OPTIMIZATION OF BIOETHANOL PRODUCTION FROM KITCHEN WASTE

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OPTIMIZATION OF BIOETHANOL PRODUCTION FROM KITCHEN WASTE

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I hereby declare that all information in this document has 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 not original to this work.

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ABSTRACT

OPTIMIZATION OF BIOETHANOL PRODUCTION FROM KITCHEN WASTE

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Kitchen waste, which is collected in large amounts from cafeterias, restaurants, dining halls, food processing plants, and household kitchens, have become a valuable material for bioprocess engineering. Due to the high carbohydrate fraction, kitchen waste has great potential to be used as a potential substrate for ethanol production. Utilization of it as a raw material in ethanol fermentation would also contribute to reduction of costs. In the first part of this study, the effect of pretreatment method and enzymatic hydrolysis on glucose production was evaluated. Dry baker's yeast, *Saccharomyces cerevisiae*, was used in fermentation experiments conducted with and without fermentation medium at pH 4.5 and 30^{o}C for 48 hours.

Close values of glucose concentration were obtained from no pretreated and hot water treated samples. The fermentation results indicated that ethanol can be produced at similar concentrations in bioreactors with and without fermentation medium addition ($p > 0.05$). Thus, it is concluded that use of kitchen wastes as is disposed and without fermentation medium in ethanol fermentation could lower the cost to a large extent.

In the second part of this study, the effects of solid load, which is proportional to the glucose concentration (10% to 20% (w/w)), inoculum level of *Saccharomyces cerevisiae* (5% to 15% (v/v)), and fermentation time (48 to 96 h) on production of bioethanol from kitchen waste were studied using Response Surface Methodology (RSM). A three-factor Box Behnken design was used. Ethanol concentration was used as a response in the resulting experimental design. High Pressure Liquid Chromatography (HPLC) method was used to determine ethanol and glucose concentrations. The statistical analysis of the constructed model developed by RSM suggested that linear effects of solid load, inoculum level, and fermentation time and quadratic effects of inoculum level and fermentation time were all significant ($p < 0.05$) on bioethanol production. The model was verified by additional runs, which were not present in the design matrix. It was found that the constructed model could be used to determine successfully the bioethanol concentration with > 90% precision. An optimum ethanol concentration of 32.16 g/L was suggested by the model with 20% (w/w) solid load, 8.85% (v/v) inoculum level and 58.8 hours of fermentation. Further study is needed to evaluate the optimal fermentation conditions in a large scale fermentation.

Keywords: Bioethanol, pretreatment, enzymatic hydrolysis, kitchen waste, dry baker's yeast, Response Surface Methodology (RSM), Box Behnken

ÖZ

MUTFAK ATIKLARINDAN BİYOETANOL ÜRETİMİNİN OPTİMİZASYONU

UNCU, Oya Nihan
Yüksek Lisans, Gıda Mühendisliği
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Kafeteryalar, restorantlar, yemekhaneler, gıda işleme tesisleri ve mutfak atıkları şeklinde toplanan yemek atıkları biyoişlem mühendisliği alanında giderek artan değere sahip hammaddelerdendir. Yüksek karbonhidrat içeriğiyle etanol üretiminde hammadde olma potansiyeline sahiptir. Etanol fermentasyonunda hammadde olarak kullanımı üretim maliyetini de önemli ölçüde düşürmeye yarayacaktır. Bu çalışmada öncelikle, önişlem yöntemleri ve enzimatik hidrolizin elde edilen glikoz üzerindeki etkisi saptanmıştır. Kuru maya (*Saccharomyces cerevisiae*) kullanılarak 4.5 pH, 30^{o}C sıcaklık 48 saat süreyle sürdürülen fermentasyon işlemi fermentasyon kimyasalları eklenerek ve eklenmeyerek uygulanmıştır. Önişlem uygulanmayan ve sıcak su işlemine tabi tutulan örneklerde birbirine yakın glikoz değerleri elde edilmiştir.

Fermentasyon kimyasalları ilavesi de etanol üretimi üzerinde etkili bir değişken olarak bulunmamıştır ($p > 0.05$). Böylece, mutfak atıklarının önişlem uygulanmamış ve fermentasyon

kimyasalları ilave edilmemiş şekliyle etanol üretiminde kullanılmasının maliyeti önemli ölçüde düşüreceği sonucuna varılmıştır.

Çalışmanın ikinci kısmında, glikoz derişimi ile doğrudan orantılı başlangıç katı madde miktarı (%10'dan %20'ye (g kuru madde/g karışım) kadar), *Saccharomyces cerevisiae* aşılama miktarı (%5'den %15'e kadar (ml maya / ml karışım)) ve fermentasyon süresinin biyoetanol üretimi üzerindeki etkileri (48'den 96 saate kadar) esas alınarak Yanıt Yüzey Yöntemi (RSM) kullanılarak incelenmiştir. Üç faktörlü Box-Behnken tasarımı kullanılmıştır. Etanol ve glikoz ölçümleri Yüksek Basınç Sıvı Kromatografi (HPLC) ile yapılmıştır. Yanıt Yüzey Yöntemi ile yapılan istatistiksel değerlendirme sonucunda elde edilen modele gore başlangıç katı madde miktarı, aşılama miktarı, fermentasyon süresi ve aşılama miktarı ile fermentasyon süresinin karesi biyoetanol üretimi üzerinde etkili bulunmuştur ($p < 0.05$). Elde edilen matematiksel modelin geçerliliği, deney tasarımı dışında kalan koşullarda yapılan ilave deneylerle kontrol edilmiştir. Öngörülen modelin etanol derişimini hesaplamada %90'dan yüksek kesinliğe ulaştığı belirlenmiştir. Ayrıca, öngörülen modele gore en yüksek etanol derişimi (32.16 g/L), %20 katı madde miktarı, %8.85 aşılama miktarı ile 58.8 saat süren fermentasyon ile elde edilmektedir. Saptanan optimum fermentasyon koşullarının büyük ölçüde denenmesi ileriki çalışmalara bırakılmıştır.

Anahtar kelimeler: Biyoetanol, önişlem, enzimatik hidroliz, mutfak atığı, kuru maya, yanıt yüzey yöntemi (YYY), Box-Behnken.

In dedication to my Beloved Family

Özkan – Türkan UNCU

and my precious Caddy

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INTRODUCTION

1.1 Global View of Municipal Solid Waste

Growth of population, increase in urbanization, and rise in life standards have contributed to an increase both in quantity and variety of solid wastes generated by industrial, domestic and agricultural activities. In 2002, the global estimation of waste generation was 13 billion tons of which 11 billion tons of this was industrial and 1.6 billion ton was municipal solid waste (MSW). By 2025, it is expected to be generated about 90 billion ton annually (Ajnavi, 2008).

Solid wastes of organic origin include: municipal wastes, horticultural wastes, agro-industrial wastes, animal wastes, farming wastes and residues. The main objective of having an effective waste management system is to maximize the economic benefits and minimize environmental pollution. Due to increasing energy demands, financial constraints and environmental problems, organizations all over the world have recommended various guidelines and worked on various projects on generation, transport, treatment, disposal and recycling of wastes.

In the metropolitan district of Jakarta (Daerah Khusus Ibukota (DKI) Jakarta, Indonesia), about 6000 tons of MSW (primarily households, industrial waste) out of 8000 tons/day waste stream are disposed of by landfilling.

The ongoing projects of Indonesia are about converting the organic fraction in MSW into combustible gases (Anonymous 1, 2005). In the United States (USA) annually landfilled wastes are approximately 248 million tons of MSW (ASME, 2003). This waste is comprised of 56% biological and 44% non-biological materials (LaRiviere, 2007). The biological fraction is considered as a renewable energy source and currently 11% of this fraction is used in waste-to-energy (WTE) facilities in the USA such as generating electricity. The total amount of MSW in China has been increased up to 130 million ton in 2000. The approximate composition of MSW is 50% organic waste, 28% inorganic waste and 22% recyclable waste. The waste management programs include landfilling, incineration and composting (Li et al., 2002).

According to OECD (2002) statistics based on seven countries from all over the world (Mexico, Greece, Japan, USA, Norway, France, Belgium), MSW includes approximately 5% metal, 7% glass, 10% plastic, 28% paper and 35-40% organic waste. The organic fraction of MSW consists of food scraps and some yard trimmings (Figure 1.1)

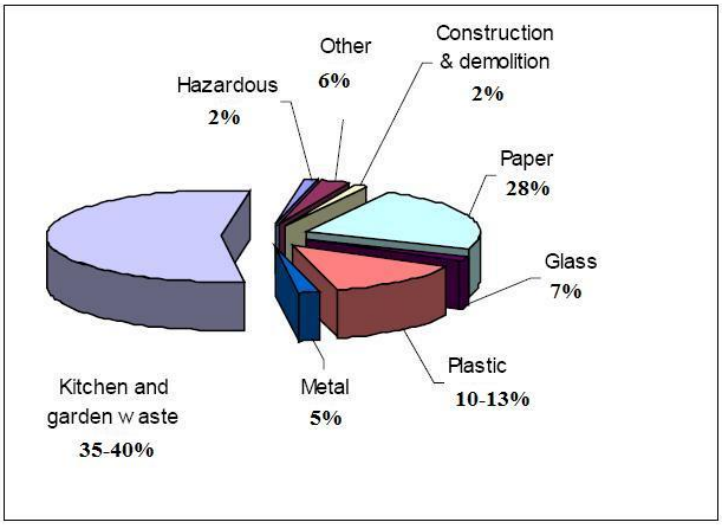


Figure 1.1 Composition of household waste stream

Organic waste is produced wherever habitation exists, mainly in the form of household kitchen and agricultural wastes. In industrialized countries, the amount of organic waste produced has been increasing each year. Although some of the kitchen and garden wastes are used in composting, majority of it goes to dumping sites and forms the most hazardous waste. Due to the organic nature of kitchen and garden wastes, elimination of them by landfilling or combusting results in unwanted situations. The landfilled waste is degraded by microorganisms and forms leachate which contains bacteria and chemical contaminants from the landfill. This leachate causes a serious hazard if reaches a watercourse. Organic matter in landfills also forms methane gas (CH_4), which is harmful to the atmosphere and hydrogen sulphur (H_2S).

Since organic waste is an unavoidable product and economies of developing countries need that all materials and resources be used to their full potential, management of organic waste is a particularly serious issue. Therefore, there exists a great need to find alternative solutions to treatment of organic wastes (yard wastes, kitchen wastes, etc).

1.1.1 Kitchen Waste and Utilization

Kitchen waste is the organic fraction of our daily food waste consisting of what is not consumed by households, and remaining portion of the foods and beverages put to the wastebins. Generally, in the USA and EU countries kitchen waste also contains high lignocellulosic matter like grass or wood straws but according to Turkey's cultural and habitative traditions households mostly contain organic food waste.

According to literature, kitchen wastes consist of fruit and vegetable peels, cooked and uncooked food, meat and bones, having a moisture content equal to or greater than 75% (Anonymous 3).

Currently, waste from residential and industrial kitchens goes to landfill each year, of which half is organic, primarily kitchen food waste. Kitchen waste makes up the single largest component of household waste going to landfill, with nearly 50 % being food scraps. When sent to landfill, food waste produces noxious leachate and greenhouse gases as it decomposes. Some cities all over the world, also collect and compost kitchen food waste to produce value-added products.

Unlike other components of household waste such as metal, glass and paper, organic waste is considered low-value and is rarely collected from recycling or processing by non-governmental sector or businesses. This can be explained by its density (it is composed predominantly of water), the cost and difficulty of transportation, the land required for processing, and relatively low-value of resultant products.

Particularly in warm climates organic waste tends to decompose quickly within a day or so. Rotting of organic waste is often responsible for the foul smell in bins, vehicles and disposal facilities. The products of decomposition are corrosive, thus containers and vehicles need to be designed by taking this fact into consideration, and cleaned frequently to reduce this problem.

The methods used in treatment of MSW include: recycling, land filling, obtaining energy from combustion, and composting. Paper, glass, plastic and metal wastes are used in recycling practices and organic waste, consisting of kitchen waste from households and food courts, is used in composting practices.

Due to high moisture content and low calorific value of the kitchen wastes, extra combustible material is needed. It results in both air pollution and increase in expenditure costs.

By growing developments of waste management, practices about finding economic and environmentally friendly solutions to these problems have begun.

Composting has been used as a strong alternative method against landfilling and combustion. Composting is the biodegradation of organic matter, such as yard and food waste. The decomposition is performed by microorganisms, mostly bacteria, but also yeasts and fungi. The organic material biodegrades substantially under specific composting conditions and converted into humus. Compost is a stable, dark brown, soil-like material which can hold moisture, air and nutrients. Contrary to popular belief compost does not smell rotten: often it will smell as fresh as a forest floor (which is, of course, naturally-made compost). Compost contains some plant nutrients including nitrogen, phosphorus and potassium (NPK), though not as much as animal manure or chemical fertilizers. Compost can also contain a range of minerals and microorganisms beneficial to plant growth. However, its main benefit is as a soil conditioner. Adding compost to soil can lessen the need for chemical fertilizers because it holds nutrients in the soil, it can also help reduce soil erosion, and improve the structure of the soil; thus benefiting drainage and plant roots.

Other ways of dealing with the organic portion of municipal waste are:
(1) Feed for animals
(2) Feedstock for anaerobic digestions

Anaerobic microorganisms thrive in environments with no oxygen. Many such microorganisms occur naturally; in the absence of air these will prevail and decompose the organic material. Anaerobic decomposition gives rise to methane. Methane is a potent greenhouse gas which over a period of 100 years is thought to be 23 times more harmful to the environment than carbon dioxide (CO_2).

Therefore, where anaerobic digestion is employed as a treatment method, it is vital that the methane is captured and used. One such example of a controlled anaerobic digestion system for organic waste is biogas digester.

These are most often used for human and animal waste, but there are examples of their successful use with organic waste. Biogas is a source of energy with one of the lowest relative carbon footprints of all. Methane can be burnt cleanly on simple stoves, producing carbon dioxide and water, making it a very clean household fuel. As with all organic waste processing techniques, one of the most significant challenges of using digesters is ensuring the quality of raw materials. Contamination from plastic, sand and soil can reduce the effectiveness of the plant, and chemical contamination could compromise the microorganisms, as well as contaminate the resultant compost.

1.1.2 Turkey's Situation

In Turkey within last few decades solid wastes have become one of the biggest environmental problems. Parallel to the increase in population in big cities and changes in the consumptional behavior, the composition of the solid wastes have also changed.

Within the years 1994 and 2003, there observed a 47.09% increase in the collected MSW . The amount of MSW collected in controlled dumping sites was 5.6% in 1994 and increased to 29.7% in 2003 (OPOCE, 2005). According to the data obtained from G.I.S. (Governmental Institute of Statistics) of Turkey, the daily generation of MSW is 1.0 kg/capita and 0.6 kg/capita of daily kitchen waste.

Thus, it makes approximately 68,000 tons of MSW daily and 28.4 million tons of annual kitchen waste. The only research done on the composition of kitchen waste was carried out by G.I.S. in 1992.

The composition given by that research is given in Table 1.1 (Neyim et al., 2003).

Table 1.1 Type and composition of MSW of Turkey in 1992

Type of MSW	**% (w/w)**
Organic waste	65.45
Ash	22.48
Recyclable Materials	12.05
Composition of Recyclable Materials	**% (w/w)**
Paper	45.48
Metal	8.62
Glass	18.46
Plastic	13.19
PET,PVC	6.15
Rubber	3.30
Textile	4.80

In Turkey a vast amount of kitchen waste generated has been stored in an uncontrolled condition at dumping sites. Extrication of MSW can not be done systematically, due to the fact that some of the MSW is collected by people from the streets and some is separated by the containers provided by ÇEVKO (Environmental Protection and Recycling of Packaging Waste Foundation).
Most of the recyclable part of MSW can not be fully assessed either, which results in economical losses.

1.2 Alternative Energy Sources vs. Ethanol

The initial stimulus for ethanol production in the mid-1970s was the drive to develop alternative and renewable supplies of energy in response to the oil embargoes of 1973 and 1979. Production of fuel ethanol has been encouraged through federal tax incentives for ethanol-blended gasoline. The use of fuel ethanol was further encouraged by the Clean Air Act Amendments of 1990, which required the use of oxygenated or reformulated gasoline (RFG). The Energy Policy Act of 2005 (P.L. 109-58) established a renewable fuels standard (RFS), which mandates the use of ethanol and other renewable fuels in gasoline. Approximately 99% of fuel ethanol consumed in the United States is "gasohol"[1] or "E10" (blends of gasoline with up to 10% ethanol). About 1% is consumed as "E85" (85% ethanol and 15% gasoline), and alternative to gasoline (Anonymous 2, 2004).

Fuel ethanol is usually produced in the United States from the distillation of fermented simple sugars (i.e. glucose) derived primarily from corn, but also from wheat, potatoes, or other vegetables. However, ethanol can also be produced from cellulosic material such as switchgrass, rice straw, and sugar cane pulp (known as bagasse).

The alcohol in fuel ethanol is chemically identical to ethanol used for other purposes such as distilled spirit beverages and industrial products. With increasing demands and decreasing sources of energy, biofuels – fuels derived from biological raw material processing- have become strong competitor of crude oil and petroleum industry.

The effectiveness of biofuels is highly subjective based on location and feedstock. Since the production of biofuels continued to be from valuable feedstocks such as ethanol from sugar cane and diesel from oil palms, they cannot replace petroleum and there is not enough land for it.

Currently the primary source of energy is the fossil fuels, which provide the power for modern life appliances like computers, electronics, cars and machinery. Fossil fuels are originated from chemical reactions and physical changes in organic matter. They have been used on earth for millions of years as energy reservoirs but unfortunately the deposit of fossil fuels is limited. Up to 1970's it is believed that fossil fuels could exist until they are exhausted, but latest investigations show that they can no longer be able to meet the demands in near future (Dresselhaus and Thomas, 2001); (Bockris, 2002).

Before the Industrial Revolution people have started to release few gases to the atmosphere, which are called greenhouse gases. They effectively store some heat in the atmosphere and cause warming of the surface. Some of them like water vapor, carbon dioxide, methane, ozone and nitrous oxide naturally occur in the atmosphere and others result from human activities (growth of population, fossil fuel burning and deforestation). They change the Earth's climate by collecting the sun's heat energy and forming a blanket and causing global warming.

The climate change also plays an important role in tendency to use less contaminating energy. The pattern of increased concentration of carbon dioxide (CO_2) and equivalents in the atmosphere are the major cause of climate change (IPCC, 2007). The CO_2 emissions play the most important role in this respect and main sources for these emissions are industrial processes and automobiles.

Another reason for climate change is the use of fossil fuels as an energy source. The global economy is highly dependent on fossil fuels like oil and coal. It is also inevitable that the costs of oil extraction and petroleum will one day exceed the costs of alternative energy sources and this will increase investments in potential alternatives. In order to decrease the dependence of fossil fuels and lower the CO_2 emissions it is crucial that the use of renewable energy be increased.
Renewable energy sources have much lower environmental impact than conventional energy technologies. They are also promising options for transportation sector in many countries due to their potential to substitute the oil derived liquid fuels.

"The 2003 biofuels directive (2003/30/EC) aims to increase the use of biofuels for transport, in particular for road transport". According the Kyoto Protocol (Japan) European Union (EU) aims to replace 5.75% of all transport fossil fuels with biofuels (bioethanol and biodiesel) by 2010 (EPA, 2002). Figure 1.2 gives the ongoing trend of contribution of biofuels usage.

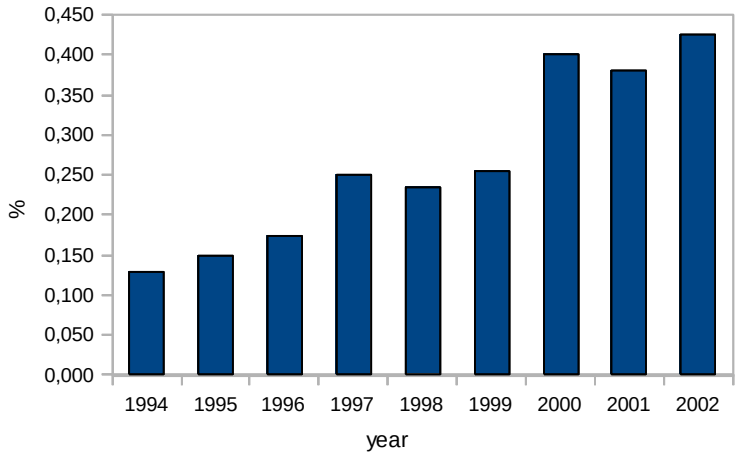


Figure 1.2 Share of biofuels in transport consumption 1994-2002

Although ethanol is a strong alternative as a biofuel, there are oppositions to it. These oppositions come from the fact that valuable feedstock in human food chain like corn and sugar cane are used in the production of ethanol. The second opposition is that the inputs for seeding, planting, and harvesting until distillation, costs much more than the energy output of ethanol (Patzek, 2007).

1.3 Ethanol as Biofuel

Ethanol is a clear, colorless, flammable and "oxygenated" fuel. It has a chemical formula of C_2H_5OH. The addition of ethanol to gasoline reduces carbonmonoxide (CO) production by providing more oxygen and promoting complete combustion. A study by Whitten et. al. (1997) showed approximately 14% CO reduction as a result of oxygenated fuel usage in winter. This enhanced its usage as a transport fuel. As a fuel, ethanol can be used mainly in four forms, which are: anhydrous ethanol (100% ethanol), hydrous ethanol (95% ethanol and 5% water), blended with gasoline (on

average 85% gasoline and 15% ethanol) and ethyl tert-butyl ether (ETBE) (Wyman and Hinman, 1990). The present car engines have to be modified to use anhydrous ethanol as a fuel oil, thus today most countries like USA and Canada use it as gasoline-blended. In the USA and Canada 10% ethanol- gasoline blend (E10) is sold as transport fuel and used widely. In Brazil with modified engines, cars are able to utilize 90% hydrous ethanol as a fuel. This favors the fact that, the water present in hydrous ethanol increases the octane number and also the heat of vaporization of ethanol (Wyman and Hinman, 1990).

Besides the advantages; being a renewable energy source, reducing dependence of petroleum fuel and crude oil, and decreasing CO_2 emissions, the ethanol industry stimulates domestic economic development.
With growing production, there observed an increase in domestic jobs and revenue. As stated before, ethanol can be used in all cars in the form of E10 since 1979, and 3,000,000 E85 ethanol-gasoline blended cars are in use currently.

Fuel ethanol (ethyl alcohol) is made by fermenting and distilling simple sugars. It is the same compound found in alcoholic beverages. The biggest use of fuel ethanol in the United States is as an additive in gasoline. It serves as an oxygenate, to prevent air pollution from carbon monoxide and ozone; as an octane booster, to prevent early ignition, or “engine knock”; and as an extender of gasoline stocks. In purer forms, it can also be used as an alternative to gasoline in automobiles specially designed for its use. It is produced and consumed mostly in the Midwest, where corn — the main feedstock for domestic ethanol production — is grown.

According to Shonnard (2003) fuel ethanol is very crucial with its four characteristics; being renewable, gasoline replacer or additive, MTBE replacer, and

reducer of carbon dioxide emissions. From the first times fuel ethanol was started to be produced the cost of production was always tried to be lowered. By improving the microorganisms used in fermentation or combining the process steps and usage of raw materials which are not in human food chain, production cost can be lowered further. The cost of ethanol / gallon is reduced up to $1.22 from $4.63. The main progress responsible for the decrease in the cost is the use of cellulosic materials in the processes.

Today ethanol competes with petroleum fuel and diesel as a transportation fuel. But it still has some disadvantages like having a lower energy density and not really being a cost competitive versus gasoline, etc.

1.3.1 Production Methods of Ethanol

Ethanol can be produced by different methods besides fermentation. It can be produced synthetically from petroleum. In 1995, about 93% of the ethanol in the world was produced by the fermentation method and about 7% by the synthetic method (Badger, 2002). The synthetic way of ethanol production so-called ethylene hydration is the primary method for industrial ethanol production, while fermentation is the primary method for ethanol production for beverages.

Industrial ethanol is typically made from petrochemical feedstocks, by the acid-catalyzed hydration of ethylene given in the following chemical reaction:

$$C_2H_4 + H_2O \xrightarrow{\text{acid (i.e. phosphoric acid)}} CH_3CH_2OH$$

Ethanol fermentation is explained in detail in the next section 1.3.2.

1.3.2 Ethanol Fermentation

Many organisms grow without using the electron transport chain. The generation of energy without the electron transport chain is called fermentation. This definition is exact and original meaning of the term is fermentation, although currently it is often used in a broader context (Shuler and Kargi, 1992).

Fermentable sugars, especially glucose, can be converted to other valuable products such as fructose, ethanol, numerous organic acids and many other products by the enzymatic hydrolysis and biochemical conversion of cellulosic substrates (Grohmann, 1993).

As the yeast cells reach a certain value the production of ethanol rather than cell growth is favored by CO_2 , which is a by-product of cell metabolism due to its displacement of oxygen originally present in the medium. The fraction of glucose used for ethanol production rather than the cell growth can be increased by keeping low level of dissolved oxygen after yeast cells reach necessary amount of population. Low glucose concentrations also favor ethanol production.
At glucose concentrations up to 100 g/L, the yeast growth is repressed, which permits the ethanol production (Atkinson and Mavituna, 1983).

The methods of ethanol fermentation include:

Separate Hydrolysis and Fermentation (SHF): In a typical bioconversion of cellulosic biomass, enzymatic hydrolysis and fermentation is performed sequentially in

separate processes. The advantage of this method is to minimize the interactions between steps.

Simultaneous Saccharification and Fermentation (SSF): SSF is a single step process which enzymatic hydrolysis and alcoholic fermentation are carried out at the same time, in the same vessel (Philippidis et al., 1993). Because the glucose produced by the hydrolysis process is immediately consumed by the microorganism, this reduces enzyme inhibition, which in turn increases sugar production rates, concentrations, and yields, and decreases enzyme loading requirements.

Direct Microbial Conversion (DMC): This process combines all three processes (enzyme production, enzymatic hydrolysis, and fermentation) in one step.
There are cost savings because of the reduced number of vessels required. However, the ethanol yields are rather low, several metabolic by-products are produced, and the organisms usually suffer from low ethanol tolerance.

1.3.3 Factors Affecting Ethanol Fermentation

A number of factors like temperature, pH, glucose and ethanol tolerance of yeast limit the production of ethanol via fermentation.

Use of high glucose concentration is one of the main ways to obtain high yields of ethanol. However, high substrate concentrations can be inhibitory to fermentation due to osmotic pressure (Jones et al., 1981). Generally, when concentration exceeds 20% (w/v) glucose, osmotic pressure becomes an important issue and adversely affects fermentation efficiency.

Xin et al. (2003) reported that at high sugar concentrations bacterial growth was inhibited and the highest concentration of 16.5% (w/v) ethanol was obtained at 35% (w/v) glucose concentration. Borzani et al. (1993) studied the effect of initial sugar concentrations on fermentation and demonstrated a logarithmic relationship between sugar concentration and fermentation time.

Sree et al. (2000) carried out a study consisting of a batch fermentation system to produce ethanol using immobilized, osmotolerant *Saccharomyces cerevisiae*. Fermentation was carried out at 30^{o}C with different initial sugar concentrations of 150, 200 and 250 g/L. The maximum amount of ethanol was observed after 48 h as 72.5, 93 and 83 g/L respectively. They stated that initial 20% of glucose is the highest limit for feasible fermentation.

Temperature also exerts a profound effect on growth, metabolism, maintenance of yeast and fermentation. Generally industrial application of ethanol fermentation was carried out at 25-30^{o}C. Mauricio et al. (1995) stated that at low temperatures of fermentation higher volatile acidity is observed. They also investigated the best temperature range for fermentation and found that fermentation carried out at 25-30^{o}C had a negative effect on survival of *Saccharomyces cerevisiae*. With *Saccharomyces cerevisiae* strains fermentation could be possible up to 35^{o}C but at higher temperatures alcohol yield decreases due to production of secondary metabolites.

Fermentation at 35-40^{o}C or higher temperatures has advantages like higher ethanol recovery but this could only be achieved with thermotolerant yeast strains.
A study by Laluce et al. (1993) investigated temperature effect on fermentation capacity of three different strains of *Saccharomyces cerevisiae* (19G, 78I and baker's yeast) in a medium containing 15% total sugar.

At 39-40^{o}C complete conversion of glucose to ethanol was observed at the end of 12 h, whereas at temperatures higher than 40^{o}C a strong inhibitory effect in all cases was observed.

In general *Saccharomyces cerevisiae* is an acidophilic organism as such it grows well under acidic conditions. The optimal pH range for *Saccharomyces cerevisiae* varies between 4.0 and 6.0 depending upon the fermentation medium. The pH affects the efficiency of ethanol fermentation by influencing the activity of plasma proteins and intracellular enzymes. If enzymes are deactivated by pH < 4.0 the yeast will not be able to grow and produce ethanol efficiently (Narenranath and Power, 2005).

Yadav et al. (1997) observed an increase in ethanol production as well as fermentation efficiency with an increase in pH from 4.0 to 5.0 and found the optimum pH for *Saccharomyces cerevisiae* species around pH 4.5. Another study carried by Yalçın and Özbaş (2008) showed that *Saccharomyces cerevisiae* worked well between a pH range of 4.0-4.5 and yielded slightly better results at pH 4.0 with fermentation of Kalecik Karası and Narince types of grapes.

1.4 Pretratment of Raw Material

Aiduan et al. (2007) states that a potential source for low-cost biofuel production is to utilize lignocellulosic materials such as crop residues, grass, sawdust, wood chips, and other solid wastes. A pretreatment method is usually needed to improve the enzymatic activity when lignocellulosic materials are used as a substrate in ethanol fermentation (Sewalt et al., 1997; Kim and Holtzapple, 2005; Sun and Cheng, 2007).

Previous studies in bioconversion of lignocellulosic materials to bioethanol in the last two decades due to its large availability and immense potential clearly indicate that in order to obtain high glucose yields, removal of lignin (Dekker, 1988) and hemicellulose (hinders the access of enzymes to cellulose), reduction of cellulose crystallinity (Goldstein, 1983), and increase of porosity (Grous et al., 1986) provide better penetration of the enzyme (Dawson and Boopathy, 2007).

Also an effective pretreatment should satisfy the following criteria:

1. Maximize fermentable sugar yield
2. Prevent degradation of carbohydrates
3. Minimize inhibiting by-product formation
4. Be energetically and economically efficient

As a result, the main role of pretreatment is to breakdown the lignocellulosic substrates to their monosaccharide compounds.

The pretreatment methods that can be applied to organic wastes are of diverse categories; physical (mechanical size reduction and pyrolysis), physico-chemical (steam explosion, ammonia fiber explosion and CO_2 explosion), chemical (ozonolysis, acid and alkaline hydrolysis) and biological methods (fungi treatment) (Mani, 2002). Alkaline and acid treatments have been successfully practiced in several studies (Cara et al., 2008; Dawson and Boopathy, 2007; Yu and Zhang, 2004). In a study performed by Dawson and Boopathy (2007), postharvest sugar cane residue was treated with acid (H_2SO_4) and alkaline (H_2O_2) solvents. They concluded that acid hydrolysis produced higher amounts of ethanol. Yu and Zhang (2004) reported high concentrations of ethanol from acid hydrolyzed cotton wastes.

Other alternative methods have been practiced, such as hot water and steam pretreatment (Laser et al., 2002).

1.4.1 Acid Hydrolysis

Acid hydrolysis is the most widely used traditional method for pretreatment of lignocellulosic material. As Goldstein (1983) cited in his work, Bracconet discovered acid hydrolysis process in 1819, when treated wood with concentrated sulphuric acid and produced glucose. This process appears to be efficient by means of accessing and breaking down of cellulose. In the study of Converse and Grethlein (1979) acid hydrolysis treatment was used for saccharification of biomass. It was determined that the time-temperature relation is a critic step to maximize glucose yields by preventing degradation.

There are two types of acid hydrolysis: dilute acid or concentrated acid hydrolysis. Dilute acid hydrolysis requires lower acid consumption at higher temperatures, which is the main advantage of this method. (Jones and Semrau, 1984). When cellulosic molecules are hydrolyzed, the reaction is followed by conversion of sugars into other products like furfural. Thus the sugar degradation not only decreases the yield but also enhances the production of by-products like furfural, which can inhibit fermentation later (Graf and Koehler, 2000).

1.4.2 Hot Water Treatment

In studies found on kitchen waste, which mostly focused on starchy fraction, no pretreatment method has been used prior to enzymatic hydrolysis (Kumar et al., 1998; Tang et al., 2008; Wang et al., 2008).

It is clear from study of Kumar et al. (1998) that starch granules swell and rupture, thus amylose and amylopectin molecules unfold and disperse into solution when hot water is used with waste material.

Under these conditions enzymes attack the starch molecules and work better in the process called liquefaction and saccharification. Gelatinization might play an important role during liquefaction process by softening starch granules and thus contributing to enzyme attack. In the study of Frederickson et al. (1998), gelatinization temperatures were stated as (onset, peak and offset) 51.6^{o}C, 56.1^{o}C and 83.6^{o}C respectively.

1.4.3 Alkali Treatment

Some bases are used for pretreatment of lignocellulosic substrates. The effect of alkali treatment depends on the lignin content of materials (McMillan, 1994). The mechanism of alkaline treatment depends on the saponification of ester bond crosslinking and porosity of lignocellulosic materials increase by removal of these crosslinks. The dilute NaOH treatment causes an increase in internal surface area, decrease in polymerization and cyrstallinity and disruption of lignin structure (Fan et al., 1987).

1.5 Enzymatic Hydrolysis

Enzymatic hydrolysis of cellulose is carried out by *cellulase* enzymes. In the hydrolysis process cellulose is broken down into reducing sugars, which are further fermented by bacteria or yeast to ethanol (Duff and Murray, 1996). *Cellulases* are a mixture of several enzymes. Three major types of enzymatic activity are found in *cellulases*: (i) *endoglucanases,(*ii) *exoglucanases* and (iii) *β-glucosidases* (Mani et al.,2002). *Endoglucanases* randomly cut cellulose polysaccharide chain and generate

oligosaccharides at various lengths. *Exoglucanases* act on the end of these chains and liberate cellobiose and glucose (Teeri, 1997).

Finally, *β-glucosidases* hydrolyze cellodextrins and cellobioses into glucose. The summary of reaction is given in Figure 1.3

Figure 1.3 Enzymatic hydrolysis of cellulose

The activity of enzymes control the rates of reactions. Cellobiose accumulation inhibits exo and endoglucanases, and *β-glucosidase* activity is inhibited by glucose accumulation.

1.5.1 Liquefaction and Saccharification

The ethanol fermenting microorganism *Saccharomyces cerevisiae* lacks amylolytic enzymes. Thus, it cannot convert starch directly to ethanol (Ang et al., 2001). Due to the fact that the main potential feedstock for ethanol production is carbohydrates, which include starch, cellulose and hemicellulose, the use of enzymes to breakdown the polymer structure and oligosaccharides is required prior to fermentation (Kim and Holtzapple, 2005).

The hydrolysis of starch is carried out in a two step process consisting of liquefaction and saccharification.

For liquefaction step, *endo-1,4-D-glucan glucohydrolase* (*α-amylase*) and in the saccharification step *amyloglucosidase* enzymes are used. The enzyme *α-amylase* isolated from *Bacillus licheniformis* is currently used in starch hydrolysis for production of ethanol with the optimum conditions of 90^{o}C and pH 6 (Richardson et al., 2002).

By liquefaction, starch molecules consisting of branched and unbranched forms of amylopection and amylose are degraded into oligosaccharides and maltoses which are then at the step of saccharification generate glucose molecules. AMG-amyloglucosidase is the other enzyme responsible for saccharification, it breaks successive bonds from the non-reducing end of the chain and produce glucose (Bernfeld, 1951).

Table 1.2 shows other enzymes used in liquefaction and saccharification of starchy materials (Fiechter, 1992); (Olsen, 2001).

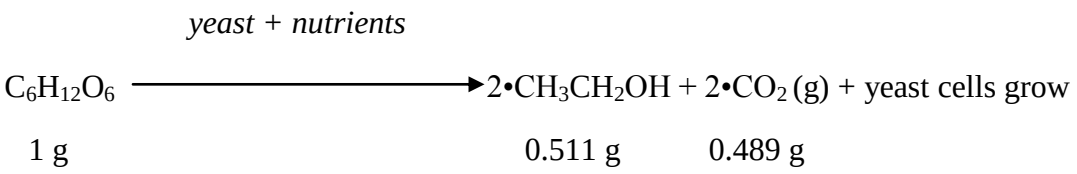
Table 1.2 Enzymes used in starch hydrolysis

Enzyme	Type	Source	Amount	Activity
α - amylase	liquefying	*B.subtilis*	0.06% (w/w) of starch	Decreases viscosity (pH 5.5, 70^{o}C)
		B.licheniformis	0.06% (w/w) of starch	Decreases viscosity (pH 5.5, 95^{o}C)
		Barley malt	0.5-1% (w/w) of grain	Decreases viscosity (60^{o}C)
β - amylase	saccharifying	Barlet malt	2% (w/w) of grain	Generates maltose (pH 5.5, 60^{o}C)
Glucoamylase (AMG)	saccharifying	*A.niger*	0.18% (w/w) of grain	Generates glucose (pH 4.5-5.0, 55-60^{o}C)

1.6 Fermenting Yeasts and their Properties

For production of ethanol from glucose, yeasts are the most commonly used microorganisms, especially *Saccharomyces* strains (Atkinson and Mavituna , 1983).

The anaerobic fermentation of ethanol from glucose is summarized by the following chemical reaction:



The theoretical yield is 0.51 g ethanol and 0.49 g carbondioxide per gram glucose fermented (neglecting the glucose consumed for the growth and maintenance of yeast). Due to various metabolic pathways for production of ethanol, other products such as glycerol, acetic acid and formic acid are also produced. However, in practice ethanol conversion efficiencies up to 90% of theoretical value have been achieved using *Saccharomyces cerevisiae* (Atkinson and Mavituna, 1983).

In batch fermentation, glucose is consumed in the production of yeast with the presence of oxygen. As long as excess glucose exists the cell growth rate increases which is ensured as the logarithmic phase of growth. The cell growth reached an upper limit where the enzymes help the oxidative pathways become saturated by sugars (Harrison, 1963). Following the logarithmic phase the yeast population reaches a maximum value where beyond that limits the rate of multiplication equals to the rate of death which is called the stationary phase. The onset time and duration of stationary phase is a function of glucose concentration, oxygen and ethanol levels in the medium. As glucose concentration decreases rapidly or comes to an end, the cell delay eventually surpasses the multiplication rate and a decrease in viable cell concentration observed which is called death phase.

The activity of *Saccharomyces cerevisiae* is inhibited by the presence of alcohols (ethanol and methanol) and acids (acetic and formic acid) and by interfering with the membrane functions. Alcohols also inhibit cytoplasmic enzymes (Erickson and Fung, 1988). Some other organisms used in ethanol fermentation and amount of ethanol that can be obtained is summarized in Table 1.3.

Table 1.3 Different types of ethanol producing strains (Kalra, 1980)

Strain	% Ethanol produced
Saccharomyces cerevisiae	5.8-11.16
Zygosaccharomyces species	4.2
S.ellipsoids	9.7
S.pombe	8.7
S.mallaeri	7.8

1.7 Optimization of Ethanol Production

Response Surface Methodology (RSM) is a statistical tool useful for collecting, analyzing, and optimizing data (Myers and Montgomery, 2002). It uses quantitative data to determine and solve multivariate mathematical equations, which determine the optimum point for a specified set of independent variables.

These constructed models consider interactions among the variables and can be used to determine how product changes with variations in the factor levels. RSM is more efficient than traditional experimental procedures because it decreases the time and cost required to determine the optimal conditions (Giovanni, 1983).
The most extensive applications of RSM are such cases where several input variables potentially influence the measure characteristic which is called the response. The input variables are called independent variables, and they are selected by the control of the researcher (Myers and Montgomery, 2002).

Basically RSM is a four-step process. First, the critical variables which are important to the process under study are identified. Second, the range of variable levels are defined. Third, the specific test samples are determined by the experimental design and then tested. Fourth, the data collected from these experiments are analyzed and interpreted by RSM.

RSM includes five assumptions in order to serve effectively:

1. The critical factors are known.
2. The range of factor levels, which influence the response is known.
3. The factors vary continuously throughout the experimental range.
4. There exists a mathematical function that relates the factors to the measured response.
5. The response, which is defined by this function, is a smooth surface.

In addition to these assumptions, theresearcher should be aware of some critical limitations:

1. Large variation in the replicates can result in misleading conclusions.

2. The independent variables may not be correctly specified or sufficiently defined resulting in an inaccurate determination of the optimum point.
3. The optimum point may not be determined by RSM because the range of variable levels tested was either too narrow or too broad to determine the optimum.
4. If statistical principles aren't followed, biased results can occur.
5. Over-reliance on the computer to conduct the experiment can lead to incomplete results. The researcher must have good interpretation and knowledge about the process to draw appropriate conclusions from the data.

In RSM, generally two types of designs are used: Central Composite Design and Box-Behnken Design.

Central composite designs are often recommended when performing sequential experiments, because these designs can incorporate information from a properly planned factorial experiment. Central composite designs have axial points outside the "cube". These points may not be in the region of interest, or may be impossible to run because they are beyond safe operating limits.

Box–Behnken designs are usually recommended when performing non-sequential experiments, that is, when planning to perform the experiment once. These designs allow for efficient estimation of the first and second order coefficients. Because Box–Behnken designs have fewer design points, they are less expensive to run than central composite designs with the same number of factors.

Also, Box–Behnken designs can be useful if the safe operating zone for the design under consideration is known. Box–Behnken designs do not have axial points, thus,

all design points will fall within the safe operating zone. In addition, Box–Behnken designs ensure that all factors are never set at their high levels simultaneously (Shuaeib et al., 2007).

1.8 Previous Studies

In studies of ethanol production, various strains of *Saccharomyces cerevisiae* have been widely used as inoculum (Laplace et al., 1992; Najafpour et al., 2004; Gardner et al., 1993). Bansal and Singh (2003) reported a comparative study of ethanol production from molasses using *Saccharomyces cerevisiae* and *Zymomonas mobilis*. They found that yeast was more ethanol tolerant. However, yeast need deliberate conditions of temperature, pH, and nutrients in order to function at high yield. In this study temperature, pH and agitation speed were kept constant since those conditions have been extensively reported in current literature (Shen et al., 2009; Torrico and Acevedo., 1988; Sharma et al., 2008; Gardner et al., 1993; Ado et al., 2009).

Studies on optimization of ethanol production have been carried out with different raw materials and process variables. Ratnam et al. (2003) reported an optimization study of ethanol production from sago starch with independent variables as temperature, pH and fermentation time in SSF (simultaneous saccharification and fermentation).

Krishna and Chowdary (2000) also performed a similar study, but they used lignocellulosic materials instead. There have been studies considering substrate concentration and inoculum size as process variables in response surface designs (Karuppaiya et al., 2009 ; Laluce et al., 2009), however none of them used kitchen waste.

A study by Wang et al. (2008) filled the gap in this subject. They studied optimization of ethanol production from kitchen garbage by response surface methodology, but the variables they investigated were temperature, pH and fermentation time.

Several studies were also carried out taking into consideration the inoculum effect on ethanol production by various organisms as well as pure culture form of *Saccharomyces cerevisiae* (Sreekumar et al., 1999 ; Neelakandan and Usharani, 2009 ; Gibbons and Westby, 1986). However, no study has been found to investigate neither the inoculum level of commercial dry baker's yeast, nor initial glucose concentration on ethanol production from kitchen wastes.

1.9 Objectives of This Study

As stated before, the raw materials used in bioethanol production are mainly coming from starchy and sugary feedstocks. The main disadvantage and future problem is that these sources can come to an end because the materials are included in human food chain. As entire world is shifting to find alternative solutions to this problem, researchers start investigating lignocellulosic materials for the purpose of ethanol production. In this study it is aimed a completely new approach from raw material point of view.

Again it is well known that waste disposal and recycling methods are very crucial for a few decades. In order to avoid environmental contamination and production of hazardous compounds – and also for economical purposes- waste treatment methods are becoming more popular. By combining these two facts, the most important point of this study is to utilize kitchen wastes as a raw material for bioethanol production.

The hydrolysis process prepares the waste for fermentation, thus there is a need to obtain fermentable sugars from carbohydrate stocks of the waste. As there are many different pretreatment methods being applied to different lignocellulosic materials, the most suitable method for this organic waste should be determined first. Another aim of this study is therefore to determine the cheapest and most effective pretreatment and hydrolysis method.

The final focus of this study is on fermentation parameters and conditions. The kitchen waste is a variable material due to its content. The main concern is the carbohydrate present in the waste. As it is very frequently investigated the temperature and pH for fermentation, this study focuses from a different point of view and aims to broaden the literature.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Kitchen Waste as a Raw Material

The kitchen waste used in pretreatment, biomass growth curves and ethanol-glucose trend determination was used in the mixed form of different food wastes as produced from its source. Therefore, the wastes were collected from several food courts of Middle East Technical University (METU), Ankara, Turkey and household kitchens.

The experimentation of RSM was carried out during ERASMUS student exchange program. The kitchen waste used in response surface experiments of this study was collected from cafeteria of Department of Agricultural Engineering, University of Bologna (UNIBO), Bologna, Italy and from three different household kitchens.

The tissue, plastic, cartoon, and glass pieces were separated if present in the waste, and remaining organic residuals were mixed and ground in a chopper to form the composite substrate for experiments. The composite waste was stored at $4^{o}C$ until use in a day or two.

The collected kitchen wastes included paprika, parsley, chicken, mushroom, iceberg, pudding, zucchini, yoghurt, French fries, pasta, pizza, cookies, coffee residues, salad, fresh and dried beans, bread, rice, wheat, potato, tomato, carrots, aubergine, lettuce, rice, corn and peels of fruits and vegetables as well as whole parts.

2.1.2 Organism

A commercial instant baker's yeast *Saccharomyces cerevisiae* was purchased from a local store and kept in a refrigerator until use. The dry weight of baker's yeast was determined as 92.89%. The instant yeast was dispersed in sterile water at room temperature at different concentrations of 5, 10 and 15 g/L (g instant bakers' yeast / liter of DI water) and added as an inoculum without any cultivation (Chiang et al., 1981).

2.1.3 Enzymes and Supplier

The enzymes used in liquefaction and saccharification steps were *α-amylase* (A6211-1MU), *amyloglucosidase (AMG)* (10115), *cellulase* (C1794-10KU), and *β-glucosidase* (49290), which were all purchased from SIGMA-Aldrich. The activity of enzymes reported by the supplier was considered in our study.

2.1.4 Fermentation Medium

To study the effect of fermentation medium on ethanol production, one set of experiments were done without addition of fermentation medium. The second set contained 6 g/L yeast extract, 1.5 g/L KH_2PO_4, 1 g/L $MgSO_4.7H_2O$, and 4 g/L $(NH_4)_2SO_4$ as fermentation medium. All chemical reagents were of analytical grade.

2.2 Methods

2.2.1 Pretreatment methods

Three pretreatment methods were used: hot water, dilute acid and a control (no pretreatment). For dilute acid treatment sulphuric acid at two concentrations of 1 and 4 % (v/v) was added to the kitchen waste. Samples were kept at 60^{o}C for 3 h in all pretreatment methods (Li et al., 2007). After these steps all flasks were followed by enzymatic hydrolysis for complete breakdown of starchy and cellulosic compounds. The glucose concentrations after pretreatments are given in Table 3.1 (Appendix.C).

2.2.2 Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out in two steps. For liquefaction of starchy waste, *α-amylase* was added (120 U/ g substrate) to the waste and kept at 95 ^{o}C for 1 hour at 100 rpm (Spindler et al., 1992). The *α-amylase* works well with pH 5.0-6.0, thus the initial pH was adjusted to 5.5 after pretreatment processes. In the second step, starch based oligosaccharides and the cellulosic fraction were processed simultaneously. The enzymes *amyloglucosidase* (120 U/g substrate) (Wang, 2008), *cellulase* (8 FPU/g substrate) and *β-glucosidase* (50 U/g substrate)(Krishna and Chowdary, 2000) were added after the liquefied mixture was cooled to 55^{o}C.

Glucose production was monitored until the glucose concentration reached a constant value. Agitation was applied at 100 rpm during enzymatic hydrolysis. To terminate the enzymatic activity, samples were boiled for 15 min at each time of sampling.

2.2.3 Fermentation

Fermentation experiments were conducted in 250 ml erlenmayer flasks with a working volume of 100 ml. The yeast was added at a ratio of 10% (v/v) to the fermentation mixture under aseptic conditions. Before inoculation, the flasks and medium were sterilized by autoclaving. Sulphuric acid (0.5 M) was used to adjust the initial pH to 4.5. The flasks were placed in a temperature controlled shaker. The temperature and agitation speed were maintained constant throughout the experiment at 30^{o}C and 150 rpm, respectively. The fermentation period was limited to 48 h.

2.2.4 Analytical Methods

2.2.4.1 Moisture Content

For determination of moisture content, gravimetric method was used (AOAC, 1984). Five grams of mixed and ground waste was initially weighed and put onto glass petries and placed in 105^{o} C oven. Before analysis, empty petries were put into 105°C oven for 30 – 60 minutes and then cooled in desiccator for 20 minutes to record their moisture free (tare) weight. During analysis petries were weighed in hourly intervals, until a constant weight was reached. When constant weight was obtained, petries were taken from oven and put into desiccator for 20 minutes to cool down to room temperature. Then final weight was recorded. Analysis was done in duplicate.

The moisture content of the sample was calculated using the following formula:

$$\%\, Moisture \;=\; [(A - B)/B] \times 100 \qquad \textbf{(Equation 2.1)}$$

where;
A = Weight of wet sample (g), and
B = Weight of dry sample (g)

2.2.4.2 Ash Content

This method (AOAC, 1984) covers determination of ash, expressed as the percentage of residue remaining after dry oxidation (oxidation at 550 to 600^{o} C). An oven dried (at 105^{o} C) sample of 1 g (with two replicates) was placed into the crucible and kept in the furnace at 575^{o}C for 6 h at which constant weight was reached. The amount of ash was calculated using the formula;

$$\% \, Ash = \left[\left(W_i - W_f \right) / \, W_i \right] \times 100 \qquad \textbf{(Equation 2.2)}$$

where;
W_i: Initial weight of the sample before placing in furnace
W_f: Final constant weight of the sample

2.2.4.3 Protein Content

For protein determination the Kjeldahl Method was used (CRA, 2001). This method is used for nitrogen determination when suitable amounts of sample, sulfuric acid and catalysts are used. For more accurate results of protein content a correction factor of 6.25 is used (CRA, 2001).

A sample of 1 g with a known moisture content was used in the analysis in two replicates. After performing the necessary steps the final protein content was calculated according to the given analytical formula:

$$\%\ N\ (dry\ basis) = \frac{(V_{0.1N\ H2SO4} - V_{blank} - V_{0.1N\ NaOH}) \times 0.0014 \times 100}{(W_s \times (100 - \%X_s)/100)}$$

$$\%\ Protein = \%N\ \times 6.25 \qquad \textbf{(Equation 2.3)}$$

where;

% N: percentage of nitrogen present in the sample

W_s: weight of the sample in g

% X_s: percent moisture of the sample

2.2.4.4 Fat Content

The total fat content of the kitchen waste was determined according to Hexane/Isopropanol (3:2) Method as suggested by Hara and Radin (1978). The ratio of 3:2 hexane/isopropanol is used to provide necessary polarity.

To each flask, 17 g of raw kitchen waste was added. The weight of the flasks and waste were determined. Six milliliter of hexane together with 4 ml of isopropanol were mixed in the flasks and allowed to soak for five minutes. Thereafter the liquid phases were decanted into empty beakers. This process of mixing and decanting were repeated three times after which the samples were left in 105^{o}C oven to dry overnight.

The flasks were then placed in a dessicator to prevent water from being absorbed. The weight was measured when the samples were cooled down to room temperature. The percent fat extracted was calculated according to Equation 2.4

$$\%\ \boldsymbol{Fat} = \left[\left(\boldsymbol{W_i} - \boldsymbol{W_f}\right)/\ \boldsymbol{W_i}\right] \times \mathbf{100} \qquad \textbf{(Equation 2.4)}$$

2.2.4.5 Total CHO's

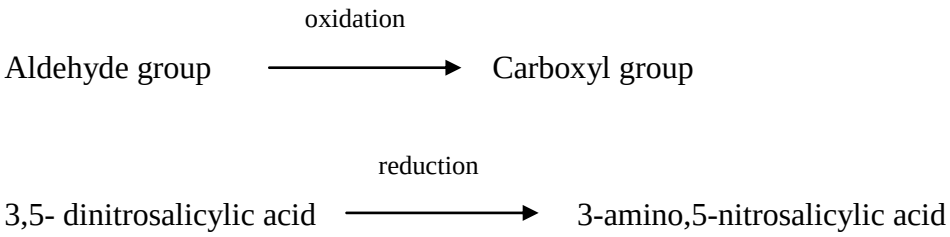
Total carbohydrate determination is a very hard and time-consuming process and there is no direct and standard method for this. Therefore total amount is found by subtracting other components of organic materials from the whole (Sahin and Sumnu, 2006).

(% *Total Solids*) = % *Protein* + % *Fat* + % *Ash* + % *Total CHO's* **(Equation 2.5)**

2.2.4.6 Reducing Sugars

Dinitrosalicylic Acid (DNS) Method is a widely used test method to determine the reducing sugar concentrations.

It tests for the presence of free carbonyl group (C=O), so-called “reducing sugars”. This involves the oxidation of the aldehyde functional group present, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions (Miller, 1959);



The samples prepared according to Dinitrosalicylic Acid Method (DNS) were analyzed using spectrophotometer. Samples were cooled to room temperature in a cold water bath, the absorbances were recorded at 550 nm (Pramanik, 2005). Although this is a convenient and relatively inexpensive method, due to the relatively low specificity, one must run blanks diligently if the colorimetric results are to be interpreted correctly and accurately.

This method was used when determining the fermentable sugar concentrations of different pretreatment methods, glucose concentrations during enzymatic hydrolysis, and glucose consumption trends in synthetic and waste media. Detailed results are given in results and discussion chapter. The concentration data of each replicate are also given in Appendix.C-F-G.

2.2.4.7 Determination of Ethanol by GC Method

Gas Chromatography Method is widely used in determination of volatile substances. For ethanol analysis GC (*SHIMADZU*, Kyoto, GC-14A #124457) was used. Ethanol solutions with 1, 3, and 5 % (v/v) were used as internal standards (Toro-Vazquez and Perez- Briceno, 1998) with quantification based on peak area.

The key parts of a gas chromatograph include: a source of gas as the mobile phase, an inlet to deliver sample to a column, the column where separations occur, an oven as a thermostat for the column, a detector to register the presence of a chemical in the column effluent, and a data system to record and display the chromatogram. In addition, some facility is needed so that temperatures of various components can be accurately known and controlled (Prat, 2003).

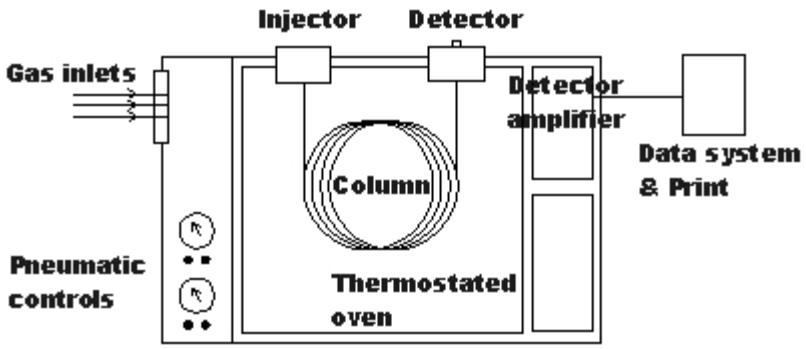


Figure 2.1 A Gas Chromatographer

Instrumentation:

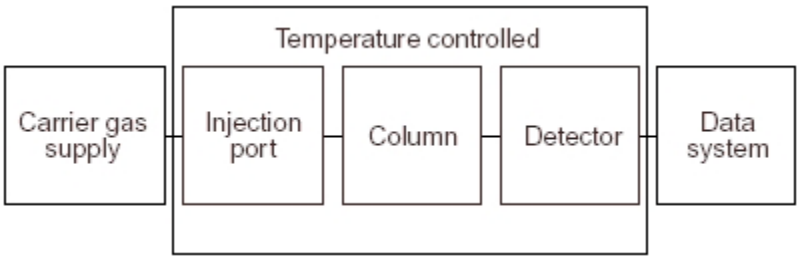


Figure 2.2 Block diagram of GC (Prat,2003)

One ml of batch cultures were collected, 10-fold diluted, microcentrifuged (6000 rpm, 5 min) and the supernatant was kept at -18^{o}C prior to subsequent analysis. The separation was carried out in a 2 m steel column packed with Propac Q at a temperature of 150^{o}C using Helium (He) as a carrier gas. Detection and quantification were carried out by a flame ionizing detector (FID) at 190^{o}C. Analysis was performed with range and polarity adjusted to 2 and 1, respectively.

2.2.4.8 Determinaton of Ethanol by HPLC

High Pressure Liquid Chromatography (HPLC) system is used in various detections of alcohols, phenolic compounds and sugars. In this study HPLC system was used to determine ethanol and glucose concentrations of fermentation experiments in response surface analysis.

The concentrations of ethanol and glucose in fermented samples of hydrolyzed kitchen waste were chromatographed on a *SHIMADZU* HPLC system equipped with a refractive index detector Rheodyne 7110 (RID-10A, *SHIMADZU*) and a Chromatopac C-R7Ae data processor with attenuation 6 (*SHIMADZU*, Kyoto, Japan). Aliquots of filtered samples (1,5 ml) were injected to quartz cuvettes and placed to the autosampler of the HPLC system. Ethanol concentration were eluted using 5mM H_2SO_4 solution as the mobile phase. The Aminex HPX 87H column (300 x 7,8mm), which was supported by de-ashing cartridge (Bio-Rad, USA) was used at 80^{0}C and a flow rate of 0,6 ml/min. The complete analysis of one run was carried out in 30 min (Liebmann et al., 2009). The integrators of the system determined the start, retention time and end of the peak and calculated the area under each peak as a function of width and height of the peak.

Concentrations of ethanol and glucose were quantified using the peak areas compared with the peak areas of standard solutions of ethanol and glucose expressed as g/L.

Samples of 1ml were taken at predetermined time intervals from 250 ml flaks where fermentation process was carried out at 30^{o}C, pH 4.5 and 150 rpm. The original samples which were taken from the fermented mixtures of hydrolyzed kitchen waste were prepared with 10-fold dilution by distilled water for HPLC analysis. Diluted samples were then filtered through 0.22 μm membrane filter and kept at 4^{o}C until analysis.

Synthetic D-glucose monomer was used to prepare the standard solutions at concentrations of 40, 80 and 120 g/L. The ethanol solutions were prepared by commercial ethanol with 99.5% purity at concentrations of 10, 20 and 30 g/L as the internal standards.

2.2.4.9 Determination of Biomass

The method used in determination of biomass concentration based on optical density measurement using a spectrophotometer (Anonymous 4). The moisture content of dry baker's yeast was determined as explained in Section 2.2.4.1 with gravimetric method given by AOAC (1984). Therefore, the cell density was quantified as grams of dry weight per liter.

One milliliter of samples (duplicated) were taken aseptically, in every 4 hour from fermentation flasks and 10-fold diluted before analysis to be consistent with the results. Solutions containing 0.1, 0.2, 0.3 and 0.4 g dry yeast / liter were used to generate the calibration curve. The blank solutions were prepared from the same diluted samples by removing the biomass after centrifugation at 6000 rpm for 5 min.

The measurement was done at 550 nm wavelength. The data of dry cell concentrations were given in Appendix E.

2.2.5 Experimental Design

2.2.5.1 Experimental Design for Bioethanol Fermentation

In this study, to determine the optimum concentration of ethanol produced, a statistical tool, Response Surface Methodology (RSM) was used (Myers and Montgomery, 2002). For the design of experiments performed by RSM, the statistical software MINITAB® 15.1 (Minitab Inc. State College, PA, USA) was used. RSM was conducted through Box-Behnken Design, which is one of the most common experimental designs used for engineering practices.

Each independent variable was included in the design at three levels rather than five levels required for a central composite design or the four levels for a San Cristobal design (Thomson, 1982). In Box Behnken design, the coding of independent variables is done by using integers (-1, 0, +1) for minimum, middle and maximum levels. The Box Behnken design with three variables consisted of a combination of the following subsets of points from full factorial, 3^3 design. Points of (±1, 0, 0), (0, ±1, 0), (0, 0, ±1) and center points (0,0,0) were included (Khuri and Cornell, 1987). All these points are used for measuring means, standard deviations, experimental errors and lack of fit.

A set of 15 experiments was carried out in two randomized replicates giving total experimental runs of 30 with 6 center runs. The independent variables selected in this study were: solid load, inoculum level and fermentation time, and coded as X_1, X_2 and X_3 , respectively. The coded and uncoded forms of independent variables are tabulated in Table 3.

The predictive model as a second order polynomial was as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \quad \textbf{(Equation 2.6)}$$

$$\beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where; Y is the response (ethanol concentration), β_o, β_i and β_{ii} are regression coefficients.

2.2.5.2 Statistical analysis and optimization

The analysis of variance (ANOVA) and regression analysis were performed to define the coefficients of the predictive model and significant terms using MINITAB® 15.1 (Minitab Inc. State College, PA, USA). The contribution of individual factor and their quadratic effects on ethanol concentration were also determined. The optimum fermentation conditions of bioethanol production were determined by Response Optimizer tool of MINITAB® 15.1 (Minitab Inc. State College, PA, USA). The model obtained by regression was maximized for ethanol concentration.

2.2.5.3 Model Verification

The constructed model was verified by conducting additional fermentation experiments, which were not present in the design matrix and comparing the results of experiments to the predicted values.

The coefficient of determination (R^2) was determined to check for the linearity between the predicted vs. experimental ethanol concentration values. Statistical difference measure test was also carried out to evaluate the performance of the model by calculating root mean square error (RMSE) and mean absolute error (MAE) values as follows:

$$RMSE = \left(\frac{1}{N} \sum_{i=1}^{N} (P_i - O_i)^2 \right)^{0.5} \quad \textbf{(Equation 2.7)}$$

$$MAE = \frac{1}{N} \sum_{i=1}^{N} |P_i - O_i| \quad \textbf{(Equation 2.8)}$$

where P_i and O_i are predicted and experimental ethanol concentrations, respectively; N is the number of data.

The root mean square error (RMSE) has two components; systematic and unsystematic errors. Their values are calculated by:

$$RMSE_s = \left(\frac{1}{N} \sum_{i=1}^{N} (\hat{P}_i - O_i)^2 \right)^{0.5} \quad \textbf{(Equation 2.9)}$$

$$RMSE_u = \left(\frac{1}{N} \sum_{i=1}^{N} (\hat{P}_i - P_i)^2 \right)^{0.5} \quad \textbf{(Equation 2.10)}$$

where $RMSE_s$ and $RMSE_u$ are systematic and systematic components of RMSE, respectively, $\hat{P}_i$ is the predicted ethanol concentration as the least square linear regression of P_i and O_i.

Total RMSE is then:

$$RMSE^2 = RMSE_s{}^2 + RMSE_u{}^2 \qquad \textbf{(Equation 2.11)}$$

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Characteristics of Kitchen Waste

The average composition of kitchen wastes used throughout the study is summarized in Table 3.1. The total dry matter is an indirect indication of main nutrients available to the yeast for growth and maintenance. The total carbohydrate content is the main focus to yield fermentable sugars. Analytical tests indicated that moisture content of the kitchen waste varied within the range 60-70% (w/w), which led to 30-40% (w/w) of total dry matter. Approximately 60% of the total dry matter was determined as carbohydrate fraction, which proved that the kitchen waste could be used as a valuable raw material for ethanol production. The protein, fat and ash content of the kitchen waste provided the required elements for functioning of yeast.

Table 3.1 Average composition of kitchen waste

Constituent	Content (% w/w)
Moisture	63.4 ± 3.8
Total Solids	35.8 ± 3.7
Protein	4.4 ± 0.24
Fat	6.8 ± 1.85
Ash	1.5 ± 0.84
Total CHO's	23.5 ± 6.63

3.2 Effect of pretreatment method on glucose production

As mentioned in materials and methods section, the kitchen waste used in this study contained various food residues in raw and cooked form as well as whole edible parts and peels of fruits and vegetables. It can be observed visually that the kitchen waste included considerable amount of both starchy and lignocellulosic materials. Therefore, in order to increase the yield of enzymatic hydrolysis a pretreatment method was used (Table 3.2). Each pretreatment method was followed by enzymatic hydrolysis performed under the same conditions. Thus, the difference in final glucose concentrations was concluded to be due to the pretreatment method. This was also proved by the initial glucose concentration determined at the end of each pretreatment method (Table 3.2). According to the data given in Table 3.2, it was found that hot water pretreated and no pretreated samples had higher initial glucose concentrations than the acid pretreated samples ($p<0.05$).

Table 3.2 Pretreatment conditions and glucose concentrations after each pretreatment

Pretreatment conditions			Glucose concentration (g/L)
Temperature (^{o}C)	Time (hour)	Chemical	
60	3	1% acid	13.1
60	3	4% acid	12.9
60	3	Hot water	22.7
NPT	NPT	NPT	24.7

[a]NPT: No pretreatment method applied; kitchen waste was directly subjected to enzymatic hydrolysis

The glucose concentrations during enzymatic hydrolysis of the wastes subjected to different pretreatment methods is given in Figure 3.1. The enzymatic hydrolysis consisted of two steps: liquefaction and saccharification respectively. Enzymatic hydrolysis process time interval was given as 6-9 h for the latter step (Wang, 2005). To determine the necessary process time for saccharification of our waste system, samples were taken hourly and analyzed for glucose concentration. The concentration of glucose increased gradually with time and reached a constant value within 6 h for all pretreatment methods. Thus, the enzymatic hydrolysis was carried out for 6 h in all subsequent experiments. Moshe (1967) found the process time as 8-10 h. However in another study by Audian et a.l. (2007) the optimum hydrolysis time was stated as 3h. The difference among results is due to the type of the raw material and difference in enzymatic hydrolysis conditions.

The highest glucose concentration was obtained as 64.7 g/L from the unpretreated samples after 6h and followed by hot water treatment as 56.7 g/L. However, these values were not found statistically different ($p>0.05$).

This result indicated that hot water pretreatment could be skipped to start right away with the enzymatic hydrolysis, being consistent with results of Tang et al. (2008) and Wang et al. (2008) and opposite the study of Laser et al (2002). The two acid concentrations (1% and 4%) also gave statistically similar results ($p>0.05$) but still lower than no pretreatment case ($p<0.05$), releasing glucose concentrations of 51.5 and 45.4 g/L, respectively. Furthermore, the glucose amount of 1% acid was the same as the amount of hot water ($p>0.05$). Thus, the acid treatment was also concluded to be an effective method as reported by Dawson and Boopathy (2007). By these findings, we showed that a two-step-enzymatic hydrolysis as described previously without any pretreatment was adequate for glucose production from kitchen wastes processed in mixed form of carbohydrate fractions prior to fermentation.

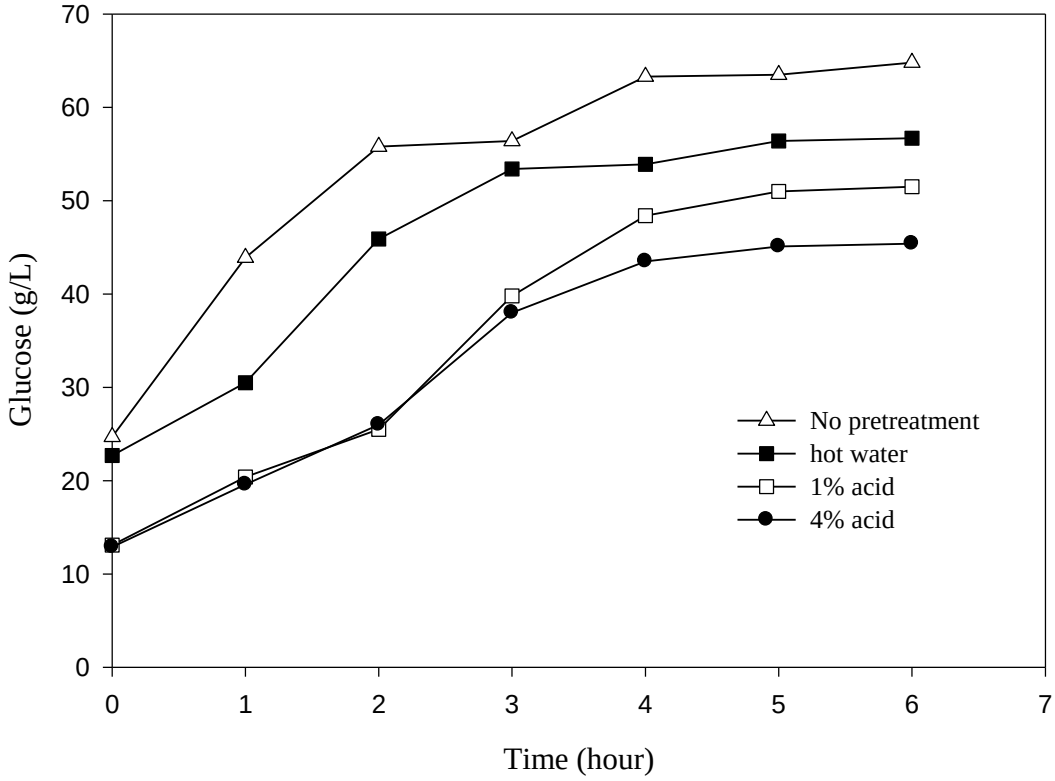


Figure 3.1 Glucose concentrations during enzymatic hydrolysis

3.3 Effect of fermentation medium on ethanol production

Following the enzymatic hydrolysis step, the saccharified mixture was subjected to batch ethanol fermentation at pH 4.5 and 30^{o}C for 48 h with agitation speed of 150 rpm. The experimental plan shown in Table 3.3 was carried out to determine the effect of fermentation medium addition on ethanol production. The initial glucose concentration, final ethanol concentration and yield values are also given in Table 3.3.

Statistical analysis of the results revealed that the ethanol concentrations and the yields were similar for the fermentation medium added and not added samples regardless of the pretreatment method applied ($p>0.05$). It was found that the highest ethanol concentration and yield values were obtained as 23.3 g/L and 0.36 g/g from no fermentation medium added and unpretreated samples, which was significantly higher than the result of the hot water pretreated and fermentation medium added samples only. This value was attributed to a productivity of about 0.49 g/L.h. These results supported the idea that fermentation of kitchen wastes in mixed carbohydrate fractions was possible without adding the fermentation medium. Thus, it can be concluded that nutrients already present in the kitchen waste were sufficient for functioning of *S. cerevisiae* to produce ethanol.

Our results are consistent with results of others. Wang et al. (2008) reported fermentation of *Saccharomyces cerevisiae* with kitchen garbage at pH values of 4-6.63 and temperatures of 26.8-40^{o}C. They obtained ethanol concentration of 22.13 g/L at 26.8^{o}C within 48 h at pH 5, which can be taken as similar conditions of our study. Their productivity for these conditions was 0.46 g/L.h, similar to our value.

It should also be noted that Wang et al. (2008) used a pure yeast culture and working volume of 150 ml for fermentation experiments. In batch experiments using *Saccharomyces cerevisiae* KF-7, Tang et al. (2008) reported 29.9 g/L of ethanol in 24 h at pH 4.5 and 30^{o}C from 64 g/L of glucose using 300 ml of working volume. This difference was explained by the yeast strain, working volume and thus the higher initial glucose amounts they used in their study. In another study of Kumar et al. (1998), mixed bakery wastes, potato chips, and grain flour was fermented by distiller's yeast at pH 5 and 30^{o}C. They obtained ethanol concentration of 245.4 g/kg from 609.95 g/kg of glucose after 15h, which gives ethanol yield of 0.40 g/g.

Therefore, it can be concluded that kitchen waste in mixed carbohydrate fractions could be successfully utilized in ethanol fermentation using dry baker's yeast.

Table 3.3 Effect of fermentation medium addition on ethanol yield

Pretreatment	Glucose before fermentation (g/L)	Ethanol (g/L)	Yield (g ethanol/g glucose)
Hot water+EH+WM	56.7	14.6	0.26
Hot water+EH+NM	56.7	17.2	0.30
NPT+EH+WM	64.8	17.4	0.27
NPT+EH+NM	64.8	23.3	0.36

[a] Results are averages of two replicates

[b] EH = Enzymatic hydrolysis, WM = With fermentation medium, NM = No fermentation medium, and NPT = No pretreatment method

3.4 Behavior of *Saccharomyces cerevisiae* in Kitchen Waste

In this section, it was aimed to see if long fermentation time (96 h) has an adverse effect on *Saccharomyces cerevisiae* growth and functioning in waste medium.

Saccharomyces cerevisiae is an aerobic microorganism, in which the multiplication and cell biomass relies on respiratory metabolism rather than fermentative metabolism (Walker, 2000).

Since yeast is propagated in aerobic conditions, until oxygen concentrations decreases, respiration predominates and energy is generated in the form of ATP and production of cell biomass is enhanced.

From the results obtained by kitchen waste fermentations performed at 30^{o}C, pH 4.5, 150 rpm and initial cell concentration of 1g/L (corresponding 10% (v/v) inoculum of *Saccharomyces cerevisiae*), it can be seen that the cell biomass concentration reached high values in waste medium fermentation (Figures 3.2 and Appendix E). It was also found that, final *Saccharomyces cerevisiae* concentration was increased with increasing solid load from 10% (w/w) to 15% (w/w). However, cell concentration was observed to have a lower value with 20% (w/w) solid load than 15% (w/w) solid load (Figure 3.2).

This might be due to Crabtree effect. In batch yeast fermentation, high sugar concentration results in Crabtree repression, which inhibits respiratory enzymes and adversely affects the production of cell biomass (Win et al., 1996).

In waste medium fermentations given in Figure 3.2, after 16 h, the stationary phase started. There cell concentration continued to increase with slight fluctuations, and the highest cell concentration was achieved at the end of 96 h fermentation with 15% (w/w) solid load (Appendix E).
But the growth of the yeast found to be insignificant in all levels of solid load in waste medium ($p > 0.05$).

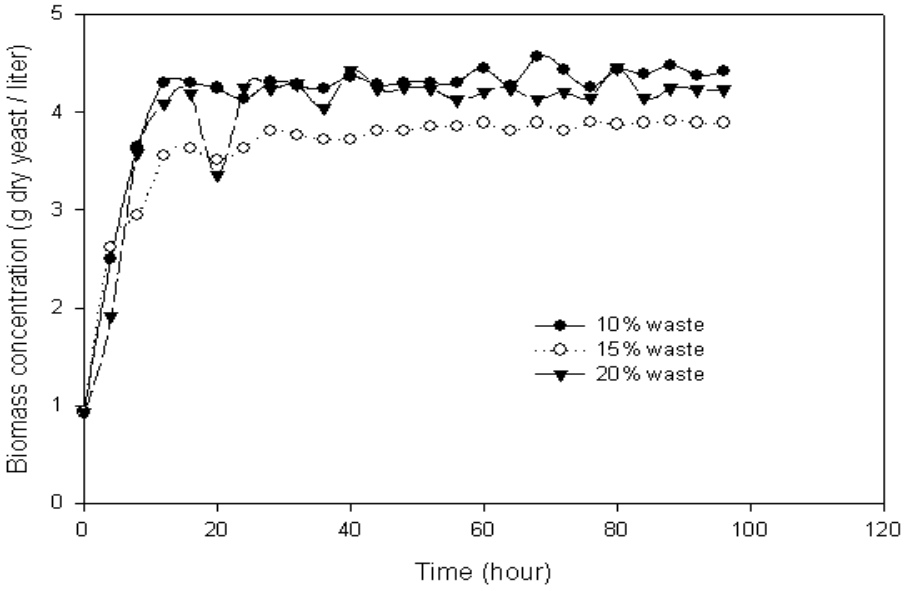


Figure 3.2 Growth of *Sachharomyces cerevisiae* in waste medium

During growth yeast has to maintain a constant intracellular pH, because there are many enzymes functioning within the yeast cell for metabolism. These enzymes work best at their optimal pH's, which are acidic due to the acidophilic nature of *Saccharomyces cerevisiae* (Narendranath et al., 2001). Therefore initial pH of fermentation experiments was adjusted to 4.5 and the final pH values were found to be 4.3 on average. Thus it was concluded that, kitchen waste provides a self-buffering capacity and constitutes a well functioning range of *Saccaromyces cerevisiae* for growth.

3.5 Glucose-Ethanol trends and determination of fermentation time

Saccharomyces cerevisiae as being a fermentative microorganism is able to use organic substrates anaerobically in alcoholic fermentation (Noor et al. 2003). During fermentation glucose is converted to ethanol by glycolysis. As stated in Chapter 1.3.3 initial sugar concentrations, temperature, pH and inoculum size are important factors affecting ethanol fermentation.

Utilization of kitchen waste as the carbon source for ethanol fermentation is a new concept in literature and little information has been found on it. In this chapter, it was aimed to find the time interval for kitchen waste fermentation conducted at 30^{o}C, pH 4.5, 150 rpm and 1g/L initial inoculum concentration. Three levels of solid load (corresponding to different initial sugar concentrations) were taken into consideration (10,15 and 20% (w/w)). The concentrations of glucose and ethanol in waste medium are given in Appendix F and Appendix G, respectively.

Results from Figures 3.3, 3.4 and 3.5 indicated that on average 80% of glucose conversion was achieved (Table 3.4). Also in all levels of solid load (10, 15 and 20% (w/w)), ethanol production increased with increasing solid load (i.e. glucose concentration).

When waste is fermented with 10% (w/w) solid load (51.3 g/L initial glucose concentration; Figure 3.3), glucose conversion was calculated as 79%. The highest ethanol yield was calculated as 0.37 g ethanol/ g glucose at the end of 72 h of fermentation. At 72th hour, the highest ethanol concentration and desired conversion were observed. Thus the productivity was calculated as 0.26 g ethanol/L.h.

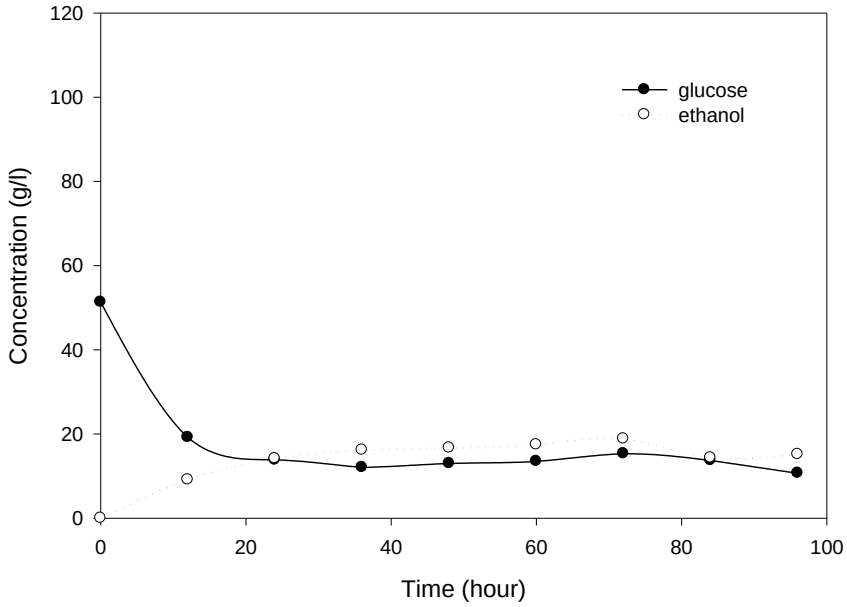


Figure 3.3 Waste medium fermentation with 10% solid load

Figure 3.4 shows the trends in waste medium fermentation with 15% (w/w) solid load (74.67 g/L initial glucose concentration and 81% conversion of glucose). The ethanol concentration reached the highest value at 72 h and gave a yield of 0.38 g ethanol / g glucose. The productivity was found as 0.39 g ethanol /L.h.

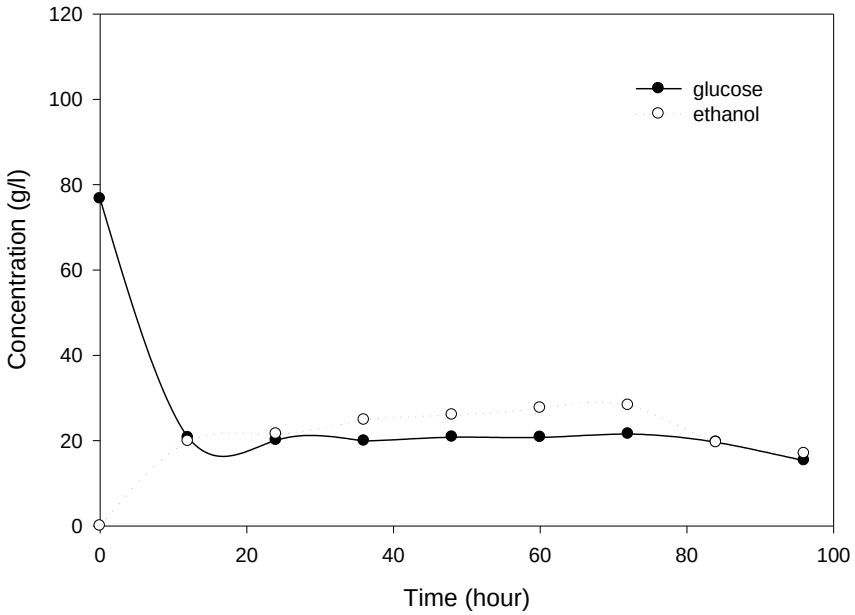


Figure 3.4 Waste fermentation with 15% solid load

Generally glucose concentrations about 20% and more resulted in an increase in osmotic pressure and therefore a decrease in fermentation capacity (Jones et al., 1981) Similarly, Sree et al. (2000) stated 20% (w/v) initial glucose concentration as the highest operable limit for successful fermentation.

In waste fermentations, the time at which the highest ethanol concentrations could be observed was prolonged to 72 h. From Figure 3.5 the trends of glucose consumption and ethanol production in waste medium can be seen at 20% solid load (105.81 g/L initial glucose concentration).

The glucose conversion was calculated as 85% and resulted in an ethanol yield of 0.39 g ethanol / g glucose. Productivity in this case was found as 0.69 g ethanol /L.h, which gave the highest value among waste medium fermentations at 10, 15 and 20% (w/w) solid load.

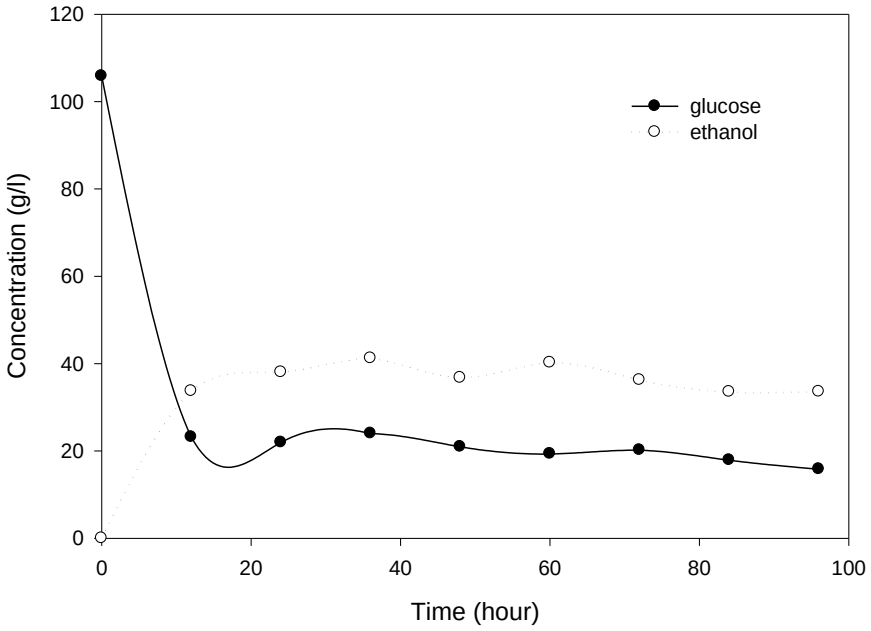


Figure 3.5 Waste fermentation with 20% solid load

As it can be seen on Table 3.4 with increasing solid load in kitchen waste, the initial glucose concentration was increased and higher conversion of glucose was achieved. Higher concentrations and better utilization of glucose –with increasing solid load- resulted in higher yields of ethanol and thus enhances the productivity.

Table 3.4 Ethanol yields of synthetic and waste fermentations

Medium	Glucose Conversion	EtOH(max) g/L	Yield	Productivity
Waste 10% solid load	79%	18.82	0.37	0.26
Waste 15% solid load	81%	28.27	0.38	0.39
Waste 20% solid load	85%	41.19	0.39	0.69

In the waste medium fermentations with all three levels of solid loads (10, 15 and 20% (w/w), the highest ethanol concentrations were observed at 72 h. The ethanol concentrations after 72 h reached stable values with slight increases up to 90-96 h. Thus, it was concluded that a 96 h of fermentation would give a good interval to observe the peak values and possible operating limits in ethanol fermentation from kitchen waste. Therefore, in determining the fermentation time levels for RSM, the highest level was taken as 96 h.

The batch of kitchen waste used in this section has the following initial glucose concentrations varying with % solid loads, shown in Figure 3.6 as “batch 2”. The batch of kitchen waste used in RSM experimentation was also given in Figure 3.6 as “batch 1”, and had different initial glucose concentrations even if it was adjusted at the same solid loads. This difference is due to the generation of kitchen waste and uncontrollable input characteristics. In each batch of kitchen waste glucose concentrations increase with increasing solid load.

Figure 3.6 Solid load and glucose concentration interaction

3.6 Optimization of Fermentation Conditions for Ethanol Production by RSM

3.6.1 Analysis of Box-Behnken Design

The independent variables investigated in bioethanol fermentation study were initial solid load (X_1), inoculum ratio (X_2) and time of the fermentation process (X_3).

For convenience of notation, actual X_i variables were coded by using the general transformation formula;

$$x_{ui} = \frac{X_{ui} - q_i}{c_i}$$

(Equation 3.6.1.1)

where;

x_{ui} : coded factor level

X_{ui} : factor level

a_i : middle value of the factor levels

c_i : the range between the two values of levels of each factor

The use of coded levels has several advantages. By using coded variables, experimental designs can be written without the need of showing the interest range for each input variable. Also, regression coefficients can be compared easily since each of variable's range is the same. The actual values and the corresponding coded values of independent variables which cover a broad spectrum of available operating conditions are given in Table 3.5.

Table 3.5 The uncoded and coded levels of independent variables used in RSM

		Coded Levels		
Operating Conditions		-1	0	1
		Uncoded Levels		
%Solid Load	(w/v)	10	15	20
%Inoculum Level	(v/v)	5	10	15
Fermentation time	(hour)	48	72	96

The results of Box Behnken experiments for studying the effects of solid load, inoculum level and fermentation time are presented in Table 3.6. The data clearly showed that the initial solid load of the fermentation mixture had a linear impact on ethanol

concentration. The highest ethanol concentration of 33.30 g/L was observed at 20% (w/w) solid load, 10% (v/v) inoculum level and 48 h of fermentation.

Table 3.6 Design matrix of RSM experiments

	INDEPENDENT VARIABLES			RESPONSE
RUN No	**%Solid Load (w/w)**	**%Inoculum (v/v)**	**Fermentation Time (hour)**	**Ethanol Conc. (g/L)**
1	15	5	96	20.21
2	20	15	72	25.08
3	10	10	96	12.83
4	15	5	96	18.94
5	10	10	96	10.28
6	15	10	72	22.01
7	20	10	96	29.86
8	10	10	48	16.80
9	15	15	48	18.68
10	20	5	72	30.68
11	10	5	72	14.99
12	10	15	72	9.53
13	20	10	96	27.54
14	15	10	72	26.01
15	10	15	72	13.01
16	10	5	72	15.72
17	20	10	48	33.30
18	15	10	72	24.71
19	15	10	72	23.21
20	15	10	72	20.70
21	15	5	48	21.25
22	15	10	72	25.83
23	10	10	48	16.96
24	20	10	48	32.32
25	15	5	48	21.36
26	15	15	96	17.21
27	15	15	96	19.50
28	20	15	72	28.68
29	20	5	72	28.48
30	15	15	48	18.44

- The glucose concentrations of given solid loads of 10,15 and 20% (w/w) are 40, 60 and 80 g/L, respectively

To find the effect of solid load (X_1), incoulum amount (X_2) and time of fermentation (X_3) on ethanol concentration, Equation 2.5 was used.

The regression equations and coefficients were determined from the analysis of Box-Behnken design. The regression models were simplified by eliminating insignificant terms. Thus, the number of terms in the regression models were reduced. Reducing the number of terms provides useful and simple models which is easier to work with. The models were reduced manually by looking at the p-value of each coefficient. The coefficient with the highest p-value was eliminated first, then elimination was continued until only significant terms remained. ANOVA tables and regression coefficients are given in detail in Appendix J.

Table 3.7 Determination of the mathematical model for RSM

Terms	Coefficients	P-Value
Constant	23.763	
Solid Load	7.861	0.000
Inoculum	-1.341	0.004
Fermentation Time	-1.421	0.003
Inoculum * Inoculum	-3.011	0.000
Fermentation Time * Fermentation Time	-1.290	0.050
Linear		0.000
Quadratic		0.000
Lack of Fit		0.411
S = 1.70258 R-Sq = 94.18%	R-Sq(pred)=91.12%	R-Sq(adj)=92.97%

The mathematical model obtained as a result of regression analysis based on the coefficient given in Table 3.7 is as follows:

$$Y = 23.763 + 7.861X_1 - 1.341X_2 - 1.421X_3 - 3.011X_2^{\,2} - 1.290X_3^{\,2} \quad \textbf{(Eq.3.1)}$$

where;

Y: Ethanol concentration (g/L)

X_1: % Solid load (w/w)

X_2: % Inoculum level (v/v)

X_3: Fermentation time (hour)

Response surface methodology was successfully used in order to determine the model representing the effects of solid load, inoculum level and fermentation time on bioethanol production. The analysis started with the full quadratic model containing all first order, second order terms and interactions of factors with each other. The results of ANOVA indicated that, the model was highly reliable (R^2 = 94.18 ; $p < 0.05$) with significant linear and quadratic effects ($p < 0.05$) but insignificant interaction effects (Table 3.7). The insignificant lack of fit (p=0.411) proved that the model fitted well to the experimental data.

These findings justified the theoretical knowledge that if the time of fermentation is taken too long, the conversion of ethanol to other chemical compounds might occur. Also the inoculum level is very delicate parameter to be determined.

If too much biomass were introduced, the fermentation wouldn't successfully proceed due to the lack of substrate and other nutrients for high yeast population as well. If too little amount of biomass is put, the biomass will mainly try to survive and increase in population thus the glucose will mainly be used for growth.

Based on the constructed model (Table 3.7 and Equation 3.1) the individual effects of solid load, inoculum level and fermentation time can be seen in Figures 3.7-9 respectively. The ethanol concentration linearly increased (Figure 3.7) from 11.98 g/L to 31.62 g/L when solid load increased from 7.5% (w/w) to 20% (w/w) having all other factors held constant at their mid-values (inoculum level 10% (v/v) and fermentation time 72 h). This is a general and well known behavior that ethanol production increases with the increasing glucose content.

In our study the kitchen waste contained about 30-40% (w/w) total dry matter and this amount decreased to 20-30% (w/w) due to dilution and removal of pulp during preparation, enzymatic hydrolysis and centrifugation. Thus, the primary solid load of fermentation mixture was 33.78 % (w/w) which was equivalent to 135 g/L of glucose. The glucose concentrations at 10, 15 and 20% (w/w) solid loads were determined approximately as 40, 60 and 80 g/L, which resulted in a working range of 4-8% (w/v) glucose. This range does not comprise a critical glucose concentration where substrate inhibition can occur. Therefore to improve ethanol production, solid load can be increased within the range of allowable maximum glucose concentrations, because glucose concentrations above 200 g/L result in a decrease in expected ethanol concentration due to product inhibition (Nquyen et al., 2009).

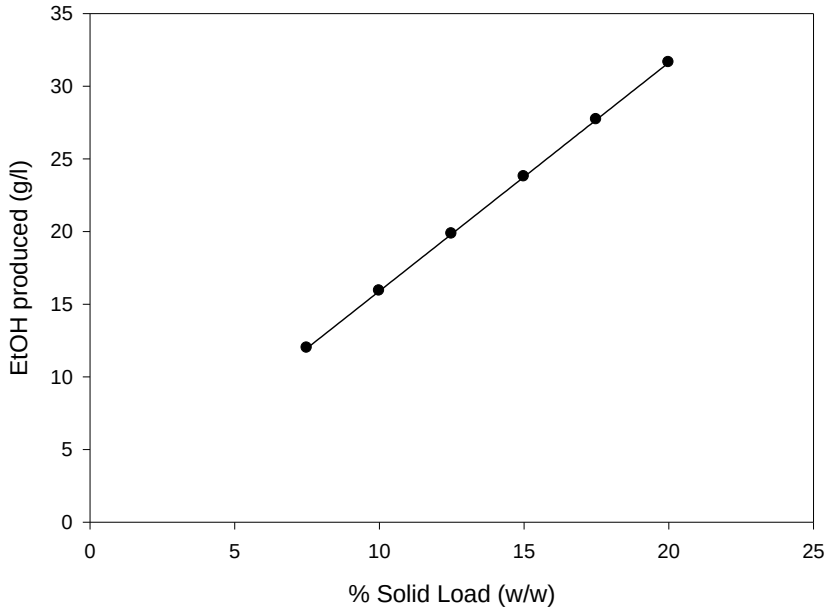


Figure 3.7 Effect of solid load on ethanol fermentation

In Figure 3.8, the ethanol concentration showed a non-linear effect with the increase of inoculum level from 5% to 15% (v/v) under the conditions of 15% (w/w) solid load and 72 h of fermentation, due to significant quadratic term at $p < 0.05$ (Table 3.7). The ethanol concentration reached a peak value of 23.76 g/L at the mid-point of inoculum level (10% (v/v) meaning 1g/L biomass concentration). Powchinda et al. (1999) stated that up to a critical amount the increase in inoculum size increased ethanol yield due to better utilization of the sugars. However, high amount of inoculum adversely affected ethanol production due to the fact that high increase in inoculum level decreased the viability of yeast population from 93 to 85% and caused inadequate development of biomass and ethanol production (Powchinda et al., 1999).

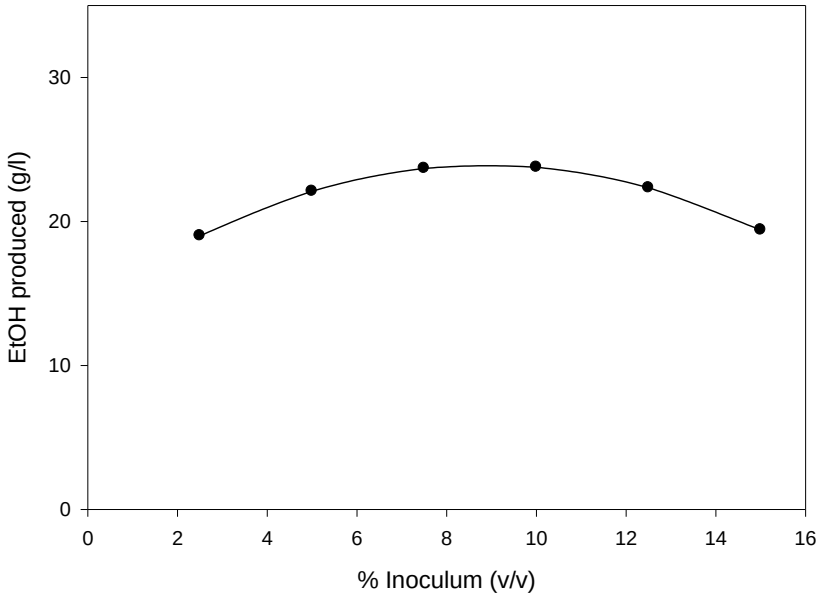


Figure 3.8 Effect of inoculum level on ethanol fermentation

Similarly, time had a nonlinear effect on ethanol production (Figure 3.9). As the fermentation time was increased to 60 h, ethanol concentration increased to 24.15 g/L. However, as the time was prolonged from 60 h to 96 h, the ethanol concentration slightly decreased. It was speculated that either insufficient glucose left in the fermentation flask or very slight increase in the acidity of the medium could have caused this change.

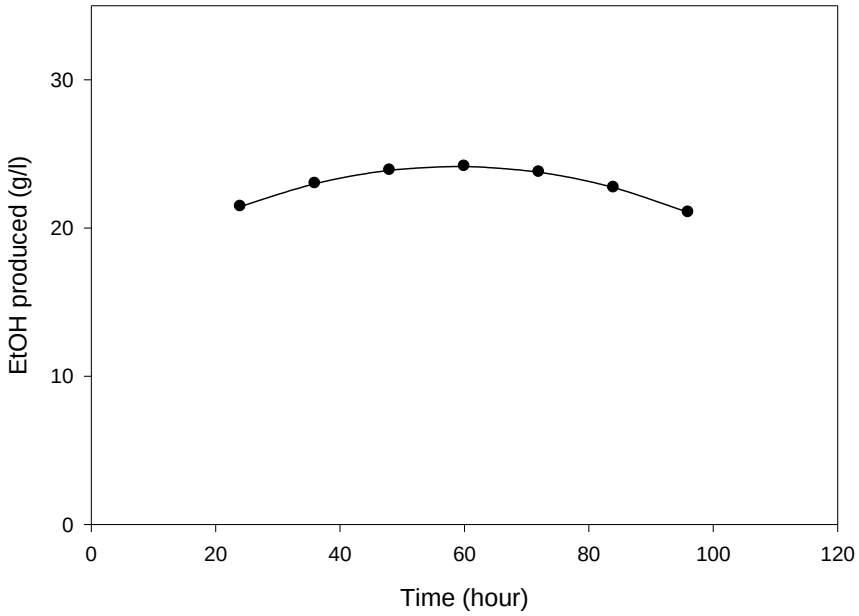


Figure 3.9 Effect of fermentation time on ethanol fermentation

The effect of interaction between solid load and inoculum level can be seen from surface plot in Figure 3.10. It can be seen that at different solid loads ethanol concentration increased with increasing inoculum level up to 10% (v/v). Ethanol concentration gradually increased with increasing solid load, independent of inoculum level.

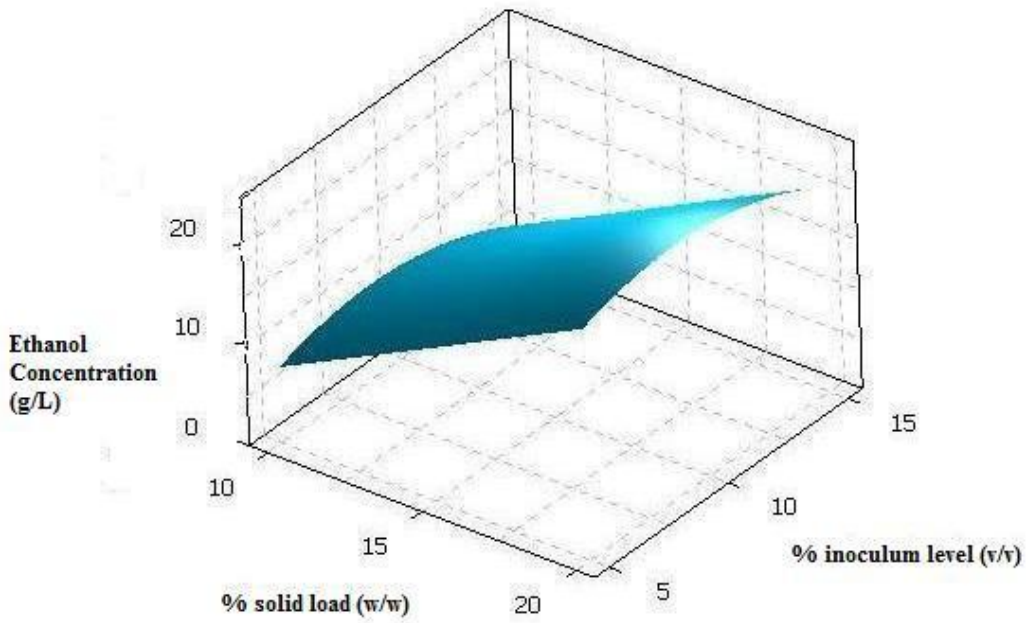


Figure 3.10 Surface plot of ethanol concentration vs % solid load ; % inoculum level (fermentation time was held at its mid-value of 72 h)

Similarly, the interaction effect of solid load and fermentation time is given in Figure 3.11. Ethanol concentration increased with increasing fermentation time but showed a slight decrease with increasing fermentation time from 72 to 96 h, independent from solid load.

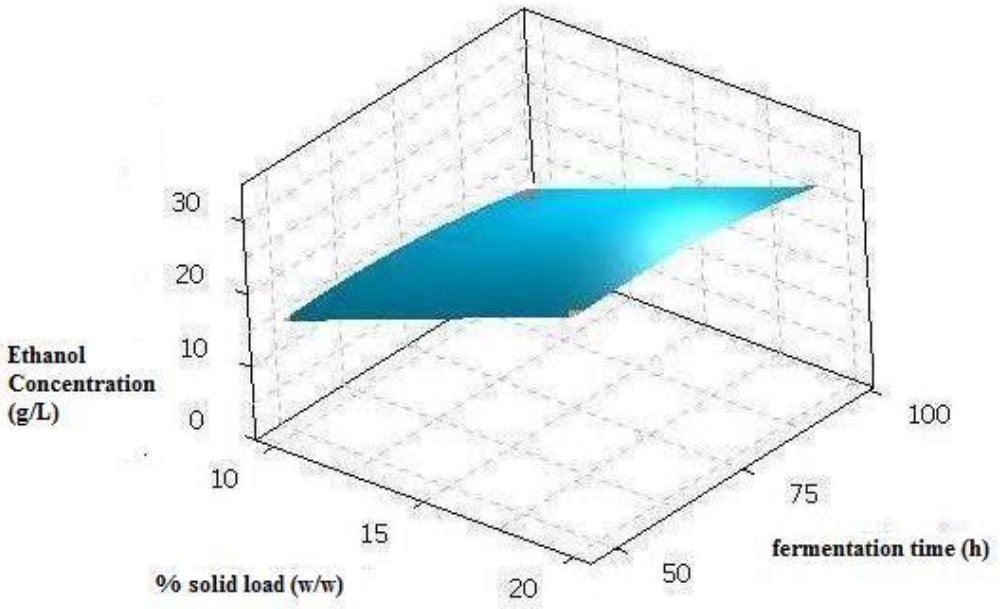


Figure 3.11 Surface plot of ethanol concentration vs % solid load; fermentation time (inoculum level was held at its mid-value of 10% (v/v))

The results of interaction effect of inoculum level and fermentation time is given in Figure 3.12. It can be seen that up to the mid-values of inoculum level and fermentation time, ethanol concentrations increased, but then showed a slight decrease with higher values of inoculum level and fermentation time.

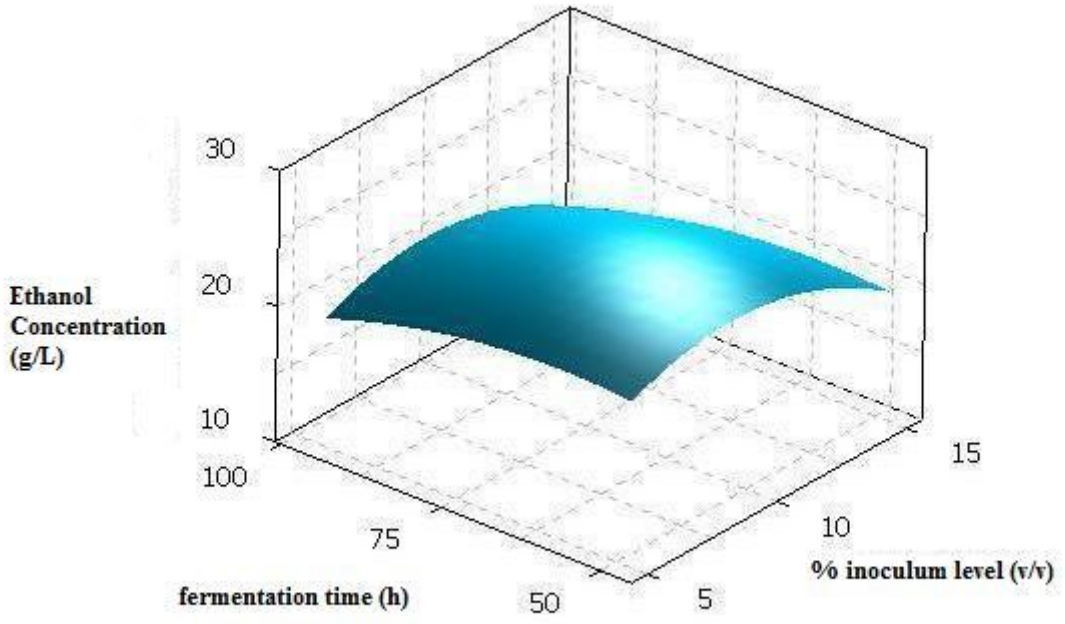


Figure 3.12 Surface plot of ethanol concentration vs fermentation time: % inoculum level (solid load was held at its mid-value of 15% (w/w))

Contour plots (Figures 3.13-15) of ethanol concentration generated using the model for various conditions indicated a comprehensive picture of the model. Ethanol concentrations at > 30 g/L could be seen on each plot at different combinations of solid load-inoculum level and solid load-fermentation time except Figure 3.15.

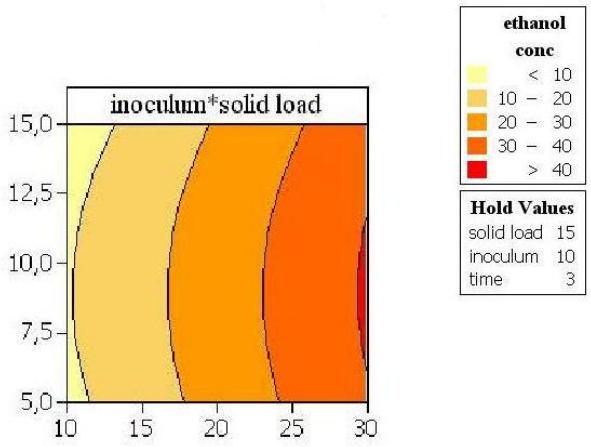


Figure 3.13 Contour plot of ethanol concentration vs % solid load; % inoculum level

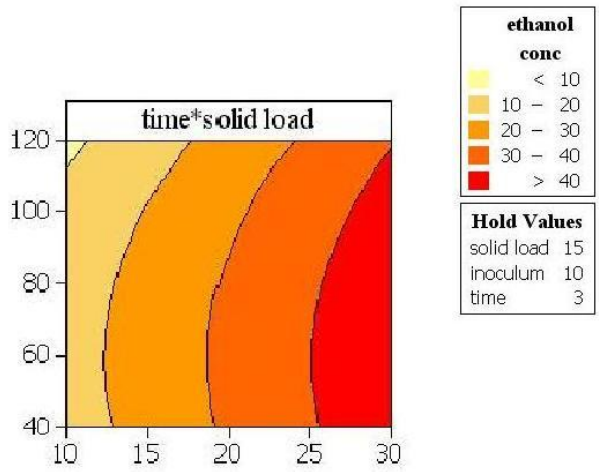


Figure 3.14 Contour plot of ethanol concentration vs % solid load; fermentation time

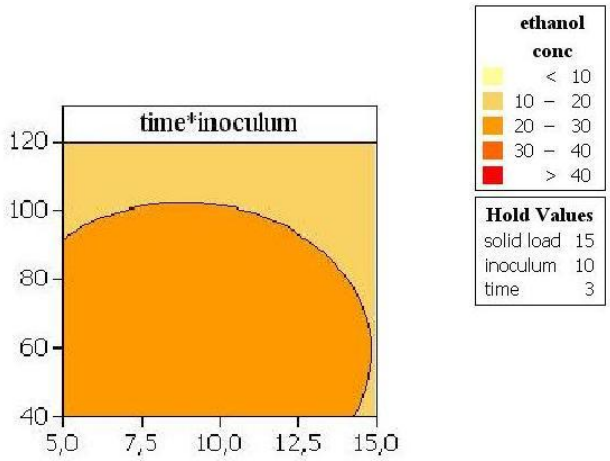


Figure 3.15 Contour plot of ethanol concentration vs % inoculum level; fermentation time

The optimum level of each factor was determined using the response optimizer tool in MINITAB® 15.1 (Minitab Inc. State College, PA, USA), to yield high concentration of ethanol. The optimum conditions were found as 20% solid load (with 80 g/L initial glucose concentration), 8.85% (v/v) yeast inoculated and 58.8 h of fermentation which yielded 32.16 g/L of ethanol. The yield of ethanol was determined as 0.16g ethanol / g dry matter or 0.40 g ethanol / g glucose according to the predicted model. Thus, 78.7% of theoretical yield was achieved. This result is slightly better than the study of Wang et al. (2008) who produced ethanol from kitchen garbage at 77% of the theoretical yield.

3.6.2 Verification of the RSM model

The constructed model was verified by additional 8 runs under different combinations of solid load, inoculum level and fermentation time (Appendix K). The results of verification experiments are presented in Figure 3.16 in terms of predicted versus

experimental ethanol concentrations. A high value of coefficient of determination (R^2=90.71) showed that the model was successful in predicting ethanol concentration.

The constructed model was also assessed using error analysis. Root mean square error (RMSE) and mean absolute error (MAE) values were calculated first and later RMSE was fractionated into systematical and unsystematical error values to determine the source of error. It was found that error was systematic ($RMSE_s$= 3.53 > $RMSE_u$=2.35), of which experimental values were slightly higher than the predicted values.

It should be noted that systematic error is not a structural weakness of the model but due to inputs such as composition of each batch of kitchen waste. In addition, glucose concentrations on different batches of kitchen waste with the same solid loads can still be different due to the varying composition of the generated wastes. This affects the final ethanol concentration but the relations with the independent factors investigated in this study and their effects on ethanol will not be affected. Therefore, it can be concluded that the verified model can be used to predict successfully the ethanol production at various combinations of solid load, inoculum level, and fermentation time.

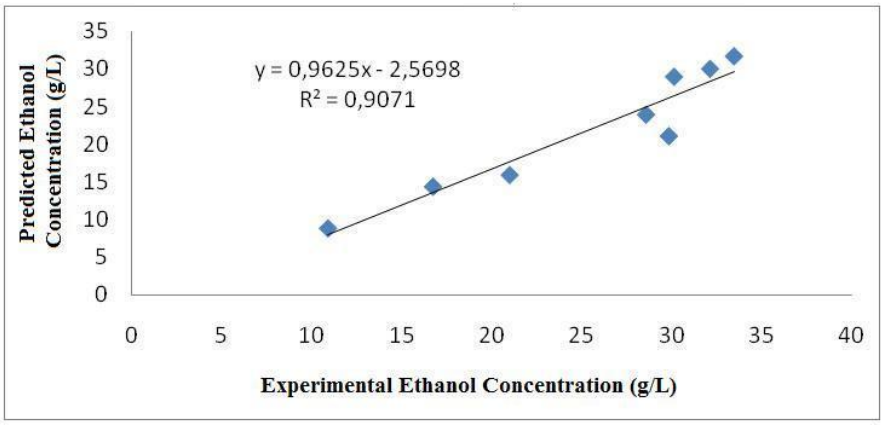


Figure 3.16 Verification of the model obtained by RSM

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

Results from the presented experimental work indicated that kitchen waste collected in mixed form have a remarkable potential of use without the need of separation according to carbohydrate fractions to produce ethanol. No pretreatment prior to enzymatic hydrolysis is needed for production of high glucose levels. Addition of fermentation medium is found to be not necessary for yeast to produce ethanol, where kitchen wastes are used as a substrate. The nutrients present in the original kitchen waste provide enough nutritive medium for *Saccharomyces cerevisiae* to produce high yield of ethanol. Thus, it is concluded that ethanol production costs could be lowered using kitchen wastes as substrate and by excluding the fermentation medium from traditional fermentation practice.

In the first part of the study, analytical methods are applied for determination of kitchen waste composition. The kitchen wastes collected had a variable range of moisture content and total dry matter. But on the average the kitchen waste has 60-70% initial moisture. By this information, it is determined that the least dry matter content of the waste is found to be 30% which is justifying the usage of kitchen waste as a raw material for bioethanol production.

The pretreatment methods investigated in this study were: dilute acid hydrolysis and hot water treatment both followed by enzymatic hydrolysis for complete conversion to glucose.

The method with the highest reducing sugar yield was enzymatic hydrolysis methods without any pretreatment. By this method the reducing sugar content was measured as 64.7 g/L before fermentation.

Another consideration in this study was the addition of fermentation medium to the fermentation mixture. The yeast used in this study was commercial instant baker's yeast (*Saccharomyces cerevisiae*) sold in supermarkets. In theory yeast needs extra organic compounds and minerals to first grow then start producing ethanol. Since the kitchen waste is a combined source of nutrients of proteins, sugars and fats besides trace amount of elements and minerals, it is found that, without addition of fermentation medium the yeast functions more efficiently.

In the present study, a RSM based Box-Behnken Design established model was also evaluated to predict ethanol production. The verification of runs showed that the constructed model can be used to predict ethanol concentration at various combinations of solid load, inoculum level and fermentation time. A maximum ethanol concentration of 32.16g/L with the optimum conditions of 20% solid load, 8.85% inoculum level and 58.8 hours of fermentation is suggested by the model. These conditions reveal a yield of 0.16 g ethanol / g dry matter (or 0.40 g ethanol / g glucose). This result is 78.7% of the theoretical yield.

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APPENDIX A

Table A.1 Batches of Kitchen Waste used in pretreatment experiments

Table A.1.1 First batch of kitchen waste

Constituent	Content (% w/w)
Moisture	68.6
Total Solids	31.4
Protein	4.4
Fat	8.8
Ash	0.9
Total CHO's	17.2

Table A.1.2 Second batch of kitchen waste

Constituent	Content (% w/w)
Moisture	60.3
Total Solids	39.7
Protein	4.7
Fat	8.7
Ash	2.7
Total CHO's	23.7

Table A.2 Batch of kitchen waste used in biomass / glucose and ethanol trends determination

Constituent	Content (% w/w)
Moisture	63.4
Total Solids	36.6
Protein	4.1
Fat	5.0
Ash	1.0
Total CHO's	26.6

Table A.3 Batch of kitchen waste used in RSM experiments

Constituent	Content (% w/w)
Moisture	61.3
Total Solids	38.7
Protein	4.4
Fat	6.4
Ash	1.2
Total CHO's	26.7

APPENDIX B

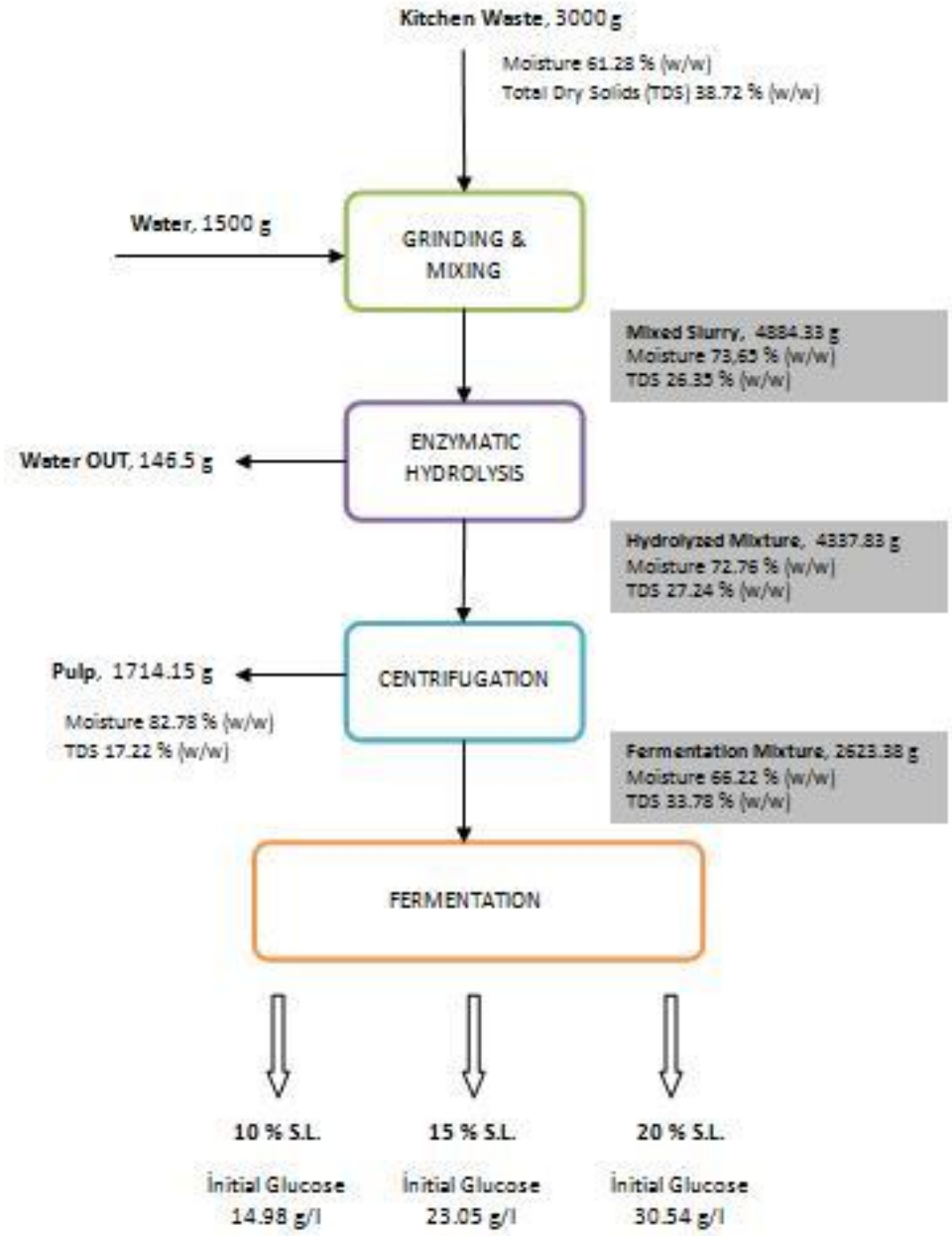

Figure B.1 Flowchart of ethanol production and related mass balances

APPENDIX C

Table C.1 Glucose concentrations during enzymatic hydrolysis

		Glucose Concentration (g/L)						
Treatment Type	**Time (h)**	**t=0**	**t=1**	**t=2**	**t=3**	**t=4**	**t=5**	**t=6**
Hot Water	Rep #1	21.69	29.69	45.37	50.66	50.82	51.14	54.34
	Rep #2	23.61	31.29	46.33	56.10	56.90	61.70	59.14
1% H_2SO_4	Rep #1	10.97	19.93	24.25	36.89	47.93	50.02	50.66
	Rep #2	15.13	20.89	26.81	42.65	48.90	51.78	52.26
4% H_2SO_4	Rep #1	11.45	19.13	24.41	26.33	43.29	43.61	46.33
	Rep #2	14.33	20.09	27.61	49.69	43.61	46.49	44.41
No pretreatment	Rep #1	24.25	43.45	51.14	52.90	62.50	62.82	63.46
	Rep #2	25.21	44.41	60.42	59.94	64.10	64.10	66.02

APPENDIX D

Table D.1 Fermentation medium effect on ethanol concentrations

	Ethanol Concentration (g/L)	
Treatment Type	**Replication #1**	**Replication #2**
Hot Water + EH[a] + FM[b]	12.99	16.24
Hot Water + EH[a] + NM[c]	15.58	18.69
EH[a] + FM[b]	16.44	18.3
EH[a] + NM[c]	22.93	23.73

[a]EH: enzymatic hydrolysis [b]FM: fermentation medium added [c]NM: without fermentation medium addition

APPENDIX E

Table E.1 *Saccharomyces cerevisiae* concentrations in waste fermentation

S.cerevisiae Concentration (g/L)						
	10% Solid Load		15% Solid Load		20% Solid Load	
Time (hour)	Rep#1	Rep#2	Rep#1	Rep#2	Rep#1	Rep#2
0	0.929	0.929	0.929	0.929	0.929	0.929
4	2.131	2.857	2.750	2.488	1.048	2.769
8	3.483	3.791	2.949	2.949	3.764	3.410
12	4.275	4.336	3.910	3.199	4.236	3.933
16	4.275	4.336	3.983	3.276	4.294	4.087
20	4.336	4.160	3.837	3.199	2.784	3.933
24	4.052	4.217	3.983	3.276	4.317	4.179
28	4.217	4.398	4.160	3.460	4.398	4.087
32	4.217	4.336	4.067	3.460	4.398	4.179
36	4.217	4.275	4.236	3.211	4.317	3.779
40	4.336	4.398	4.160	3.287	4.578	4.275
44	4.160	4.398	4.236	3.376	4.398	4.087
48	4.336	4.275	4.160	3.460	4.317	4.179
52	4.336	4.275	4.160	3.545	4.486	3.998
56	4.275	4.336	4.236	3.460	4.317	3.933
60	4.395	4.509	4.236	3.545	4.486	3.933
64	4.275	4.275	4.067	3.545	4.486	3.998
68	4.555	4.578	4.067	3.545	4.486	3.779
72	4.421	4.444	4.140	3.545	4.578	3.837
76	3.998	4.509	4.532	3.649	4.463	3.837
80	4.486	4.378	4.236	3.222	4.651	4.256
84	4.398	4.378	4.236	3.545	4.463	3.837
88	4.628	4.336	4.532	3.299	4651	3.837
92	4.486	4.275	4.236	3.545	4.463	3.998
96	4.555	4.275	4.236	3.545	4.463	3.998

APPENDIX F

Table F.1 Glucose Concentrations of waste medium fermentation (replication 1)

Glucose Concentration (g/L) **REP #1**			
Time (hour)	**10% Solid Load**	**15% Solid Load**	**20% Solid Load**
0	51.300	74.672	105.811
12	18.481	22.625	23.783
24	15.121	20.833	22.439
36	14.897	19.302	21.580
48	13.366	18.780	21.132
60	13.291	18.145	21.020
72	12.619	17.996	20.796
84	12.395	15.905	18.145
96	10.678	14.897	15.905

Table F.2 Glucose concentrations of waste medium fermentation (replication 2)

Glucose Concentration (g/L) **REP #2**			
Time (hour)	**10% Solid Load**	**15% Solid Load**	**20% Solid Load**
0	51.300	78.667	105.811
12	19.863	25.612	25.538
24	15.718	23.409	24.156
36	14.598	22.849	22.289
48	14.001	22.289	19.452
60	13.366	22.028	19.190
72	12.545	19.937	17.585
84	10.827	17.174	17.585
96	10.678	15.718	15.718

APPENDIX G

Table G.1 Ethanol concentrations of waste medium fermentation (replication 1)

Ethanol Concentration (g/L) **REP #1**			
Time (hour)	**10% Solid Load**	**15% Solid Load**	**20% Solid Load**
0	0	0	0
12	5.74	23.18	34.41
24	13.09	26.14	37.18
36	14.93	30.84	44.00
48	17.27	28.56	37.52
60	19.73	30.70	39.70
72	20.99	32.07	40.45
84	15.24	28.01	33.91
96	20.45	29.83	32.94

Table G.2 Ethanol concentrations of waste medium fermentation (replication 2)

Ethanol Concentration (g/L) **REP #2**			
Time (hour)	**10% Solid Load**	**15% Solid Load**	**20% Solid Load**
0	0	0	0
12	12.47	16.51	32.98
24	15.26	16.96	38.85
36	17.40	18.84	38.40
48	16.12	23.63	35.96
60	15.11	24.56	40.71
72	16.83	23.68	32.01
84	13.38	20.11	33.11
96	9.85	16.06	34.12

APPENDIX H

Table H.1 Regression results of ANOVA

Estimated Regression Coefficients for ethanol conc (g/l)

Term	**Coef**	**SE Coef**	**T**	**P**
Constant	23,763	0,5783	41,088	0,000
solid load	7,861	0,4256	18,468	0,000
inoculum	-1,341	0,4256	-3,150	0,004
ferm.time	-1,421	0,4256	-3,339	0,003
inoculum*inoculum	-3,011	0,6247	-4,820	0,000
ferm.time*ferm.time	-1,290	0,6247	-2,065	0,050

S = 1,70258 PRESS = 106,172
R-Sq = 94,18% R-Sq(pred) = 91,12% R-Sq(adj) = 92,97%

Analysis of Variance for ethanol conc (g/l)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	1125,68	1125,68	225,135	77,67	0,000
Linear	3	1049,71	1049,71	349,902	120,71	0,000
Square	2	75,97	75,97	37,985	13,10	0,000
Residual Error	24	69,57	69,57	2,899		
Lack-of-Fit	7	21,58	21,58	3,083	1,09	0,411
Pure Error	17	47,99	47,99	2,823		
Total	29	1195,25				

APPENDIX I

Table I.1 Ethanol concentrations of experimental and predicted values.

Independent variables			**EtOH Conc.(g/L)**	
Solid load	**Inoculum**	**Time**	**Experimental**	**Predicted**
10	10	3	20.99	15.90
10	5	2	16.73	14.36
10	15	4	10.9	8.84
15	10	2	28.56	23.89
15	10	4	29.83	21.05
20	5	3	32.12	29.95
20	10	3	33.45	31.62
20	10	4	30.12	28.91