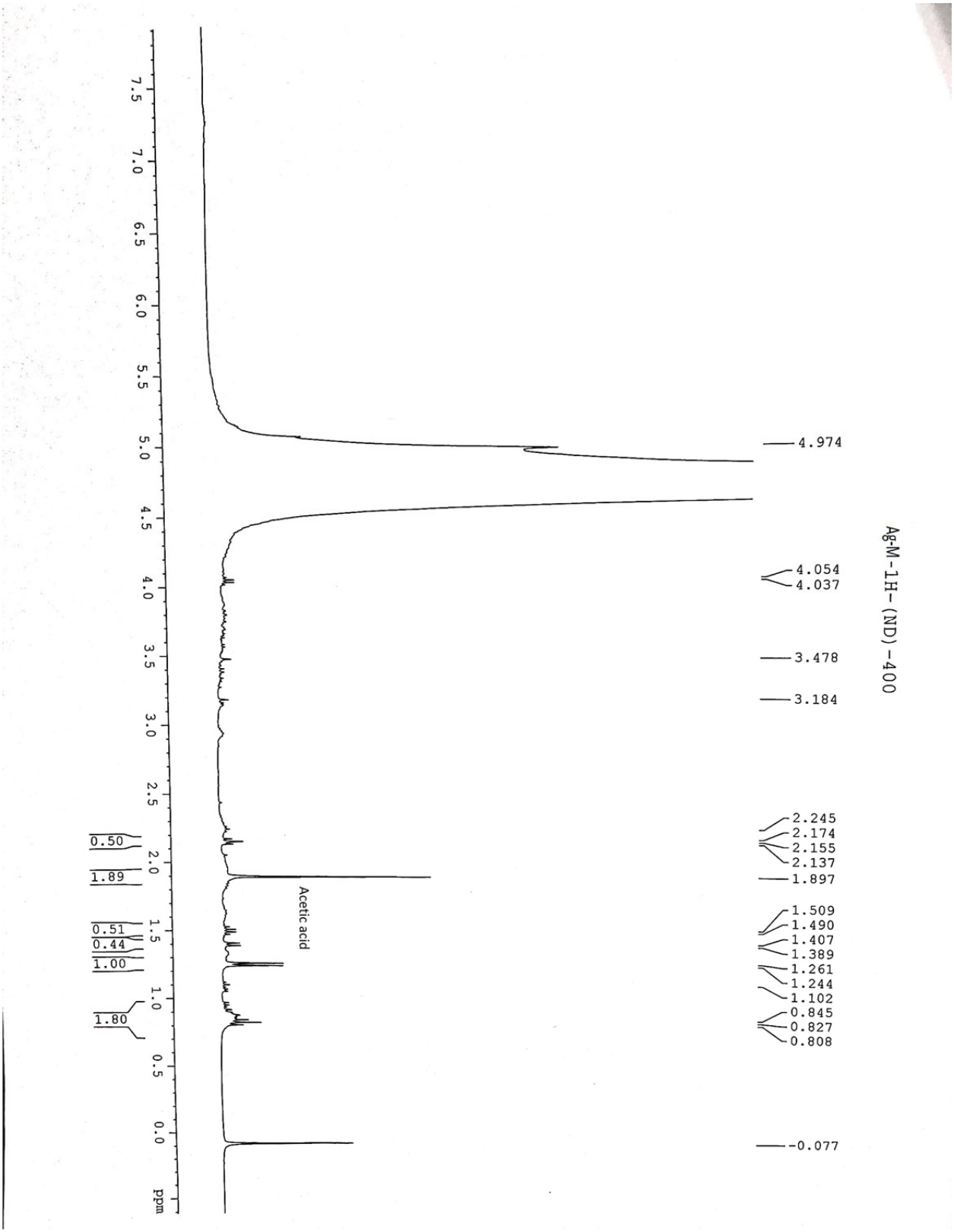

Figure 14: NMR of Mixed consortia (Ag-M

6.2 Calibration curve for quantification of total reducing sugar in the pretreated samples using DNS method

For the estimation of glucose or TRS concentration in all samples a correlation between OD and Glucose concentration has been established by preparing a calibration curve.

Table 3: Glucose concentration and OD value

Sl No.	Glucose Concentration (%)	OD value at 540nm
1	0.1	0.186
2	0.2	0.384
3	0.4	0.919
4	0.6	1.503
5	0.8	1.951

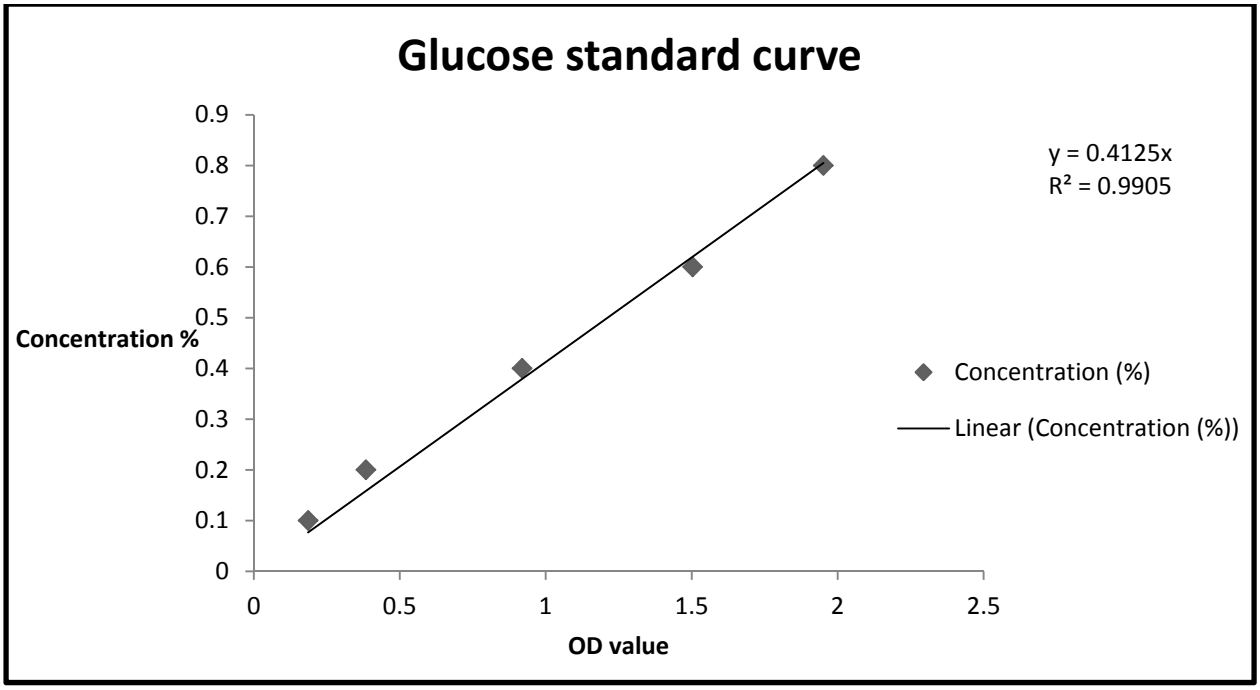

Figure 15: Glucose standard curve

As seen from the figure, a linear plot is obtained and R^2 value came out to be 0.9905 which is acceptable. The equation is y = 0.4125x where y represents concentration and x represent OD value.

From the standard curve, we can find out the unknown concentration whose O.D is available by the formula:

Concentration = {*(0.4125*OD value)* * Dilution factor}

6.2.1 Rice Straw Pretreatment and release of TRS

Rice straw (RS) has been selected as the lignocellulosic feedstock for production of VFAs and was pretreated using acid mediated hydrothermal pretreatment for extraction of simple sugars as total reducing sugar (TRS).

Table 4: Acid pretreatment of rice straw with different acid concentration and autoclave time

Set No.	Acid Concentration	Autoclave time	OD value	Glucose %
1A	1% H2SO4 w/w	15 minutes	0.968	39.93
1B	1% H2SO4 w/w	30 minutes	1.268	52.3
2A	0.5% H2SO4 w/w	15 minutes	0.842	34.73
2B	1% H2SO4 w/w	30 minutes	0.856	35.31

From the above table it has been observed that 1% H2SO4 acid solution and 30 minutes autoclave time gave the best result. Therefore it has been chosen for all the experiments performed using rice straw hydrolysate.

6.2.2 Acid pretreatment of rice straw with pyrolysed and gasified char of mustard press cake (MPC).

Table 5: Acid pretreatment of rice straw with pyrolysed and gasified char of mustard press cake (MPC)

Sl No.	Sample Designation	OD	Conc. (1/100D) (%)	TRS (%)
1	0.5 N NaCl-RSP + Acid solution 1%	0.991	0.6747719	67.47719
2	DW-RSP + Acid solution 1%	0.949	0.6461741	64.61741
3	1 N NaCl-RSP + Acid solution 1%	1.098	0.7476282	74.76282
4	0.5 N NaCl-RSP + MPC-Cg + Acid solution 1%	0.504	0.3431736	34.31736
5	1 N NaCl-RSP + MPC-Cg + Acid solution 1%	0.477	0.3247893	32.47893
6	DW NaCl-RSP + MPC-Cg + Acid solution 1%	0.601	0.4092209	40.92209
7	R-RSP + Acid solution 1%	0.832	0.5665088	56.65088
8	R-RSP + MPC-Cg + Acid solution 1%	0.381	0.2594229	25.94229
9	R-RSP + DW	0	0	0
10	DW-RSP + Acid solution 1% + MPC-Cp	0.396	0.2696364	26.96364
11	0.5 N NaCl-RSP + Acid solution 1% + MPC-Cp	0.373	0.2539757	25.39757
12	1 N NaCl-RSP + Acid solution 1% + MPC-Cp	0.332	0.2260588	22.60588
13	DW-RSP + MPC-Cp	-0.009	-0.0061281	-0.61281
14	0.5 N NaCl-RSP + MPC-Cp	-0.004	-0.0027236	-0.27236

15	1 N NaCl-RSP + MPC-Cp	-0.006	-0.0040854	-0.40854

To check whether any enhancement of TRS release from RS is possible during the pretreatment, some combination of catalysts, as represented in the above table has been used. From the above table it can be observed that rice straw treated with 1N NaCl initially and then with 1% Acid solution gave around 74% of glucose. However, in present experimental studies the raw rice straw (R-RSP) has been pretreated using 1% acid solution and 30 minutes autoclave time.

6.3 The trends of biomass and pH of Ag-M on glucose and hydrolysate

Growth curves of mixed consortia on two carbon substrates glucose and rice straw hydrolysate have been compared to check the acid production potential of the Ag-M.

Table 6: Comparison of cell biomass of 0.5 g/L glucose and 0.5 g/L hydrolysate

Time (Hr)	0.5 g/L Glucose	0.5 g/L Hydrolysate
0	0.573	0.424
2	0.9166	0.66
4	1.113	1.19
6	1.59	1.48
8	1.71	2.521
10	1.77	2.853
12	2.63	3.14

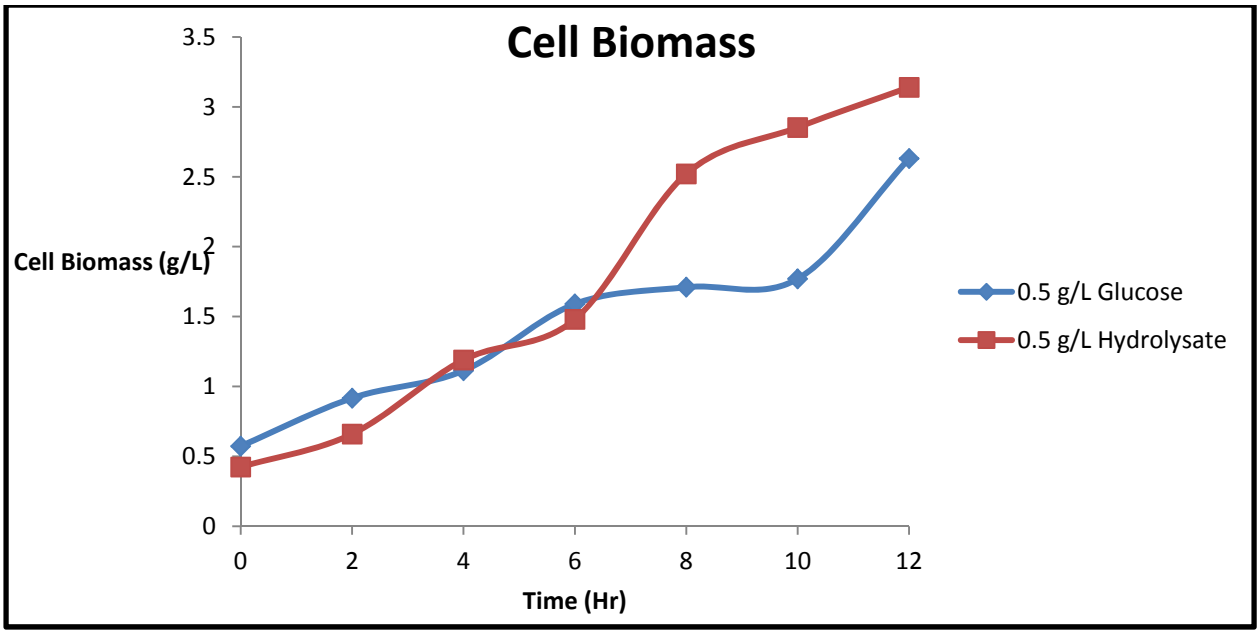

Figure 16: Biomass concentration curve of 0.5g/L glucose and 0.5g/L hydrolysate

From the above graph, it can be seen that mixed consortia Ag-M can utilize both the substrates and grow well. Growth curve of mixed consortia on 0.5g/L glucose and 0.5g/L rice straw hydrolysate shows an increasing trend of cell biomass with respect to increasing time. In this case, the biomass production in hydrolysate was slightly higher than the value obtained using glucose. This indicates that the mixed culture Ag-M is capable of using the TRS present in the hydrolysate, despite the presence of some inhibitory compounds (Furfural, HMF etc.).

Table 7: pH trend of 0.5 g/L glucose and 0.5 g/L hydrolysate

Time (Hr)	pH (Glucose)	pH (Hydrolysate)
0	7	7
2	6.8	6.5
4	6.5	6.2
6	6.1	5.5
8	5.5	5.2

10	5	5
12	5	4.8

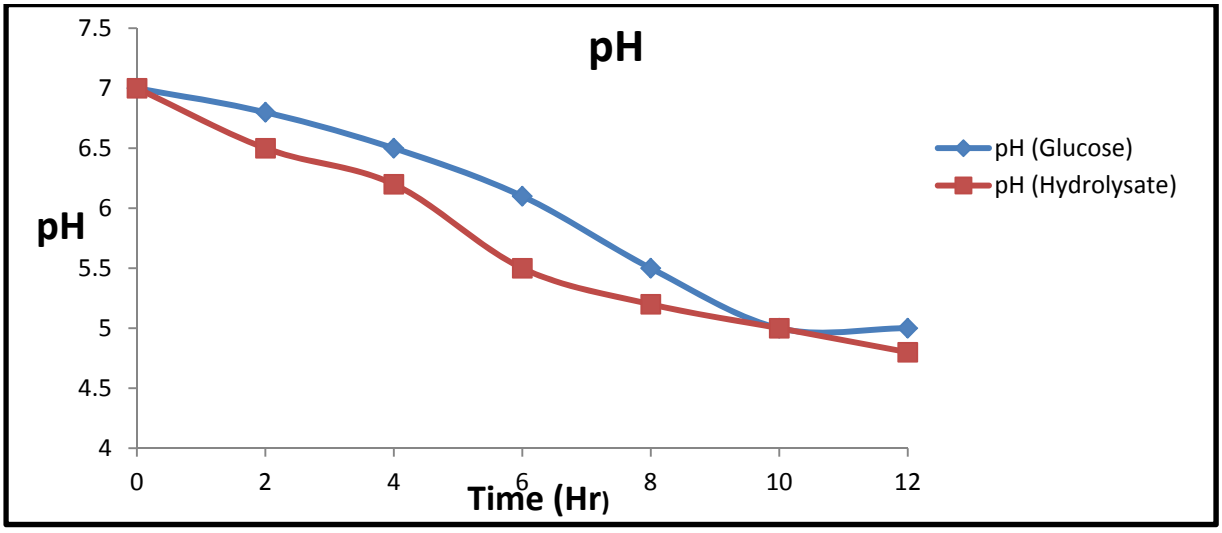

Figure 17: pH curve of 0.5g/L glucose and 0.5g/L hydrolysate

From the above graph it can be observed that the pH fall from 7 to 5 and 4.8 respectively on substrates glucose and rice straw hydrolysate, according to the biomass growth obtained. This indicates that the acidic products generated in the present system are mainly growth associated.

6.4 The trends of biomass and pH of Ag-M on glucose, hydrolysate and their combination.

Four different set of carbon substrates have been used for the studies on the trends of biomass growth and pH of Ag-M.

- Glucose 5 g/L
- Hydrolysate5 g/
- 1 g/L Glucose + 4 g/L Hydrolysate
- 2 g/L Glucose + 3 g/L Hydrolysate

Table 8: Cell biomass concentration against time for different combinations of carbon substrates

Time (h)	Cell Biomass concentration (g/L)			
	Glu (5g/L)	*Hyd (5g/L)*	*1g/L Glu + 4g/L Hyd*	*2g/L Glu + 3g/L Hyd*
0	0.4245	0.4245	0.4245	0.4245
2	0.5633	0.4833	0.5466	0.55
4	0.7933	0.54	0.6266	0.7233
6	1.04	0.7333	0.8666	0.9166
8	1.1533	0.9066	1.02	1.08
10	1.9733	1.6166	1.6933	1.8766
12	2.1133	1.8633	1.9766	2
24	2.2933	1.9633	2.0066	2.0366

From the time histories of biomass using all types of substrates, i.e., solely glucose or hydrolysate or their mixtures, it appears that up to 6h all plots show exponentially increasing trends. No lag phase is visible in any of the biomass time history, as represented in Figure… After 6h of exponential growth there is a plateau up to 8hr, observed for all cases. Another increasing trend appears from 8h to 12 h, after which the biomass growth slows down and enters the stationary phase for the pure hydrolysate and glucose-hydrolysate combinations. However, in case of pure glucose a small increase of biomass is observed.

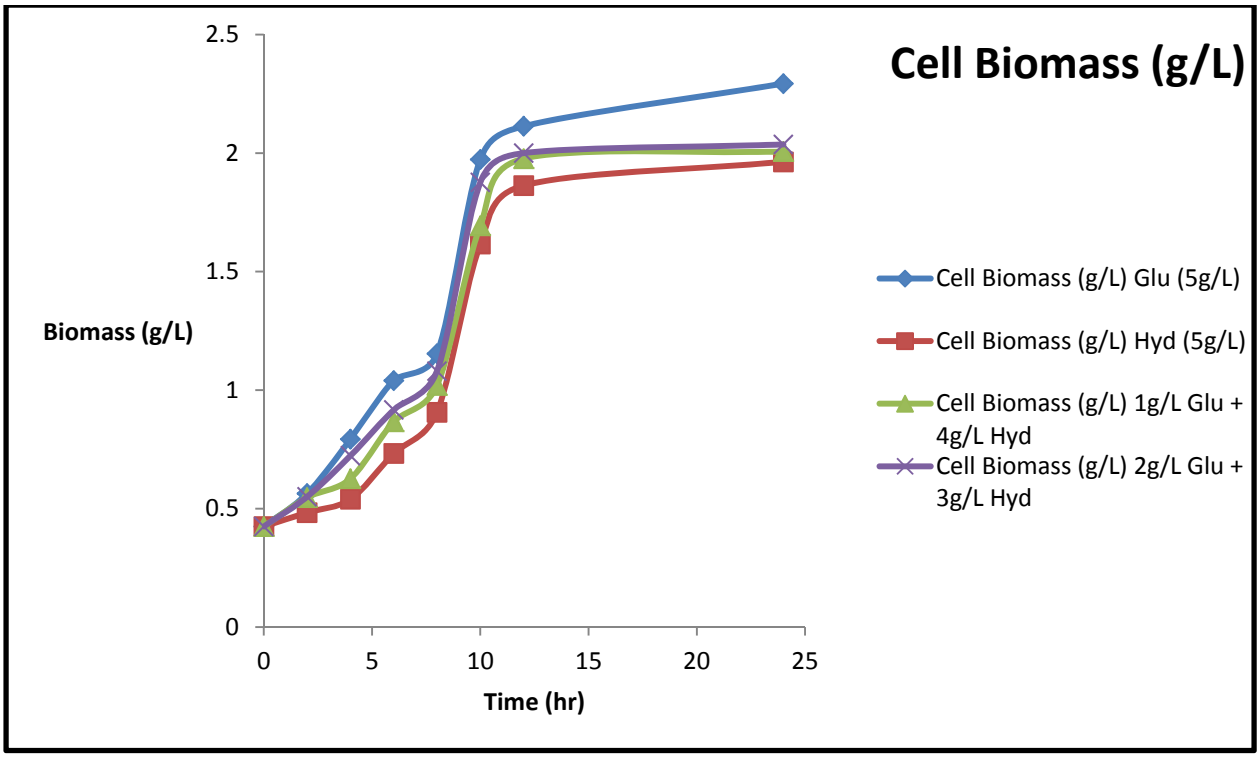

Figure 18: Biomass concentration curve of 5g//L glucose, 5g/L hydrolysate, 1g/L Glc + 4g/L Hyd and 2g/L Glc + 3g/L Hyd

The time histories of the substrate consumption in the pure glucose and mixed glucose-hydrolysate systems show sharp decrease in concentration of carbon substrate, namely, reducing sugar, until 6h for all cases. This is in agreement with the exponential growth of biomass for all cases. At 8^{th} h of growth in pure hydrolysate and glucose-hydrolysate combinations, an increase of C-substrate concentration is observed. For the pure glucose system a similar trend was observed at 10^{th} hr. This may be due to the phenomenon of lysis of a fraction of microbial cells by another fraction of cells present in the mixed culture of Ag-M, which is known as 'cryptic growth' and is a common phenomenon in growth of mixed cultures having limited supply of substrates. Another reason for this sudden increase in sugar concentration may be the lysis of comparatively larger complex sugar parts (xylan) by the hydrolytic microbes of the mixed culture that was not completely degraded during the pretreatment. For the case of pure sugar system the former phenomenon can be the sole reason. Substrate consumption ceased at 12hr in the pure hydrolysate and glucose-hydrolysate systems, probably due to the fall in pH and

presence of inhibitors that was produced during the hydrothermal pretreatment of RS. In case of pure glucose, substrate consumption continued until 24hr. All plots of substrate consumption were fully in agreement with respective biomass growth patterns.

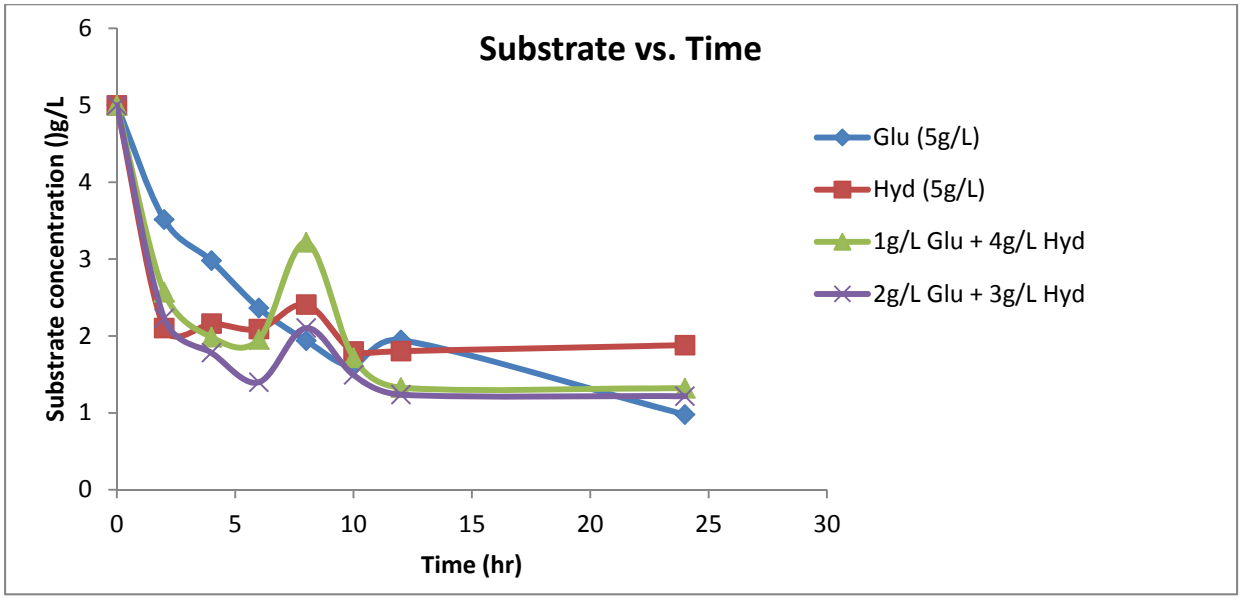

Figure 19: Substrate concentration curve of 5g//L glucose, 5g/L hydrolysate, 1g/L Glc + 4g/L Hyd and 2g/L Glc + 3g/L Hyd

Table 9: pH against time for different combinations of carbon substrates

Time	**pH**			
	Glu (5g/L)	*Hyd (5g/L)*	*1g/L Glu + 4g/L Hyd*	*2g/L Glu + 3g/L Hyd*
0	7	7	7	7
2	6.1	6.2	6.4	6.3
4	5.7	5.9	6	5.8
6	5.2	5.4	5.5	5.3
8	5	5.2	5.3	5.1
10	4.8	5	4.9	4.9

12	4.5	4.8	4.6	4.6
24	4.5	4.7	4.6	4.6

In accordance with the biomass growth and substrate consumption by the the acidogenic mixed culture Ag-M, the pH of the medium fall sharply for all cases. The pH reached least low value in case of pure glucose system, followed by the glucose-hydrolysate systems and pure hydrolysate system. Fall of pH was significant for all systems, indicating successful production of VFAs by the mixed culture.

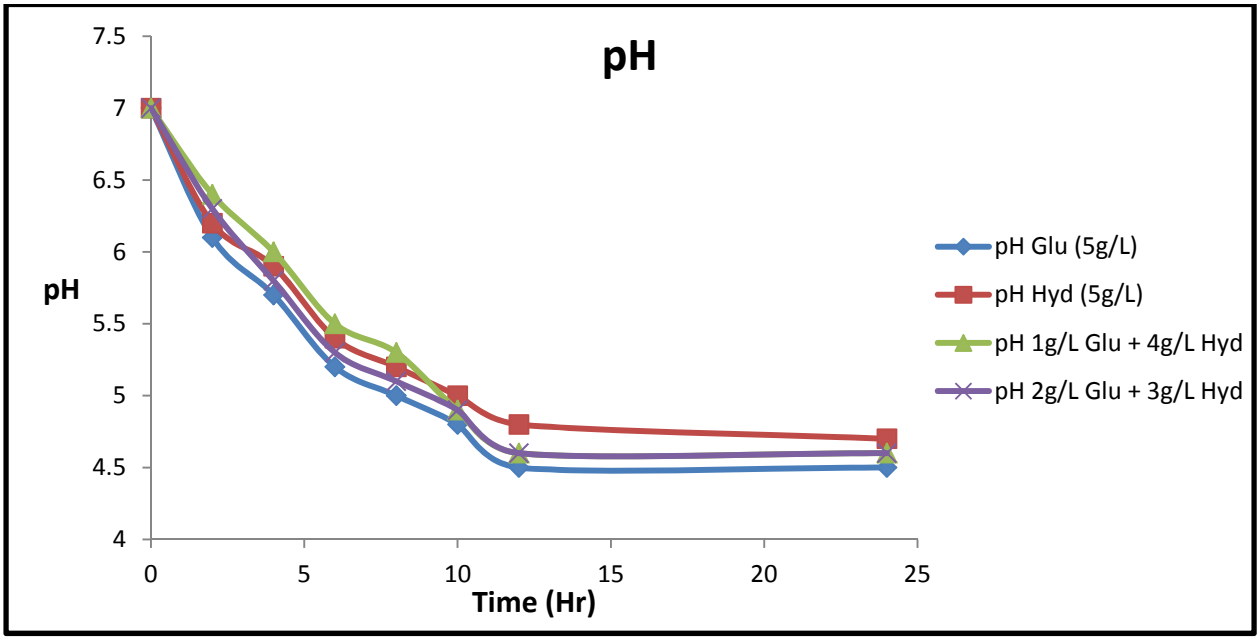

Figure 20: pH curve of 5g//L glucose, 5g/L hydrolysate, 1g/L Glc + 4g/L Hyd and 2g/L Glc + 3g/L Hyd

6.5 Evaluation of Growth Kinetic parameters μ_{max} and K_S for Ag-M in different substrates.

The mixed consortia Ag-M used for the growth in different concentrations of glucose and hydrolysate. The growth curves obtained using different range of substrate concentrations have been used for the calculation of the Growth Kinetic parameters μ_{max} and K_S.

Table 10: Range of substrate concentration

Range of substrate concentration	
Glucose 1g/L	Hydrolysate 1g/L
Glucose 2g/L	Hydrolysate 2g/L
Glucose 3g/L	Hydrolysate 3g/L
Glucose 5g/L	Hydrolysate 5g/L

Table 11: Cell Biomass Concentration against time for different glucose concentrations

Time (hr)	**Cell Biomass Concentration(g/L)**			
	Glu 1g/L	*Glu 2g/L*	*Glu 3g/L*	*Glu 5g/L*
0	0.4133	0.4133	0.4133	0.4133
2	0.5466	0.5566	0.5603	0.5701
4	0.76	0.78	0.8033	0.8266
6	0.95	0.9733	1.003	1.0266

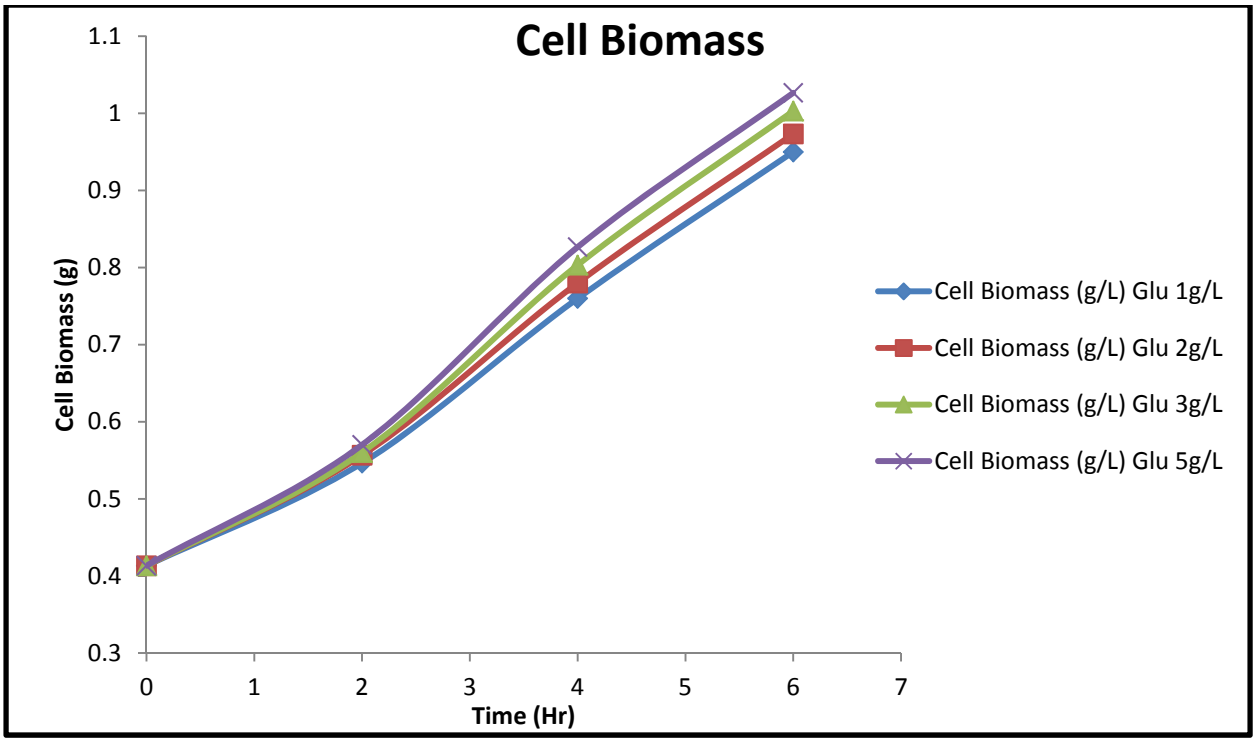

Figure 21: Biomass concentration curve of 1g/L glucose, 2g/L glucose, 3g/L glucose and 5g/L glucose

Table 12: Cell Biomass Concentration against time for different hydrolysate concentrations

Time (h)	**Cell Biomass (g/L)**			
	Hyd 1g/L	*Hyd 2g/L*	*Hyd 3g/L*	*Hyd 5g/L*
0	0.4133	0.4133	0.4133	0.4133
2	0.45	0.4766	0.502	0.517
4	0.62	0.6766	0.7166	0.7466
6	0.8466	0.8966	0.9266	0.96

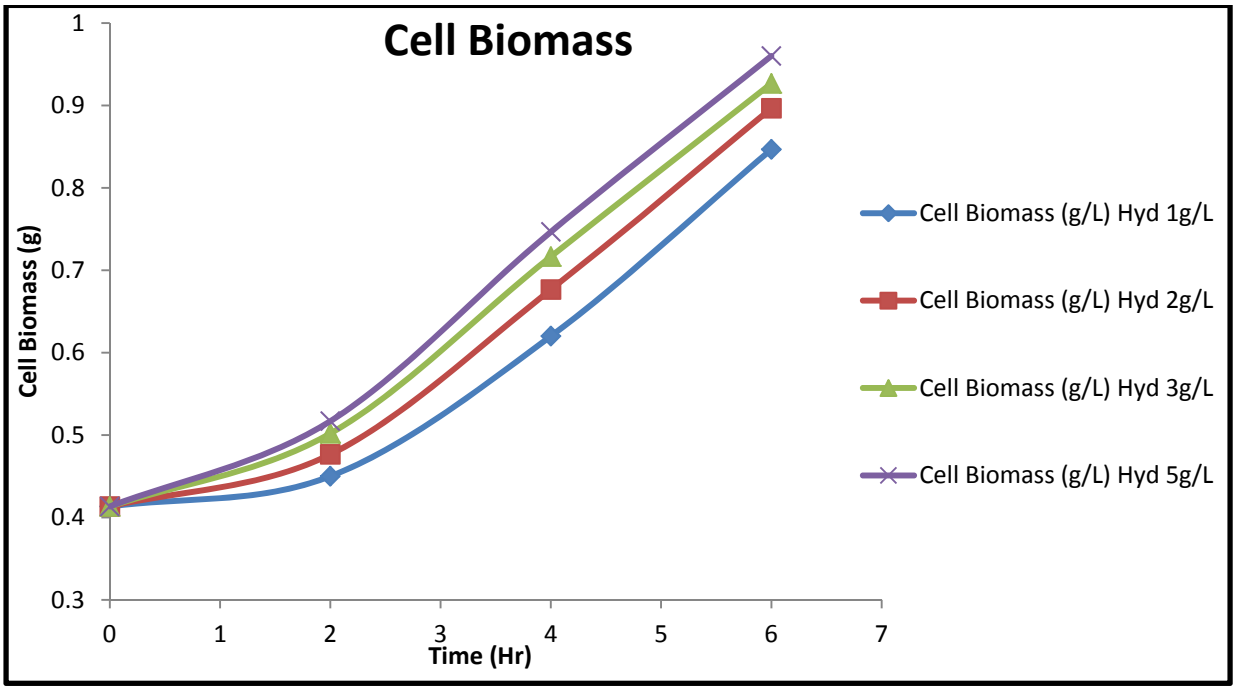

Figure 22: Biomass concentration curve of 1g/L hydrolysate, 2g/L hydrolysate, 3g/L hydrolysate and 5g/L hydrolysate

Table 13: For the substrate glucose, this table shows the data of 1/μ and 1/S of 0 and 2 hours

S (g/L)	**u(0hr, 2hr)**	**1/u**	**1/S**	**1/u**
Glu 1g/L	0.13886	7.20149	1	7.20149
Glu 2g/L	0.16496	6.06207	0.5	6.06207
Glu 3g/L	0.18689	5.35074	0.33	5.35074
Glu 5g/L	0.20258	4.93632	0.2	4.93632

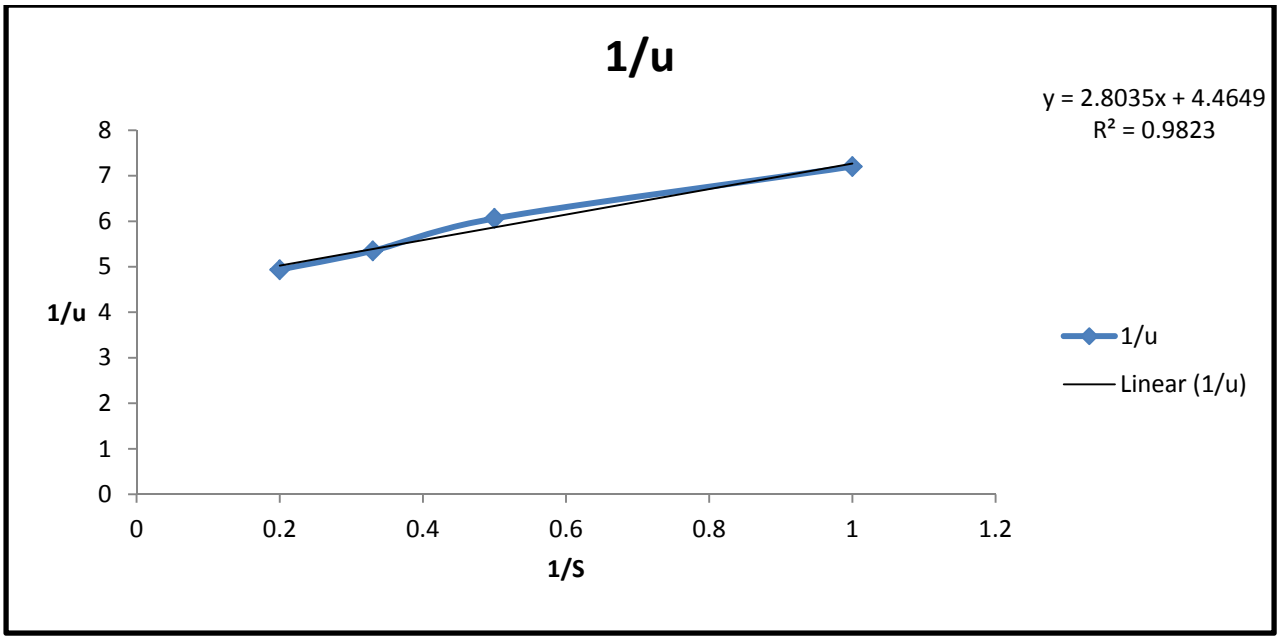

Figure 23: The plot of 1/μ against 1/S for the substrate glucose

This is showing the plot of 1/μ Vs. 1/S. This curve represents the rearranged form of

Monod's equation given by,

$$\frac{1}{\mu} = \frac{Ks}{\mu m} * \frac{1}{S} + \frac{1}{\mu m}$$

The intercept of this curve gives the value of $\frac{1}{\mu m}$ as

$\frac{1}{\mu m}$ =4.4649

μm= 0.2239 h^{-1}

The slope of this curve gives the value of $\frac{Ks}{\mu m}$ as

$\frac{Ks}{\mu m}$= 2.8035

Ks = 2.8035 * μm

Ks1= 0.6277

Table 14: For the substrate hydrolysate, this table shows the data of 1/μ and 1/S of 0 and 2 hours

S (g/L)	**u(0hr, 2hr)**	**1/S**	**1/u**
Hyd 1g/L	0.04251	1	23.5238
Hyd 2g/L	0.0713	0.5	14.0252
Hyd 3g/L	0.096908	0.33	10.31906
Hyd 5g/L	0.111469	0.2	8.971104

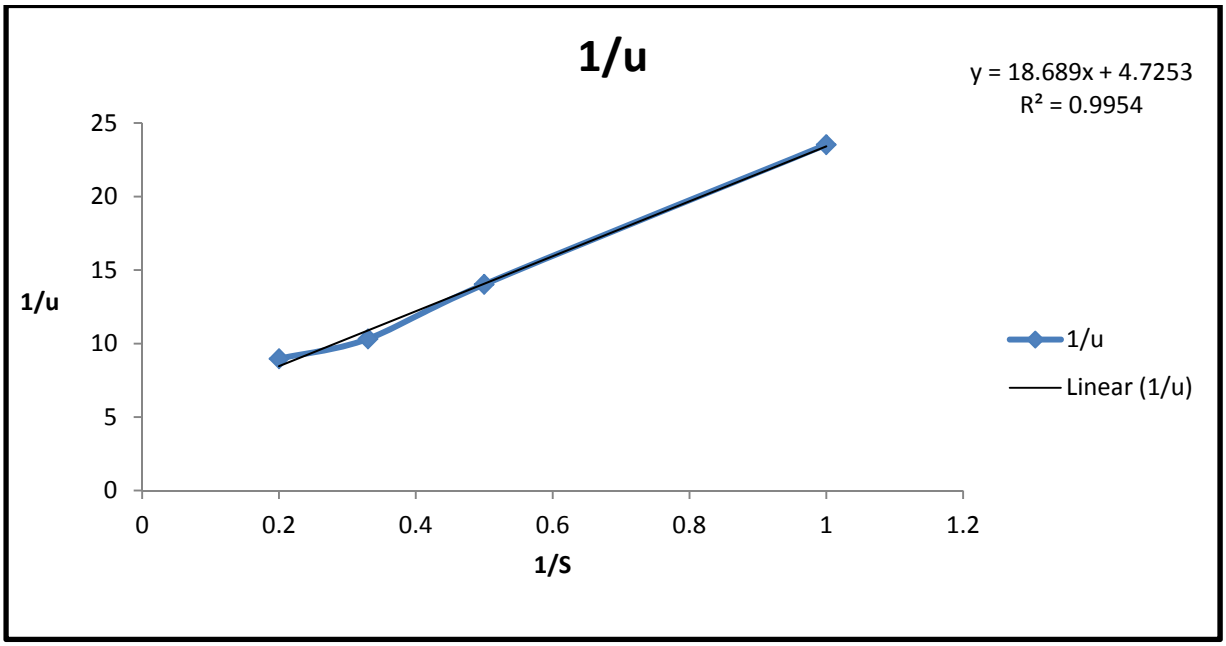

Figure 24: The plot of 1/μ against 1/S for the substrate hydrolysate

This is showing the plot of 1/μ Vs. 1/S. This curve represents the rearranged form of

Monod's equation given by,

$\frac{1}{\mu} = \frac{Ks}{\mu m} * \frac{1}{S} + \frac{1}{\mu m}$

The intercept of this curve gives the value of $\frac{1}{\mu m}$ as

$\frac{1}{\mu m}$ =4.7253

μm= 0.2116 h^{-1}

The slope of this curve gives the value of $\frac{Ks}{\mu m}$ as

$\frac{Ks}{\mu m}$= 18.689

Ks = 18.689* μm

Ks2= 3.9545

6.6 Prediction of growth pattern of Ag-M on mixed substrate (glucose + hydrolysate) systems

Values for the model equation

- μ_{m1}= 0.2239 h^{-1}
- μ_{m2}= 0.2116 h^{-1}
- k_{s1}= 0.6277
- k_{s2}= 3.9545
- $a_{12=}$ 0.15
- $a_{11=}$ 6.66

Prediction of the biomass growth and pH in the presence of mixed carbon substrates (glucose + hydrolysate) in batch systems using MATLAB.

Table 15: Prediction of biomass growth for Glc 2g/L and Hyd 3g/L in batch systems using MATLAB

Time (h)	X (g/L) Predicted	X (g/L) Experimental
0	0.4245	0.4245
2	0.5266	0.5466
4	0.6359	0.6266
6	0.7434	0.8666
8	0.8426	1.02

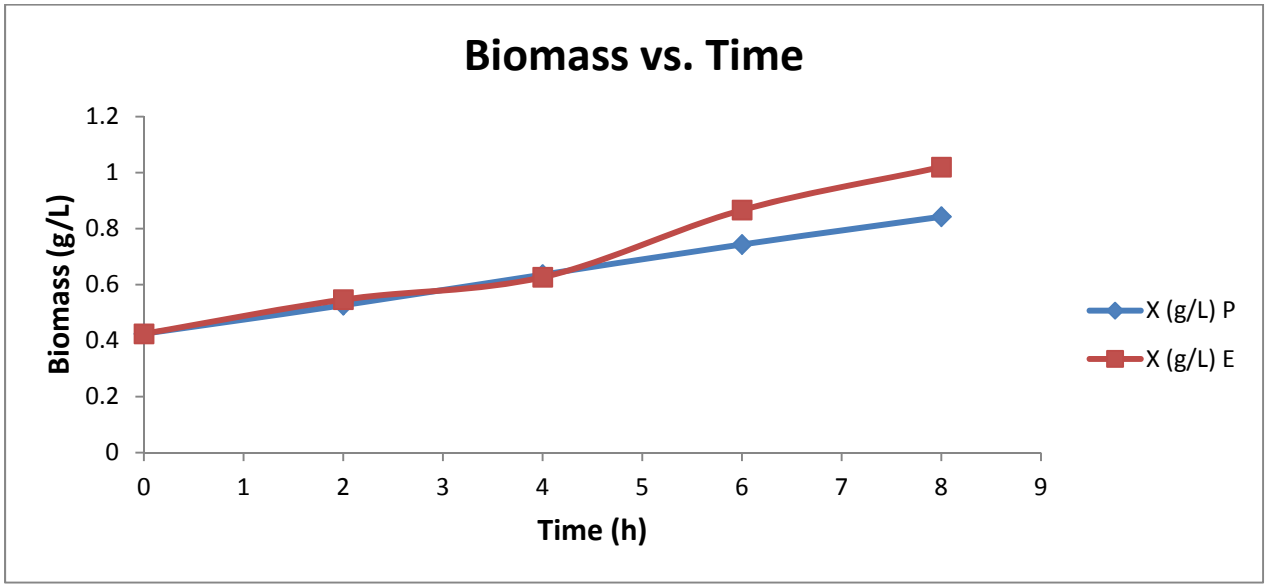

Figure 25: The plot of biomass concentration of predicted against experimental for the substrate combination of Glc 2g/L and Hyd 3g/L

Table 16: Prediction of pH for Glc 2g/L and Hyd 3g/L in batch systems using MATLAB

Time (h)	pH (Predicted)	pH (Experimental)
0	7	7
2	6.7	6.4
4	6.39	6
6	6.08	5.5
8	5.8	5.3

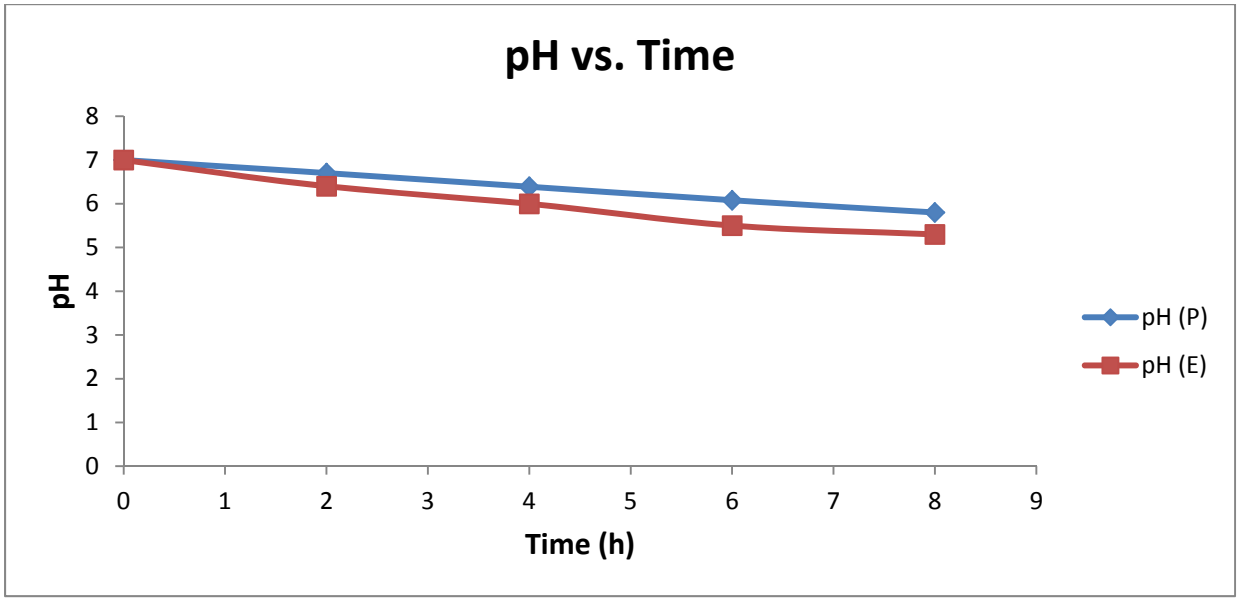

Figure 26: The plot of pH trend of predicted against experimental for the substrate combination of Glc 2g/L and Hyd 3g/L

Table 17: Prediction of biomass growth for Glc 1g/L and Hyd 4g/L in batch systems using MATLAB

Time (h)	X (g/L) Predicted	X (g/L) Experimental
0	0.4245	0.4245
2	0.524	0.55
4	0.6331	0.7233
6	0.7499	0.9166
8	0.8778	1.08

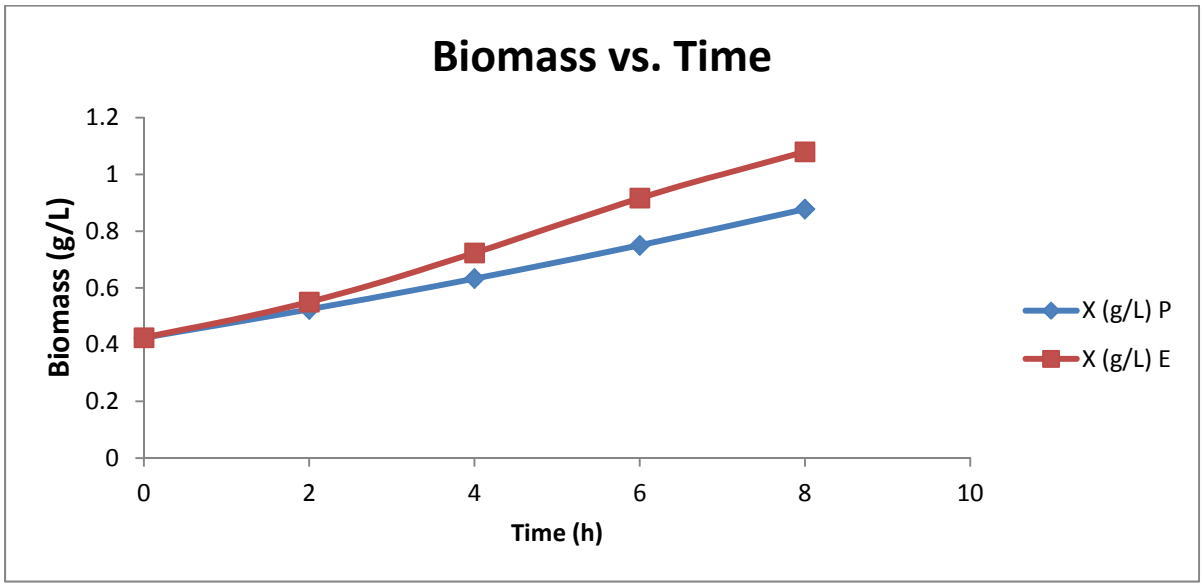

Figure 27: The plot of biomass concentration of predicted against experimental for the substrate combination of Glc 1g/L and Hyd 4g/L

Table 18: Prediction of pH for Glc 1g/L and Hyd 4g/L in batch systems using MATLAB

Time (h)	pH (Predicted)	pH (Experimental)
0	7	7

2	6.71	6.3
4	6.39	5.8
6	6.05	5.3
8	5.68	5.1

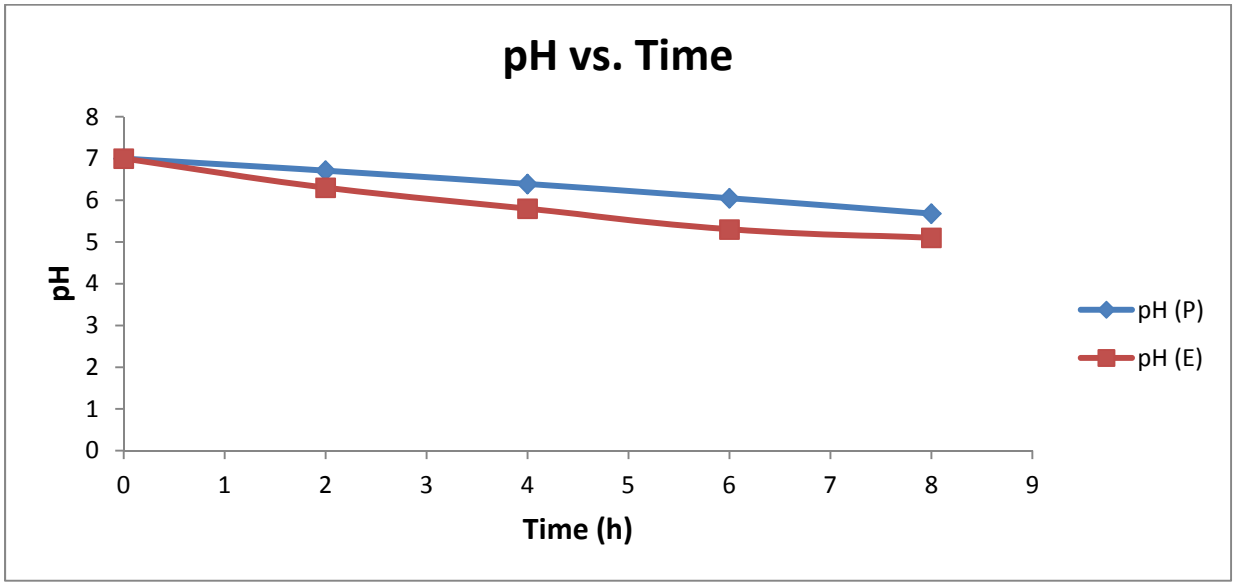

Figure 28: The plot of pH trend of predicted against experimental for the substrate combination of Glc 1g/L and Hyd 4g/L

The time history plots using experimental and predicted values of concentration of biomass and pH in all cases have been found to be in good agreement. This confirms the validity of the model for the prediction of biomass growth and pH in the present system of mixed carbon substrates.

Specific growth rates of mixed strain using glucose-hydrolysate combination have been graphed against concentration of reducing sugar in hydrolysate and glucose as independent variables in Surface plots and 3-D plots using MATLAB.

% Surface plot:

```
[X Y]=meshgrid(0:1:5);
Z=((X.*0.2239)/(0.6278+X+Y.*6.66))+((Y.*0.216)/(3.9545+Y+X.*0.15));
surf(X,Y,Z)
xlabel('x');
ylabel('y');
zlabel('z');
%title('title')
```

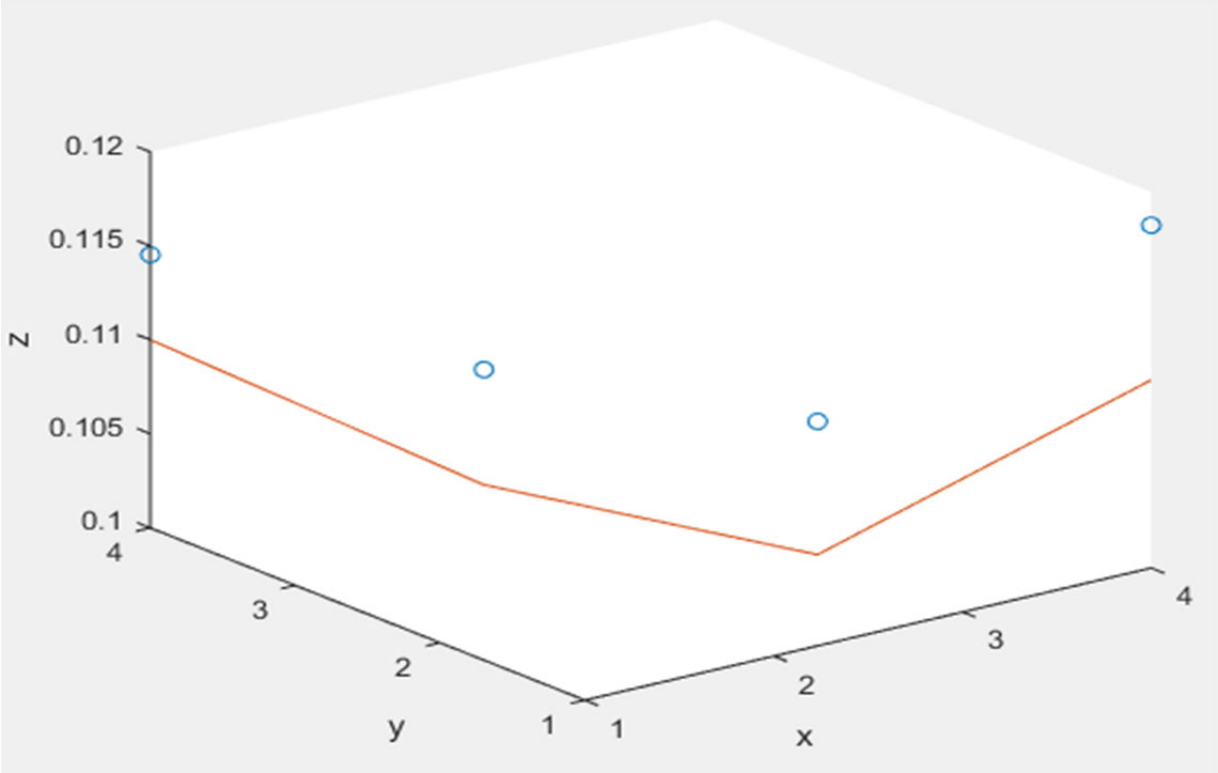

Figure 29: Surface plot of specific growth rates of mixed strain using glucose-hydrolysate combination

X= glucose concentration, g/L; y = concentration of reducing sugar, g/L; Z= μ, h^{-1}

the mesh plot shows the effect of two substrate (glucose and hydrolysate) on the specific growth rate of Ag-M. The experimental and predicted results show good agreement.

% 3D plot:

```
clear
```

```
clc
close all
x = [1 2 3 4];
y = [4 3 2 1];
z = [0.114528 0.109131 0.107086 0.11823];
z1=[0.110000 0.1030000 0.100000 0.1100];
figure
%plot3(x,y,z)
plot3(x,y,z,'o')
plot3(x,y,z1,'-')
plot3(x,y,z,'o',x,y,z1,'-')
xlabel('x');
ylabel('y');
zlabel('z');
```

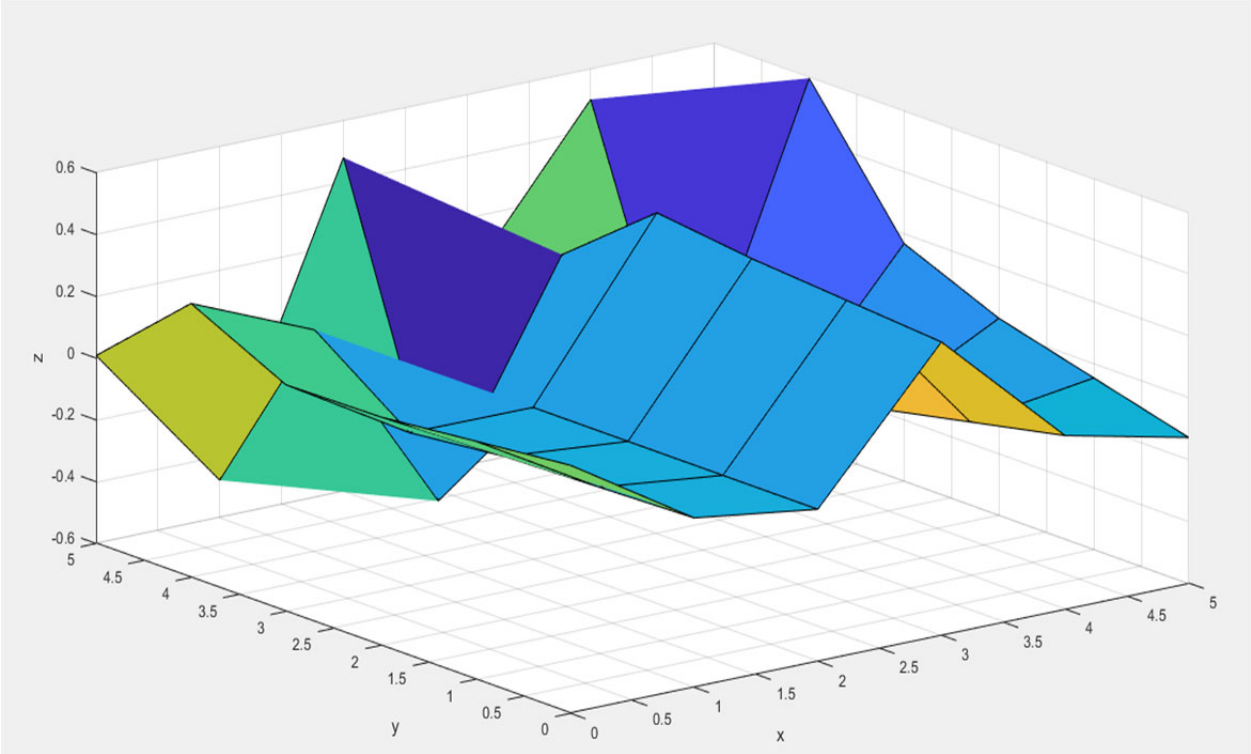

Figure 30: The 3D surface plot is showing the effect of the two substrate concentrations (x and y axes) on the specific growth rate (Z axis) of Ag-M

The 3D surface plot is showing the effect of the two substrate concentrations (x and y axes) on the specific growth rate (Z axis) of Ag-M. From the figure it can be seen that specific growth rate attains the maximum value of 0.6 /h when glucose concentration is 1 g/L and hydrolysate concentration is 4 g/L.

% Function file (2,3):

```
function dydt = acm(t, y)
umax1=0.2239;
umax2=0.2116;
Ks1=0.6278;
Ks2=3.9545;
A1=0.2334;
A2=0.1035;
A3=-2.898;
a12=6.66;
a21=0.15;
dydt = zeros(4,1);
dydt(1) = (umax1*y(2)*y(1))/(Ks1+y(2)+a12*y(3))+(umax2*y(3)*y(1))/(Ks2+y(3)+a21*y(1));
dydt(2) = -(1/A1)*(umax1*y(2)*y(1))/(Ks1+y(2)+a12*y(3));
dydt(3) = -(1/A2)*(umax2*y(3)*y(1))/(Ks2+y(3)+a21*y(1));
dydt(4) =
A3*((umax1*y(2)*y(1))/(Ks1+y(2)+a12*y(3))+(umax2*y(3)*y(1))/(Ks2+y(3)+a21*y(1)));
end
```

Script file (2, 3):

```
tspan = [0: 1: 8];
Y0 = [0.4245 2 3 7];
[T, Y] = ode45(@(t, Y) acm(t, Y), tspan, Y0);
[T Y]
plot(T,Y)
xlabel('Time (h)');
```

ylabel('Concentration (g/L), pH');

% Result (2, 3):

Table 19: Predicted and experimental values of 2g/L glc and 3g/L hyd

Time (h)	X (g/L)	Glc (g/L)	Hyd (g/L)	pH
0	0.4245	2	3	7
1	0.4728	1.96	2.6231	6.85
2	0.524	1.9106	2.2403	6.71
3	0.57776	1.8489	1.8618	6.55
4	0.6331	1.7715	1.4996	6.39
5	0.6905	1.6735	1.1662	6.22
6	0.7499	1.5492	0.8726	6.05
7	0.812	1.3923	0.6264	5.87
8	0.8778	1.1975	0.4305	5.68

% Plot (2, 3):

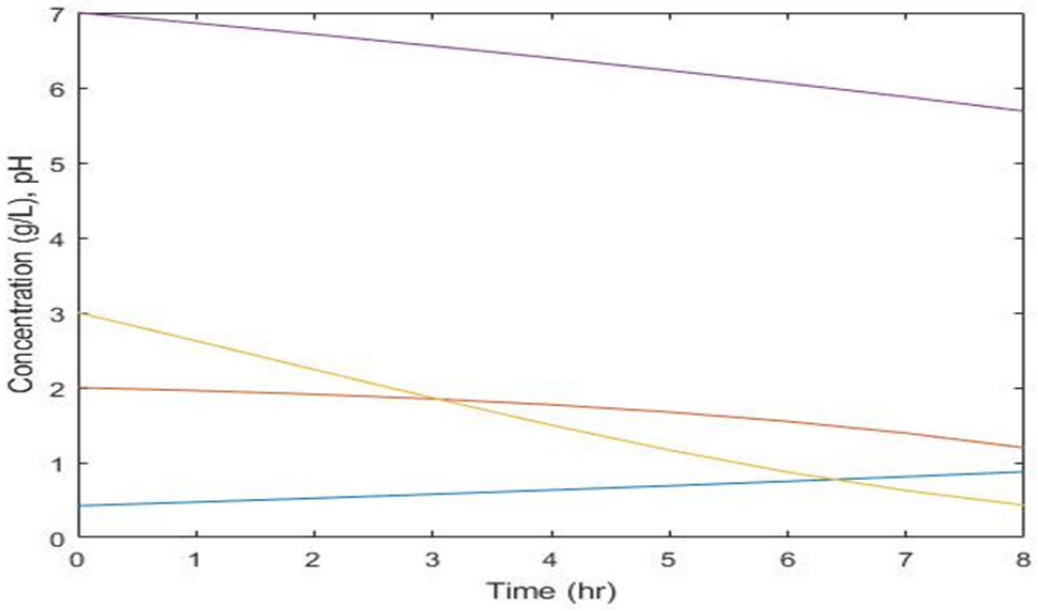

Figure 31: The time graph plotted against concentration of combination of substrates glucose 2g/L and hydrolysate 3g/L

% function (1, 4):

```
function dydt = acm(t, y)
umax1=0.2239;
umax2=0.2116;
Ks1=0.6278;
Ks2=3.9545;
A1=0.2334;
A2=0.1035;
A3=-2.854;
a12=6.66;
a21=0.15;
dydt = zeros(4,1);
dydt(1) = (umax1*y(2)*y(1))/(Ks1+y(2)+a12*y(3))+(umax2*y(3)*y(1))/(Ks2+y(3)+a21*y(1));
dydt(2) = -(1/A1)*(umax1*y(2)*y(1))/(Ks1+y(2)+a12*y(3));
dydt(3) = -(1/A2)*(umax2*y(3)*y(1))/(Ks2+y(3)+a21*y(1));
dydt(4) =
A3*((umax1*y(2)*y(1))/(Ks1+y(2)+a12*y(3))+(umax2*y(3)*y(1))/(Ks2+y(3)+a21*y(1)));
end
```

% Script file (1, 4):

```
tspan = [0: 1: 8];
Y0 = [0.4245 1 4 7];
[T, Y] = ode45(@(t, Y) acm(t, Y), tspan, Y0);
[T Y]
plot(T,Y)
xlabel('Time (h)');
ylabel('Concentration (g/L), pH');
```

% Result (1, 4):

Table 20: Predicted and experimental values of 1g/L glc and 4g/L hyd

Time (h)	X (g/L)	Glc (g/L)	Hyd (g/L)	pH
0	0.4245	1	4	7
1	0.4742	0.984	3.5555	6.85
2	0.5266	0.9643	3.0936	6.7
3	0.5809	0.9397	2.625	6.55
4	0.6359	0.9087	2.1635	6.39
5	0.6904	0.8694	1.7252	6.24
6	0.7434	0.8191	1.3266	6.08
7	0.7942	0.7547	0.9813	5.94
8	0.8426	0.6728	0.6978	5.8

% Plot (1, 4):

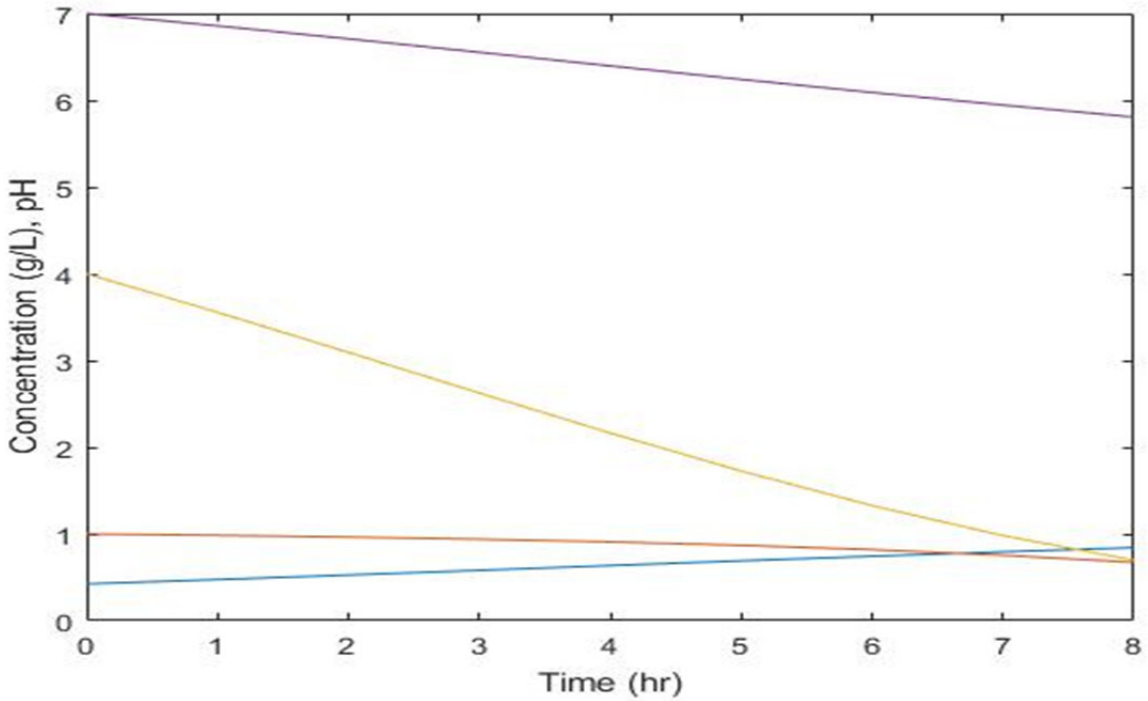

Figure 32: The time graph plotted against concentration of combination of substrates glucose 1g/L and hydrolysate 4g/L

6.7 Morphological analysis of the microorganisms used in the interactive microbial system

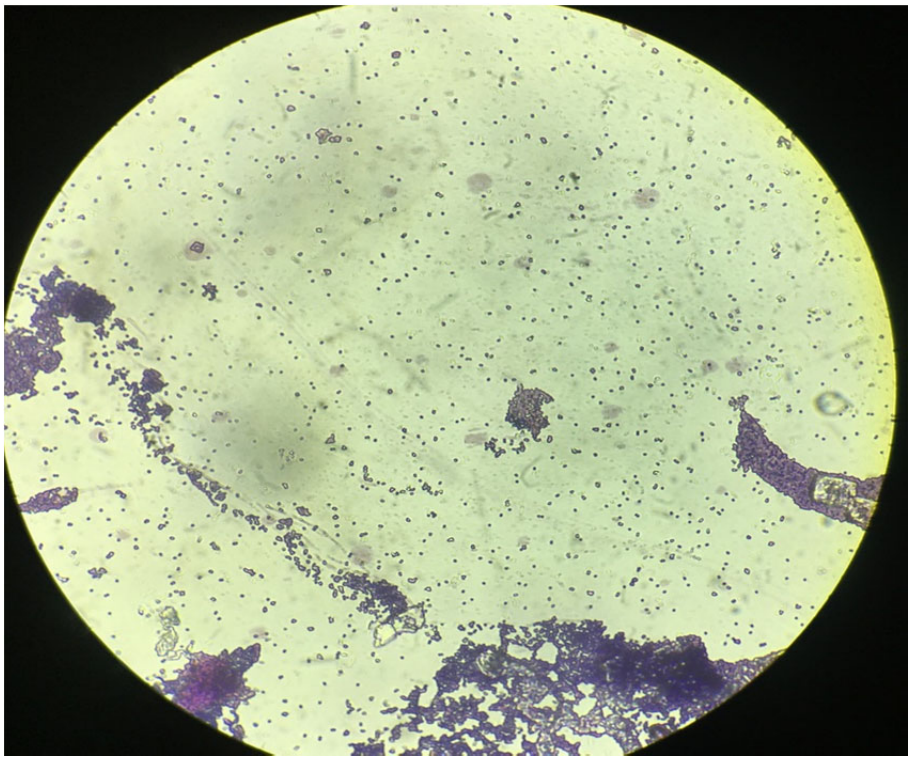

Figure 33: Microscopic observation of the mixed consortia after gram staining procedure.

As visible from the figure, rod shaped bacterial species are stained purple after gram staining procedure which indicates that the species is Gram positive as it retains the colour of the primary stain crystal violet. Few gram negative bacteria can also be seen.

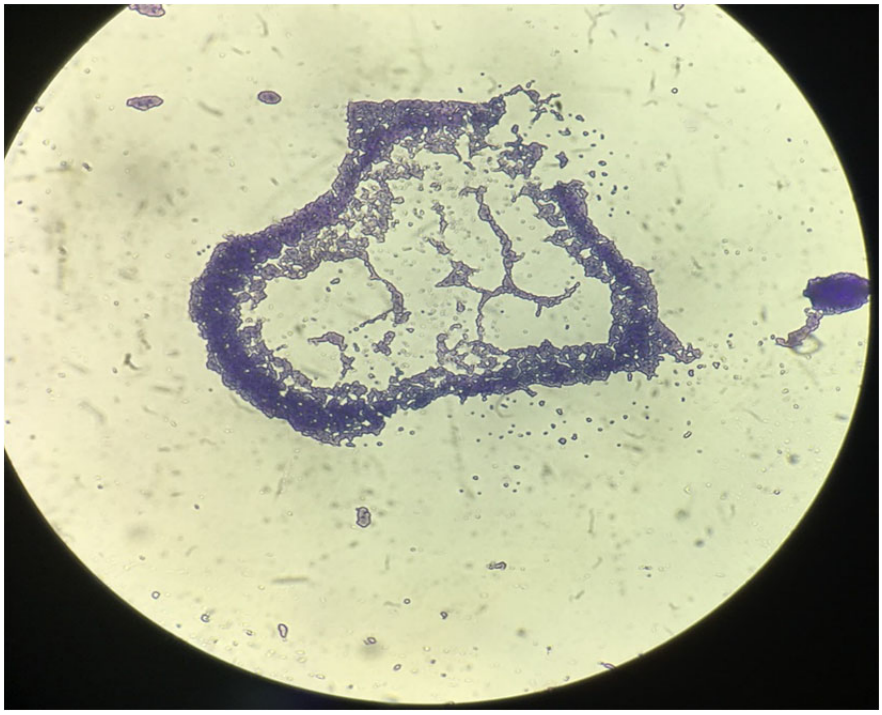

Figure 34: Microscopic observation of the** Clostridium acetobutylicum **sp. after gram staining procedure.

As visible from the figure, rod shaped bacterial species are stained purple after gram staining procedure which indicates that the species is Gram positive as it retains the colour of the primary stain crystal violet. Thus, it clearly depicts that the given bacterial species is Clostridium sp. (comparatively checked from literature).

6.8 Investigation of the survival and growth of a solventogenic bacterium on growth medium amended with the acidic effluent of the acidogenic consortium.

Five different sets of different combinations were prepared and they were monitored for 72hours for the growth of a solventogenic bacterium.

Table 21: Different sets of different combinations for the growth of solventogenic bacterium

Sl no.	Description	Effluent of acidogenic consortium	Total Amount of RCM
1	Set 1	3ml	30ml
2	Set 2	5ml	30ml
3	Set 3	10ml	30ml
4	Control	0	30ml
5	*Clostridium acetobutylicum* culture	0	30ml

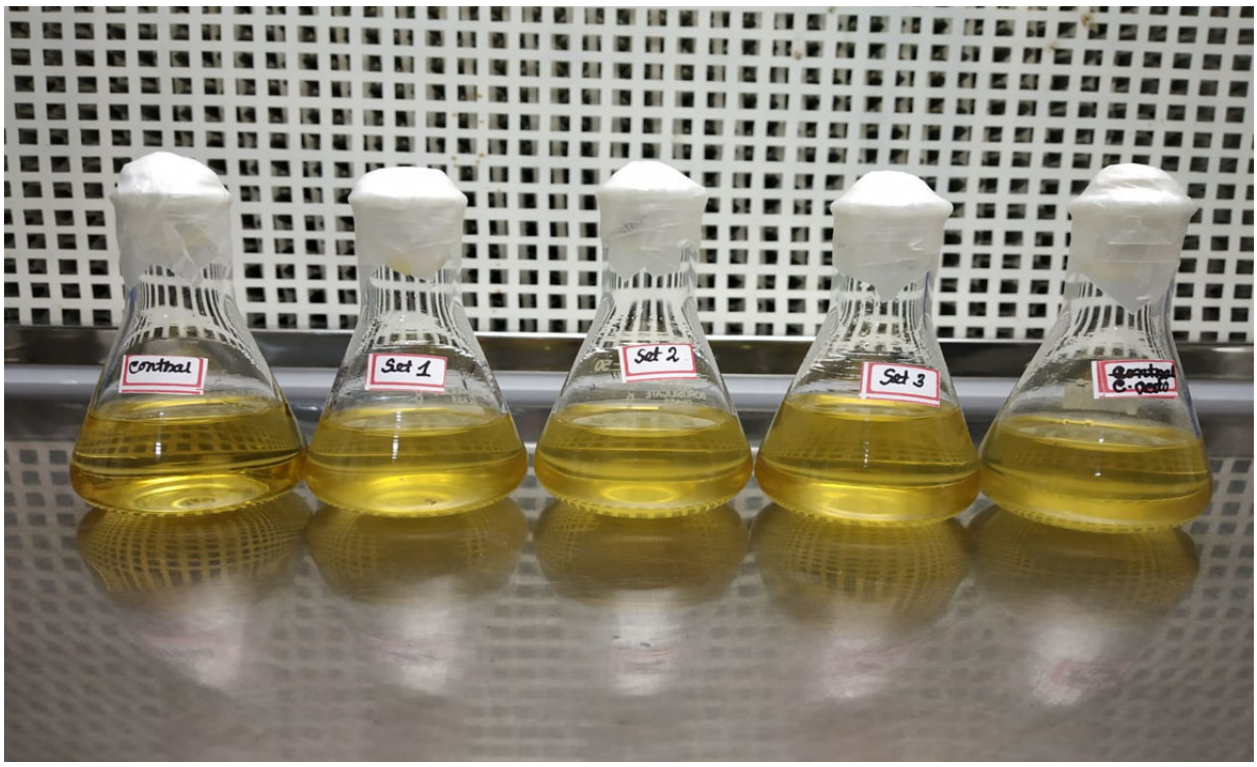

Figure 35: Different sets inoculated in RCM containing effluent of acidogenic consortium and Clostridium acetobutylicum inoculate.

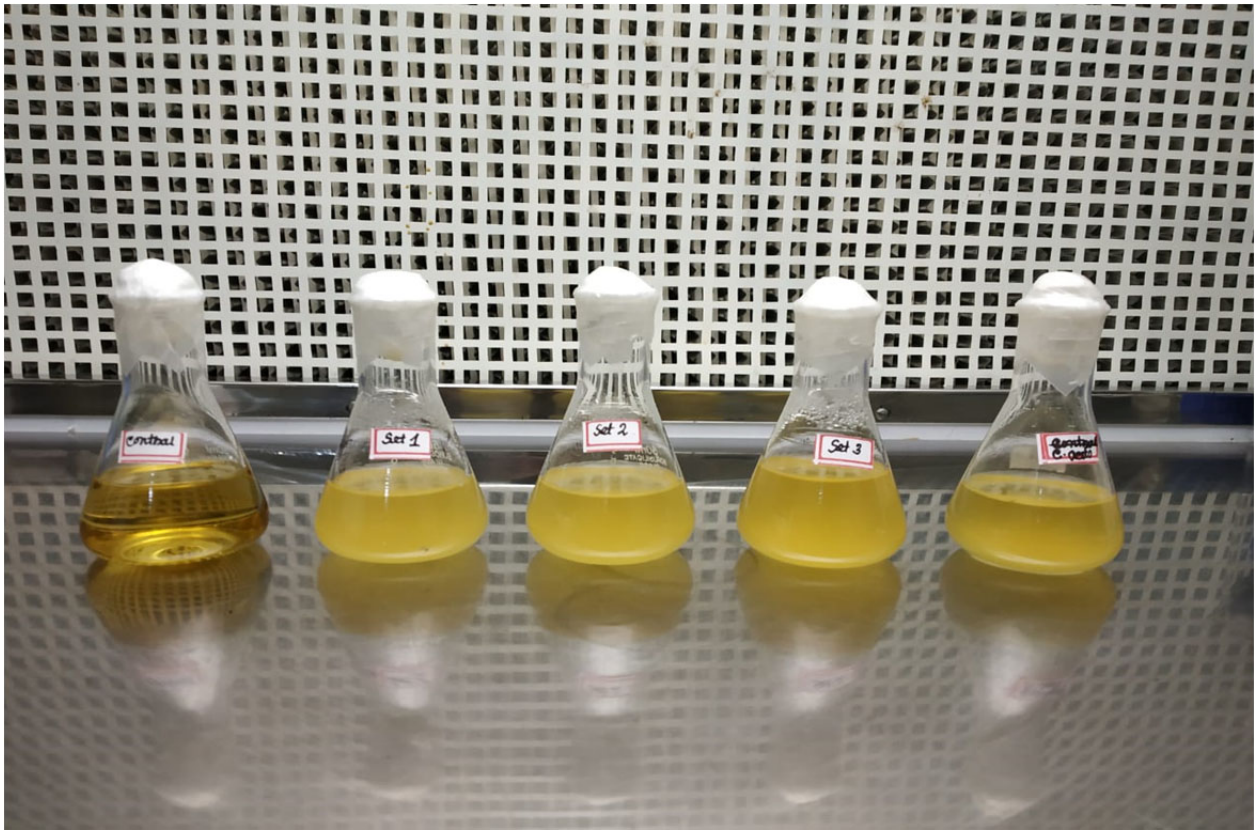

Figure 36: After 72hours it has been observed that Clostridium acetobutylicum grew well in set 1, 2, 3 and adapted in effluent of acidogenic consortium.

From the depiction of the figures 34 and 35 it can be seen that in all of the sets 1,2 and 3, in which effluent from Ag-M growth medium was added, C. acetobutylicum grew clearly. In comparison to the control the turbidity of the three sets was visibly much high after 72 hrs of growth. This primarily highlights that the acidic products of Ag-M does not have any inhibitory effects on C. acetobutylicum.

Table 22: OD and pH observation from different sets for the growth of solventogenic bacterium

Sl No.	Description	OD value at 600nm	pH
1	Set 1	0.861	5.5

2	Set 2	0.893	4.8
3	Set 3	0.983	4.8
4	*Clostridium acetobutylicum*	0.875	4.8

The OD values of the set 1, 2 and 3 was quite close to the values obtained for C. acetobutylicum alone, however, the value for set 3, which was having the maximum amount of Ag-M effluent was found to be a bit higher than that of c. acetobutylicum alone. pH fall of the three sets and that of C. acetobutylicum were also comparable and found to be in the same range. Since, the effluent of Ag-M does not inhibit the growth and survival of C. acetobutylicum,it is expected that the two microbial parts, if co-cultured, will grow synergistically and function together. In that system the alcohol yield may be enhanced due to the supply of the additional acidic products by the Ag-M to the C. acetobutylicum.

Conclusion

- Fusion of dilute sulphuric acid and steam explosion alters the lignin structure and removes the hemicellulosic portion which in turn increases the amount of available reducing sugar for biofuel production
- 1% dilute sulphuric acid at 0.1 % loading when autoclaved for 30 minutes yielded the best result. Hence it has been chosen for all the experiments performed using rice straw hydrolysate.
- The acid production potential of the isolated acidogenic mixed consortium was accessed by monitoring the fall of system pH which was found to be 4.5- 5 that indicates the consortium to be acidogenic.
- The growth kinetics of mixed consortia in both glucose and hydrolysate medium follows the monod kinetics. It gives maximum specific growth rate values in dual substrate i.e glucose and hydrolysate μ_{m1}= 0.2239 h^{-1} and μ_{m2}= 0.2116 h^{-1} respectively.
- The Substrate saturation constant of mixed consortia in glucose and hydrolysate are k_{s1}= 0.6277 and k_{s2}= 3.9545 respectively.
- A summative type of model has been found and specific growth rates of mixed strain using glucose-hydrolysate combination have been graphed against concentration of reducing sugar in hydrolysate and glucose as independent variables.
- The time history plots in Surface plots and 3-D plots using MATLAB have been found to be in good agreement using experimental and predicted values of concentration of biomass and pH in all cases.
- The survival and growth of a solventogenic bacterium on growth medium amended with the acidic effluent of the acidogenic consortium has shown positive growth.

References

1. Bhatia SK, Kim J, Song H-S, Kim HJ, Jeon J-M, Sathiyanarayanan G, et al. Microbial biodiesel production from oil palm biomass hydrolysate using marine Rhodococcus sp. YHY01. Bioresour Technol 2017;233:99–109.
2. Palmer JD, Brigham CJ. Feasibility of triacylglycerol production for biodiesel, utilizing Rhodococcus opacus as a biocatalyst and fishery waste as feedstock. Renew Sust Energy Rev 2016;56:922–8.
3. Corral Bobadilla M, Lostado Lorza R, Escribano García R, Somovilla Gómez F, Vergara González E. An improvement in biodiesel production from waste cooking oil by applying thought multi-response surface methodology using desirability functions. Energies 2017;10:130.
4. Demirbas A. Biodiesel from municipal sewage sludge (MSS): challenges and cost analysis. Energy Sources Part B 2017;12:351–7.
5. Bhatia SK, Bhatia RK, Yang YH. An overview of microdiesel — a sustainable future source of renewable energy. Renew Sust Energy Rev 2017;79:1078–90.
6. Huang YF, Chiueh PT, Lo SL. A review on microwave pyrolysis of lignocellulosic biomass. Sust Environ Res 2016;26:103–9.
7. Le RK, Wells Jr T, Das P, Meng X, Stoklosa RJ, Bhalla A, et al. Conversion of corn stover alkaline pre-treatment waste streams into biodiesel via Rhodococci. RSC Adv 2017;7:4108–15.
8. Nhien LC, Long NVD, Lee M. Novel heat–integrated and intensified biorefinery process for cellulosic ethanol production from lignocellulosic biomass. Energy Convers Manage 2017;141:367–77.

9. Jönsson, L.J., Martín, C., 2016. Pretreatment of lignocellulose: formation of inhibitory byproducts and strategies for minimizing their effects. Bioresour. Technol. 199, 103.
10. Bankar SB, Survase SA, Ojamo H, Granstrom T. Biobutanol: the outlook of an academic and industrialist. RSC Adv 2013;3:24734–57
11. UN Report; Sustainable Bioenergy: A Framework for Decision Makers; April 2007.
12. Review of EU Biofuels Directive; Public consultation exercise; April- July 2006.
13. Chisti Y; Biodiesel from Microalgae; Biotechnology Advances 25 (2007) 294–306.
14. Schmetz E, Ackiewicz M, Tomlinson G, White C, Gray D; Increasing Security and Reducing Carbon Emissions of the U.S. Transportation Sector: A Transformational Role for Coal with Biomass; National Energy Technology Laboratory.
15. B. Bharathiraja et.al "Biobutanol – An impending biofuel for future: A review on upstream and downstream processing tecniques", Renewable and Sustainable Energy Reviews, 2017.
16. Lu, J.; Sheahan, C.; Fu, P.C. Metabolic engineering of algae for fourth generation biofuels production. Energy Environ. Sci. 2011, 4, 2451–2466.
17. Kim, N. J., Lim, S. J., & Chang, H. N. (2018). Volatile Fatty Acid Platform: Concept and Application. Emerging Areas in Bioengineering, 173-190.
18. Abd-Aziz, S., Ibrahim, M. F., & Jenol, M. A. (2018). Biological Pretreatment of Lignocellulosic Biomass for Volatile Fatty Acid Production. Emerging Areas in Bioengineering, 191-201.
19. Vasudevan, D., Richter, H., & Angenent, L. T. (2014). Upgrading dilute ethanol from syngas fermentation to n-caproate with reactor microbiomes. Bioresource technology, 151, 378-382.

20. Gildemyn, S., Molitor, B., Usack, J. G., Nguyen, M., Rabaey, K., & Angenent, L. T. (2017). Upgrading syngas fermentation effluent using Clostridium kluyveri in a continuous fermentation. Biotechnology for biofuels, 10(1), 83.
21. Holtzapple, M & Lonkar, Sagar & Granda, Cesar. (2015). Producing biofuels via the carboxylate platform. 111. 52-57.
22. Samanta Koruri, S., Chowdhury, R. & Bhattacharya, P. Arch Microbiol (2015) 197: 955.
23. Bekatorou, Argyro, et al. "Downstream extraction process development for recovery of organic acids from a fermentation broth." Bioresource technology 220 (2016): 34-37.
24. Lappa, Katerina, et al. "Continuous acidogenesis of sucrose, raffinose and vinasse using mineral kissiris as promoter." Bioresource technology 188 (2015): 43-48.
25. Kandylis, Panagiotis, et al. "Acidogenesis of cellulosic hydrolysates for new generation biofuels." Biomass and Bioenergy 91 (2016): 210-216.
26. Syngiridis, Kostas, et al. "Favouring butyrate production for a new generation biofuel by acidogenic glucose fermentation using cells immobilised on γ-alumina." Bioresource technology 161 (2014): 118-123.
27. Bhatia, Shashi Kant, et al. "Current status and strategies for second generation biofuel production using microbial systems." Energy Conversion and Management 148 (2017): 1142-1156.
28. Weimer, Paul J., Michael Nerdahl, and Dane J. Brandl. "Production of medium-chain volatile fatty acids by mixed ruminal microorganisms is enhanced by ethanol in co-culture with Clostridium kluyveri." Bioresource technology 175 (2015): 97-101.

29. Tsafrakidou, Panagiota, et al. "Acidogenic fermentation of wheat straw after chemical and microbial pretreatment for biofuel applications." Energy conversion and management 160 (2018): 509-517.
30. Gildemyn, Sylvia, et al. "Upgrading syngas fermentation effluent using Clostridium kluyveri in a continuous fermentation." Biotechnology for biofuels 10.1 (2017): 83.
31. Grassi, M. C. B., et al. "New contributions for industrial n-butanol fermentation: An optimized Clostridium strain and the use of xylooligosaccharides as a fermentation additive." Biomass and Bioenergy 119 (2018): 304-313.
32. Kim, Sung Bong, et al. "Sugar recovery from rice straw by dilute acid pretreatment." Journal of Industrial and Engineering Chemistry 18.1 (2012): 183-187.
33. Ghosh, Shiladitya, Ranjana Chowdhury, and Pinaki Bhattacharya. "Mixed consortia in bioprocesses: role of microbial interactions." Applied microbiology and biotechnology 100.10 (2016): 4283-4295.
34. Hollister, Emily B., et al. "Mesophilic and thermophilic conditions select for unique but highly parallel microbial communities to perform carboxylate platform biomass conversion." PloS one 7.6 (2012): e39689.
35. Zuriarrain, Andoni, et al. "Quantitative determination of lactic and acetic acids in cider by 1H NMR spectrometry." Food Control 52 (2015): 49-53.