The Direct Production of Glucose from Glycogen under Microwave, Sonochemical and Hydrothemal heating using Different Catalysts

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Ph.D. Thesis

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This work was carried out under the supervision of

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This dissertation is dedicated to my family who always wanted me to go in the way of knowledge.
Thank you for the friendship, tremendous love and the support you have given me, and for always believing with me.

Bar-Ilan University

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DEGREE AWARDED: Doctor of Philosophy in Chemistry

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TITLE OF THESIS: Development of Direct Production of Glucose from Glycogen under Microwave, Sonochemical and Hydrothemal heating using Different Catalyst Assisted.

This is to certify that the thesis entitled **"The Direct Production of Glucose from Glycogen under Microwave, Sonochemical and Hydrothemal heating using Different Catalysts"** submitted by Miri Klein to Bar-Ilan University, Israel for the award of the degree of Doctor of Philosophy is a bona fide record of research work carried out by her under my supervision. The content of this thesis, in full or in part, has not been submitted to any other research organization for the award of a degree or a diploma.

Signature of supervisor

Prof. Aharon Gedanken

Date: April 2015

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Abbreviations

heteropoly acids (HPA's)

$HPW_{12}O_{40}\cdot nH_2O$ (HPW) and $H_4SiW_{12}O_{45}\cdot nH_2O$ (HSiW)

DLS - dynamic light scattering

FTIR - fourier transform infra-red

h- hour

min- minute

^1H NMR Spectroscopy - proton nuclear magnetic resonance spectroscopy

HRSEM - high-resolution scanning electron microscope

MW - microwave

NPs- nanoparticles

SEM - scanning electron microscope

Sono - sonication

TGA - thermogravimetric analysis

XRD - X-ray diffraction

wt- weight

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Abstract

The demand for alternate transportation fuels is continuously increasing due to the limited and diminishing supply of fossil-based fuels. Biomass is an attractive alternative feedstock for the production of biofuels. This study focused on examining the possibility of producing glucose from glycogen. Glycogen is an appropriate, more viable and advantageous feedstock for glucose production than lignocellulosic biomass, as it needs no additional pretreatment. The current research presents the development of a new, optimized, and qualitative method for glucose production from glycogen in order to provide a solution for fossil fuels industry from the economic and ecological point of view. The presented methods are based on novel techniques like microwave irradiation, sonochemistry and hydrothermal treatment as a driving force for the biomass hydrolysis reaction using acidic catalysts. This combination of the special instrumentation and the various catalysts has demonstrated excellent catalytic activity.

In the first stage, microwave-assisted technique was applied, for the first time, to the acidic hydrolysis of glycogen. The optimal conditions for the hydrolysis reaction (yield of glucose – 62 wt %) were identified: microwave irradiation time – 10 min and concentration of acid – 1 M HCl. Microwave irradiation has dramatically reduced the reaction time from more than 6 h (at 80 °C under an oil bath) to 10 min.

However, the use of HCl at industrial scale production of glucose could be corrosive. Acid concentration and recycling will also add to the process cost, making the process economically unfeasible. The aim of the second part of this work is to identify a reusable and non-corrosive catalyst for the bulk production of glucose. For this propose heteropoly acids (HPA's) are used. HPA’s are known as effective homogeneous and heterogeneous solid acid catalysts with industrial applications. Unique features of heteropoly acids include: stronger acidity compared to many mineral acids i.e. phosphoric acid, non-corrosive, easy to separate, reusable, and greener than conventional acid catalysts. Complete conversion of glycogen to glucose is achieved by using $HPW_{12}O_{40}\cdot nH_2O$ (HPW) and $H_4SiW_{12}O_{45}\cdot nH_2O$ (HSiW) as catalysts for the hydrolysis under optimized hydrothermal conditions (2.4 % mass fraction of catalyst, 100 °C and 2 h reaction time). The reusability of the catalyst (HPW) was demonstrated. In addition to

carrying out the glycogen hydrolysis in an autoclave, it was done also under microwave irradiation at higher mass fraction of the heteropoly acids (10.5 %). Under these conditions, glycogen could be completely converted to glucose. Sonication of an aqueous solution of glycogen in the presence of HPW and HSiW also yielded glucose. Thus, heteropoly acids are efficient, environmentally friendly and reusable catalysts for the conversion of glycogen to glucose.

However, as the heteropoly acid is water soluble, it's separation after the hydrolysis is time consuming. The third part of this work deals with the development of a supported heteropoly acid catalyst HSiW/Graphene to enable the separation process of the catalyst from the product easier. For this purpose, silicotungstic acid (HSiW) was deposited on graphene by an ultrasound-assisted procedure. The catalyst (HSiW/G) was characterized using a variety of physico-chemical methods. Homogeneous distribution of HSiW on the surface of graphene was demonstrated. The catalytic activity of HSiW/G was studied for the hydrolysis of glycogen by hydrothermal treatment. The yield of glucose (66 wt %) is obtained. This yield was about 8 times higher than that obtained with the same amount of bare HSiW. The stability of the HSiW/G even after 3 repeated reactions was confirmed, which is of great importance for its industrial application. We have attributed the enhancement of catalytic activity to the presence of special interaction between the graphene support and HSiW, and also to the appearance of hydrophobic cavities on the surface of graphene. The formation of these cavities facilitates the anchoring of glycogen to the catalyst surface and promotes the attack of protons that leads to selective, rapid, and efficient hydrolysis.

Furthermore, in addition to glycogen, we have investigated the feasibility of using *ficus religiosa* leaves for the production of bioethanol. Under the process conditions (8 min microwave irradiation, 1 M HCl), 10.1 wt % glucose yield could be obtained from the leaves. A microwave based hydrolysis process yielded higher glucose content compared to the conventional hydrothermal process (4.1 wt %). 3 wt % (dry wt. basis) of ethanol could be produced from the leaves of *ficus religiosa.*

To conclude, the potential of biomass, especially glycogen, as a feasible feedstock for the production of glucose is demonstrated. A variety of acid catalysts (HCl, HPA's and HSiW/G) and different methods for the hydrolysis of glycogen were evaluated to

produce glucose in a fast and environmentally friendly manner. Glucose is a precursor to a variety of fine chemicals as well as for bioethanol. Moreover, the developed methods can be used for the hydrolysis of cellulosic biomass such as cellulose or *ficus religiosa* leaves.

1. Introduction

1.1. Transition to renewable energy

Electric power plays a great role wherever people live. Energy is a key factor for the development and social prosperity of any country. Modern society's demand for energy is continuously increasing and became a prime concern. Due to industrial growth and extensive use of electrical gadgets, electricity consumption is increasing day by day. Energy is the most important issue of the 21st century. Currently, the world energy demand are mainly met by the fossil fuels[1], the supply of which is quite unstable and uncertain.

Conventional energy sources based on oil, coal, and natural gas have proven to be highly effective drivers of economic progress[2]. However, the present reserve of fossil fuel energy sources will be depleted in a few decades due to high demand and extravagant consumption. Petroleum, natural gas and coal are generally referred to as fossil fuels[3]. Global energy consumption has increased at a geometric average of 5.6 percent from 1973 onward. In terms of its growth, energy demand will rather increase rapidly by one-third from 2010 to 2035, where expectedly both India and China will need highest energy supply (from external sources) in the world, at a rate of around 50 percent during that period (Figure 1)[4]. Therefore, shifting to renewable energy can help reduce the greenhouse gas emissions. This limits future extreme weather and climate impacts, and ensures reliable, timely, and cost-efficient delivery of energy. Investing in renewable energy can have significant dividends for energy security [2,4].

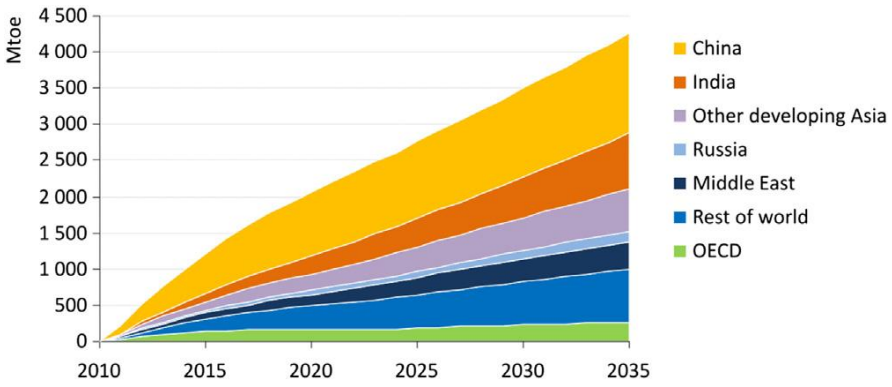

Figure 1: Growth of primary energy demand [4].

Renewable energies are energy sources that are continually replenished by nature and derived directly from the sun (such as thermal, photo-chemical, and photo-electric), indirectly from the sun (such as wind, hydropower, and photosynthetic energy stored in biomass), or from other natural movements and mechanisms of the environment (such as geothermal and tidal energy). Renewable energy does not include energy resources derived from fossil fuels, waste products from fossil sources, or waste products from inorganic sources[2]. Currently, around 18 percent of the global total energy consumption is based on renewable energy resources. Figure 2 demonstrate the distribution of the energy resources of the world [4].

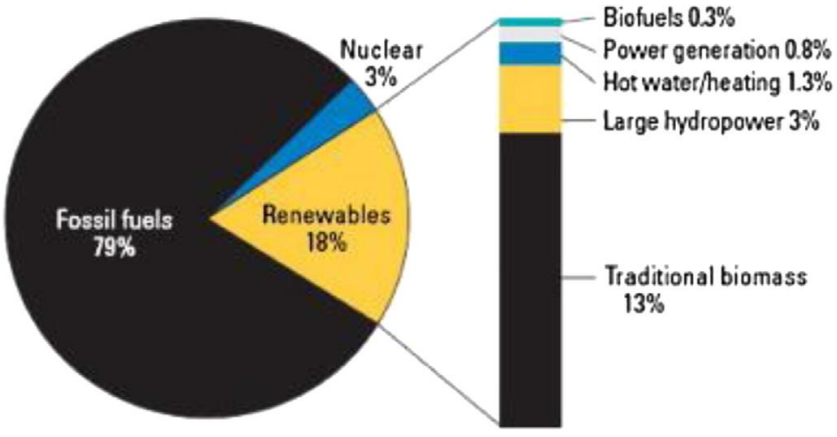

Figure 2: Energy resources of the world [4].

Renewable energy can provide a host of benefits to society, as shown in Figure 3. In addition to the reduction of carbon dioxide (CO_2) emissions, governments have enacted renewable energy policies to meet a number of objectives including the implementation of local environmental and health benefits, facilitation of energy access, particularly for rural areas, advancement of energy security goals by diversifying the portfolio of energy technologies and resources, and improving social and economic development through potential employment opportunities [2].

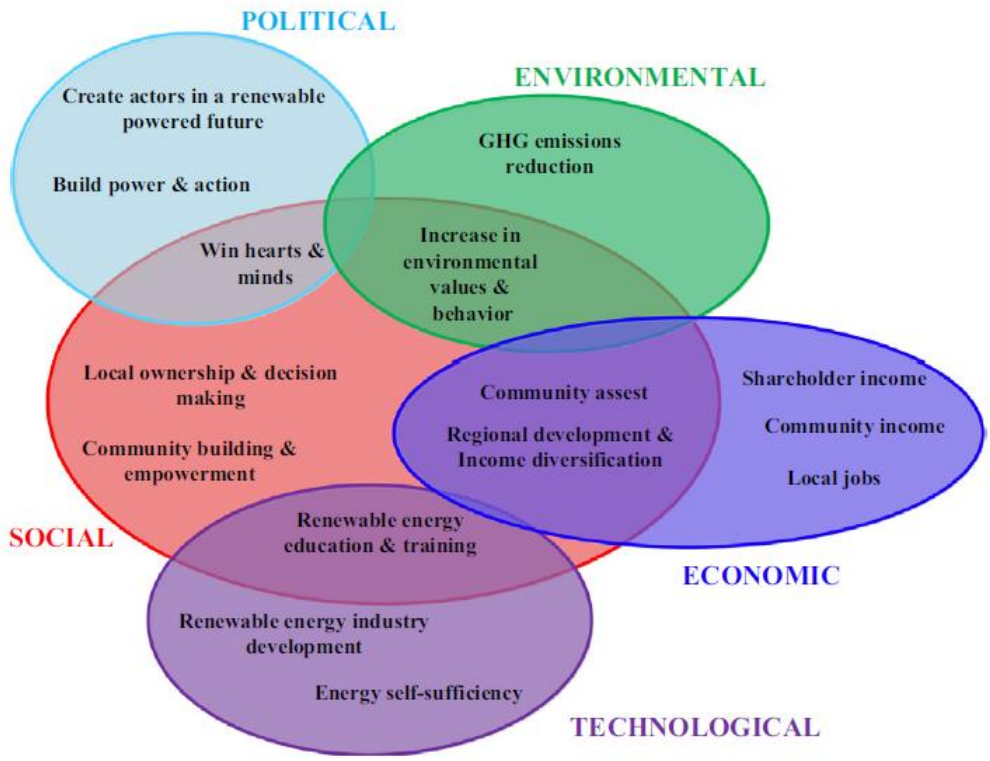

Figure 3: Global benefits of renewable energies production

For now European Union is generating 71 percent of its electricity from renewable energy sources. Developing countries can also focus on the use of solar, wind, biomass, and hydropower by taking advantage of favorable geographical locations. But there is still lack of adequate feasibility studies [5].

1.2. Biofuels as alternate energy sources

Biofuels refer to the energy content in solid, liquid and gaseous products derived from biological raw materials (biomass). Biofuels for transportion represent the major fraction of bioenergy production worldwide [6]. Developing biofuels has many advantages, such as efficient utilization of renewable resources, enhanced energy security and energy supply diversification, enhanced rural agriculture development and investment in rural areas, reduced greenhouse gas emissions, and increased jobs and improved livelihood. Global production of biofuels has been growing steadily from about 20 billion liters (125 million barrels) in 2001 to over 110 billion liters (692.5 million barrels) in 2011. The global biofuel production is projected to reach 222 billion liters by 2021, with ethanol and biodiesel share of 81 % and 19 %, respectively [7].

Sugar, starch and oil crops are currently the main feedstocks for (liquid) biofuels. Other feedstocks that are expected to become important for the production of biofuels are dedicated energy crops, including short rotation crops (SRC, e.g. willow, poplar, eucalyptus) and perennials (switch grass, miscanthus), and lignocellulosic wastes and agricultural and forestry residues. In addition, organic residues like manure are applied for the production of gaseous biofuel [8]. Biofuels are primarily produced from food crops with high content of sugar and starch, such as corn and sugarcane to produce ethanol, and oil seeds to produce biodiesel. These first generation technologies have been the first significant step of transition away from the traditional fossil fuels. It has then moved forward to the next generations of biofuels produced from non-food biomass, including residues of crops or forestry production (e.g. forest thinning, sawdust, etc.), dedicated energy crops (e.g. switch grass, poplar, and miscanthus), lignocellulosic fraction of municipal and industrial solid waste, and algal biomass [6,9].

Several developed countries have adopted mandatory biofuel policies and set biofuel targets to enhance energy security and contribute to climate change mitigation and rural agricultural development. Mandates and incentives for blending biofuels with fossil fuels contribute significantly to the ongoing growth in biofuel production and use [7]. Important objectives of biofuel supporting policies among others are, reducing the level of dependence on fossil oil imports, increasing energy security, increasing resilience against fossil oil price fluctuations, and reduction of greenhouse gas emissions. The main drawback of producing biofuels is that there are extensive agricultural areas that were

once used to grow food for sustenance, which are now aimed at producing fuel. This process causes a significant increase of food prices around the world [10].

1.3. Bioethanol as substitute to gasoline

Bioethanol and biodiesel are the two most promising biofuel products used as replacement fuel in transportation[11]. The bioethanol, unlike fossil fuels, as clean, renewable and environment friendly fuel has gained more attention. Among the advantageous properties of bioethanol as fuel energy, higher octane number (108), evaporation enthalpy, and flame speed and wider range of flammability are worth mentioning [12]. Burning ethanol instead of gasoline reduces global warming emissions by 20% from corn ethanol and 85% from cellulosic ethanol while entirely eliminating the release of acid rain-causing sulfur dioxide. However, bioethanol production involves many processes such as pretreatment, fermentation, recovery and there fining process[13].

1.3.1. Bioethanol production stages:

1.3.1.1. Hydrolysis

The conversion efficiency of biomass to ethanol depends on the extent of carbohydrate saccharification as yeast cells cannot ferment starch or cellulose directly into bioethanol [21]. A mixture of α-amylase, β-amylase, and glucoamylase of various origins is more effective for substrate with higher molecular weight [22]. Small fermentable sugars (e.g. maltose, amylose, glucose, maltose syrups, and fructose) can be produced in hydrolysis process. With the hydrolysis process, the glycosides bonds are broken by the absorption of water molecules and simple sugar is obtained as shown on Figure 4.

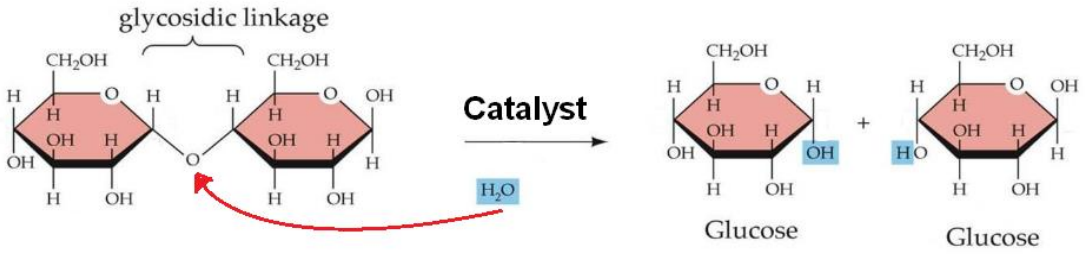

Figure 4: Schematic representation of hydrolysis process by the absorption of water molecules.

Numerous studies were done on the carbohydrate hydrolysis process, some enzymatic and some acidic hydrolysis [23]. Jianguo Liu *et al.* demonstrated bioconversion of cellulose to glucose. The saccharification was carried out with freely suspended cellulase from Aspergillus niger as the biocatalyst. The influence of solution pH, temperature, NaCl concentration, presence of cellobiase, cellulose-toenzyme ratio and stirring speed on reducing sugar production was examined. The results showed that the addition of an appropriate amount of NaCl or cellobiase had a positive effect on reducing sugar formation. The operating conditions identified to of achieve the maximum conversion rate of cellulose in the enzymatic membrane bioreactor were temperature 55 °C, pH 7.0, 50 mM NaCl, 2.25 g /L cellobiase, cellulose to cellulase concentration ratio 4, and stirring speed 1,200 rpm [24]. Leonardo Tupi Caldas Pereira *et al.* investigated enzymatic hydrolysis of sugar cane bagasse. Hydrolysis experiments were carried out using a blend of cellulases, b-glucosidase, and xylanases. Hydrolysis were performed with an enzyme load of 10 FPU/g (dry weight) of bagasse over 36 h with periodic sampling for the measurement of the concentration of glucose and reducing sugars. The resulting sugar syrups contained 22 g/l of glucose, which corresponded to 45% cellulose conversion [25].

Qiang Jin *et al.* investigated the hemicellulose hydrolysis of corn stover in spray flow through reactor with dilute sulfuric acid concentrations (10-30 kg m^{-3}) at relatively low temperatures (90-100 °C). The results indicate that the rates of xylose formation and degradation are sensitive to flow rate, temperature and acid concentration. Over 90% of the xylose monomer yield and below 5.5% of degradation product (furfural) yield were observed in this reactor [26]. Stijn Van de Vyver *et al.* prepared sulfonated silica/carbon nanocomposites which were successfully developed as reusable, solid acid catalysts for the hydrolytic degradation of cellulose into high yields of glucose [27].

Acidic ionic liquids also used for the hydrolysis process. Feng Jiang *et al.* catalyzed cellulose hydrolysis over a variety of acidic ionic liquids. It is found that the hydrolysis activity is directly associated with the acidity of catalysts. They found that the acidic ionic liquids readily catalyze the cellulose hydrolysis in the solvent of [Bmim]Cl in the range of 80–120 °C, resulting in the dominant productions of glucose and 5-hydroxymethylfurfural (HMF) [28].

Microwave irradiation technique has been extensively exploited for accelerating biomass hydrolysis process leading to fast release of fermentable sugars from polysaccharides. Gedanken *et al.*, have exploited the potential of microwave irradiation for the conversion of a variety of renewable biopolymers like starch, cellulose, and glycogen to glucose in a fast acid catalyzed process using microwave irradiation [29,30].

The sugars obtained from hydrolysis process are then subjected to fermentation for bioethanol production.

1.3.1.2. Fermentation

Bioethanol is produced from sugar by fermentation process. The feedstock is added to the fermentation vessel along with microorganism, nutrients, and other ingredients at the beginning of fermentation followed by recovery of ethanol [12,31]. The selection of most suitable mode of fermentation mainly depends on the kinetics of the microorganisms used and the nature of feedstock.

Several factors, especially, temperature, pH, fermentation time, agitation rate, initial sugar concentration, and inoculum size, have an impact on the fermentation process as well as on the ethanol yield. It was also reported that ethanol production depends on fermentation temperature and to some extent its concentration increases with the increase in temperature [12,32]. However, high temperature is considered as a stress factor for microorganisms, which is unfavorable for their growth. They produce heatshock proteins in response to the high temperature and inactivate their ribosomes [12,32]. The initial sugar concentration is an important influencing parameter as it has the direct effect on fermentation rate and microbial cells. Generally, fermentation rate will be increased with the increase in sugar concentration up to a certain level. But excessively high sugar concentration will exceed the uptake capacity of the microbial cells leading to a steady rate of fermentation [12].

The immobilization of yeast or the fermenting organism for the bioethanol production has been greatly explored as a strategy to overcome the substrate and product inhibition and to improve the ethanol tolerance. Among this approach, the most explored are immobilization of yeasts in/on adequate matrices such as calcium alginate, k-carragenan gel, polyacrylamide- alumina, wooden chips, PVA gel, orange peel, etc. The

immobilization is often combined with the choice of an appropriate process mode, such as continuous or semi-continuous fermentation and enables easier biomass separation and recirculation or its repeated use [33].

1.3.2. Bioethanol classification

Bioethanol is commercially produced by fermenting monomer sugar [34]. The bioethanol can be produced from all kinds of renewable materials such a scorn, sorghum, cellulose and algae biomass. On the basis of the raw material used for its production, bioethanol is divided into first, second and third-generation bioethanol. The classification of bioethanol is shown in Figure 5 [13] .

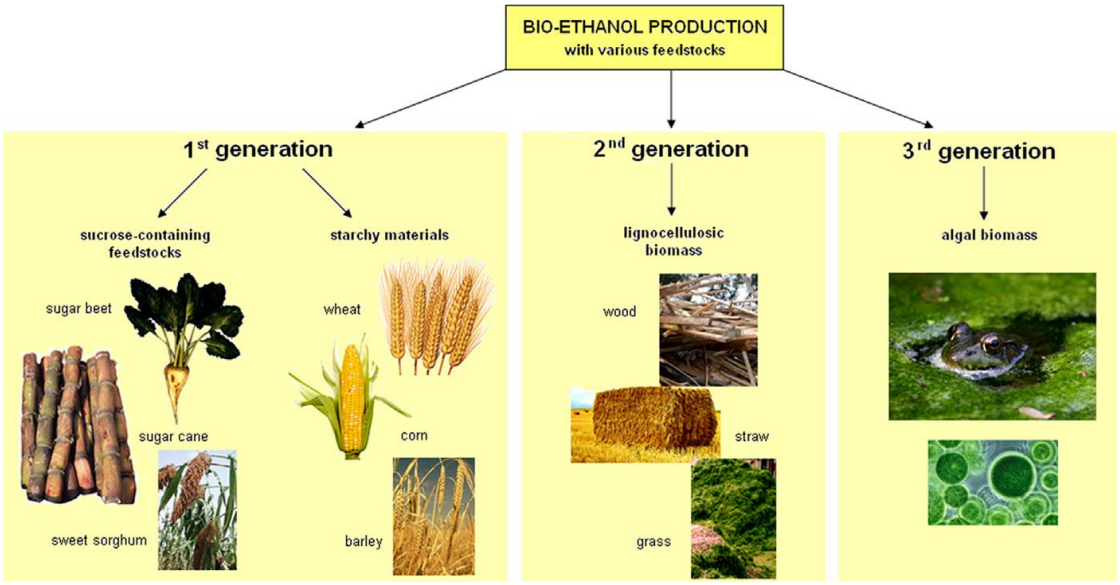

Figure 5: Classification of bioethanol [13]

1.3.2.1. First-generation bioethanol

The first-generation bioethanol production from food crops such as corn, grain, or sugar cane is well established and the industry is growing throughout the world [13].

In the US, the grain corn is the major source for ethanol production. Unlike the cane sugar which is extensively used for bioethanol production in Brazil [35,36], ethanol originating from sugar cane is less expensive as the soluble sugars could be directly extracted from the stalks [36]. Maize is another alternative energy crop, which is currently considered as a feedstock for bioethanol production in the North Central and Midwest United States [37].

However, the use of these staple food crops as feedstocks is not ideal because of the high price of raw materials, which accounts for almost 40–75% of total ethanol production cost [13].

1.3.2.2. Second-generation bioethanol

The second-generation bioethanol is derived from lignocellulosic feedstock. Among potential bioenergy substitutes, lignocellulosic biomass has been acknowledged as the primary source of biofuels and due to its abundance, lignocellulosic is the only imaginable viable source of fuels. Cellulosic and hemicellulosic polysaccharides are produced by plants at an annual production rate of 7.2×10^{10} and 6×10^{10} tons, respectively. Agricultural wastes and dry leafy plant biomass consisting of cellulose, hemicellulose and lignin are cheap and abundant feedstock for bioethanol production [38,39] . The second generation biofuel from these lignocellulosic feedstock including by-products (cereal straw, sugarcane bagasse, forest residues), wastes (organic components of municipal solid wastes), and dedicated feedstock (purposely-grown vegetative grasses, short rotation forests and other energy crops) would also need land in competition with food and fiber production. However, energy yields (in terms of GJ/ha) from these crops are likely to be higher than those of first-generation biofuels crops or their products [40].

The conversion of lignocelluloses to bioethanol is more challenging than the first-generation bioethanol process because of the complex structure of plant cell walls, Currently, no commercial-scale cellulosic ethanol plants are in operation largely because of the high price of production, which is almost twice that of corn ethanol [13,41] .

1.3.2.3. Third- generation biofuel

The search for alternative sources continued further to reduce the food cropland competition until using algae - as a sustainable and rich source of biofuel which is known as third generation biofuel [40].

Algae are photosynthetic, eukaryotic organisms that do not develop multicellular sex organs. All algae contain green chlorophyll; however, they are masked by photosynthetic pigments that give them a distinguishing color that is used to identify key divisions [42].

There are several advantages of algae bioethanol production that have combined to capture the interest of researchers and entrepreneurs around the world. These include the following: (a) Algae bioethanol production need not compete with food production in either land or water: both marine and freshwater algae may be used. Additionally, they also need not compete with people for food [43]. (b) The content of carbohydrate in the algae cell is abundant; the carbohydrates such as starches and sugars can be fermented to produce bioethanol [40]. (c) Algae have the advantages of having no lignin and low hemicellulose levels, which result in an increased hydrolysis efficiency and fermentation yields, thus they can reduce the cost of the bioethanol production [44]. (d) Algae have the ability to take up CO_2 from the atmosphere and power plants, and with the use of appropriate technology options, algae bioethanols can yield GHG reductions relative to fossil and other biobased fuels [45,46]. (e) Algae grow rapidly and can be easily grown in various aquatic environments such as fresh water, saline water, or municipal waste water [47]. (f) The microalgal cells have a very fast productivity and harvesting cycle (1–10 days) compared with other feedstock (harvest once or twice a year) and thus provide enough supplies to meet ethanol production demands [40,48].

Like cellulosic ethanol, bioethanol production from algae requires pretreatment, hydrolysis, fermentation, and distillation. In order to produce sugars from the algae biomass, pretreatment is designed to help separate cellulose, hemicellulose, and lignin so that the complex carbohydrate molecules in the algae cell can be broken down by enzyme-catalyzed hydrolysis into their constituent simple sugars. Then the fermentable sugars can be fermented into ethanol by ethanol-producing microorganisms and finally recover and purify the ethanol to meet fuel specifications [34].

1.3.3. Glycogen as a feedstock for bioethanol production

Just as sugars are stored in plants as cellulose and starch, glucose is stored in animal cells as glycogen [49]. The glycogen molecules are heavily hydrated, because they have many exposed hydroxyl groups available to hydrogen-bond with water (Figure 6). Glycogen is a polymer of (α1→4)-linked subunits of glucose, with (α1→6)-linked branches, but glycogen is more extensively branched (on average, every 8 to 12 residues) and more

compact than starch. Glycogen is especially abundant in the liver, where it may constitute as much as 7% of the wet weight, it is also present in skeletal muscle [50].

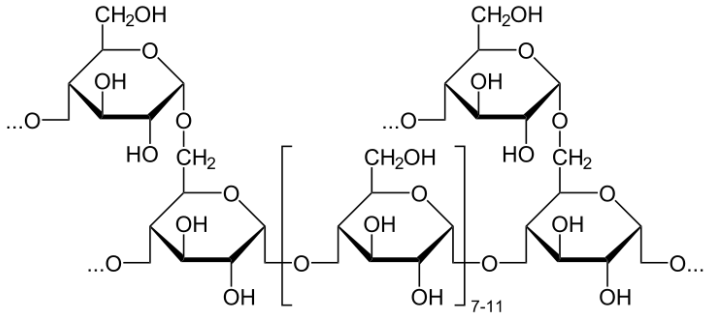

Figure 6: Chemical structure of glycogen, a rich and abundant source of glucose

Because each branch in glycogen ends with a nonreducing sugar unit, a glycogen molecule has as many nonreducing ends as it has branches, but only one reducing end. When glycogen is used as an energy source, glucose units are removed one at a time from the nonreducing ends. Degradative enzymes that act only at nonreducing ends can work simultaneously on the many branches, speeding the conversion of the polymer to monosaccharides.

Glycogen is an appropriate, more viable and advantageous feedstock for glucose production rather than lignocellulosic biomass, as it needs no additional pretreatment. The synthesis of glycogen from CO_2 by photosynthesis, and subsequently converted to glucose, has recently become a challenge that will make glycogen an abundant and renewable feedstock for glucose [39].

1.3.4. *Ficus religiosa* leaves as a feedstock for bioethanol production

Ficus religiosa (Fig. 7) is important traditional medicinal plant that is known worldwide. It is a fast-growing fuel wood tree of Indian forests, and distributed throughout India [52].

Figure 7: *Ficus religiosa* (A) *Ficus religiosa* tree (B) dark green leaf and, (C) leaf venation [54].

Ficus religiosa leaves that fall from the tree are generally a waste whose disposal is an issue from both environment and economic view points. Moreover, the leaves are a rich source of holocellulose. Till date, the feasibility of bioethanol production from these leaves has not been evaluated and has been one of the objectives of the study.

העתקת יפה את הקטעים אולם הסבירי לי בצורה אינטיליגנטית איך כל המלל קשור למחקרך? וכי את מאמינה שקורא אינטיליגנטי יאמין שאת כתבת את הקטע?

1.3.5. Current bioethanol production processes

Ethanol has been produced by anaerobic yeast fermentation of simple sugars since early recorded history. These fermentations used the natural yeast found on fruits and the sugars of these fruits to produce wines. Beer fermentations made use of the amylases of germinating grain to hydrolyze the grain starches to ferment sugars. Current practices utilize bacterial and fungal amylases to efficiently hydrolyze grain or tuber starch to glucose for fermentation to ethanol [57]. Ethanol can be produced by biologically catalyzed reactions. The carbohydrates are first broken down to simple glucose sugars by acids or enzymes (amylases), which can be then fermented to ethanol. In this stage, the hemicellulose fraction of biomass is broken down into various sugars, e.g. xylose and

glucose. Conventional organisms can not ferment many of the sugars derived from hemicellulose into ethanol with reasonable yields. However, recently new technologies capable of efficiently convert hemicelluloses into ethanol are under development [57].

For instance, using starch crops such as wheat for bioethanol production resulted in considerable high ethanol concentration in reduced fermentation time [58]. In that case, slurries containing 300 g/L of raw wheat flour were initially liquefied using 0.02 g α-amylase/g starch at 95 °C for 2 h, followed by saccharification using two different levels of amyloglucosidase activity and simultaneous fermentation by Saccharomyces cerevisiae at 35 °C for 21h, reaching a final ethanol concentration of 67 g/L. Wooley *et al.* obtained bioethanol yield of ca. 250 liter/ton by simultaneous saccharification and co-fermentation [59]. The feedstock was pretreated with cellulase at 30 ºC for 7 days [59].

Biological processing offers a number of advantages for converting biomass into biofuels. First, the enzymes used in bio-processing are typically capable of catalyzing only one reaction, and so formation of unwanted degradation products and by-products is avoided [60]. Additionally, material not targeted for conversion can pass through the process unchanged and be used for other applications [60].

1.3.6. State of the art production and utility of Bioethanol

As petroleum supplies 97% of the energy consumed for transportation. Therefore, industry and governments worldwide have been actively identifying, developing and commercializing technology for alternative transportation fuels over the past 20 years [61].

In the US most fuel ethanol is produced from corn by either dry-grind (67%) or wet-mill (33%) process. The continuous increase in bioethanol production in the US is depicted in Figure 8, reaching the world leader position in the year 2006 [57].

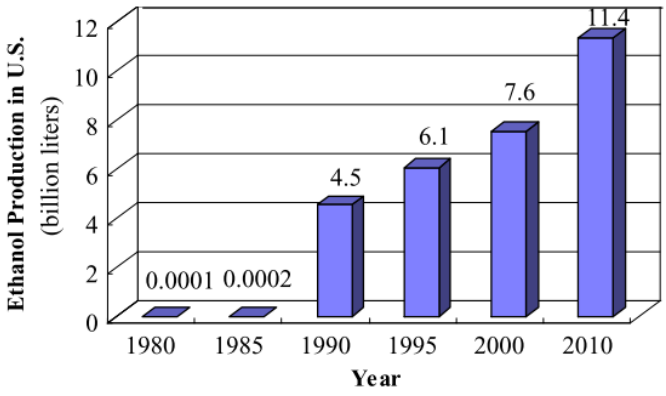

Figure 8: Ethanol production capacity in the U.S. [57]

Brazil also has shown an important potential for developing modern biomass energy carriers [62]. Brazil is the world's second largest producer with a peak production of almost 30 million m^3 in 2010 [62]. In addition, the bioethanol producing cost in the country (nearly US $0.20 per liter) is one of the lowest throughout the world [57].

In France, most of the bioethanol is produced from sugar beet. In Sweden, a fully integrated pilot plant for ethanol production from softwood, comprising both two stage dilute acid hydrolysis and the enzymatic process, was taken into operation in mid 2004. This plant has a maximum capacity of 2 ton (dry matter) wood per day [65]. In summary, for the technology for ethanol production to be sustainable the feedstock should be non-food-plant sources and the production should be demand based[61]. מזמן לא קראתי דוקטורט כל כך לא אינטיליגנטי

1.4. Catalytic hydrolysis of biomass for the production of fermentable sugars

1.4.1. Homogeneous and heterogeneous catalysts in biomass hydrolysis

The quest for sustainable and clean energy resources based on biomass degradation is becoming much more important [66]. Typically, hydrolysis reactions are very slow, it requires several days to attain the equilibrium in the absence of catalyst. Therefore, the reaction is catalyst driven. Mineral acids, such as H_2SO_4, HCl and HI, and strong organic acids, such as HCOOH, can be utilized as homogeneous catalysts [67,68]. The homogeneous catalysts typically have a single type of active site and they are water soluble [69]. The basic problem of homogeneously catalyzed processes is the separation of the product

phase from the catalyst, which is soluble in it. The process stages necessary to achieve this usually include thermal operations such as distillation, decomposition, transformation, and rectification, which normally lead to thermal stresses on the catalyst. These can cause decomposition reactions and progressive deactivation during the lifetime of the catalyst. Furthermore, thermal separation processes seldom give quantitative recovery of the catalyst, which causes loss of productivity through loss of metal [70]. Furthermore, at higher catalyst concentrations equipment corrosion can occur [67].

Recently, homogeneous catalysts are used in industry in biphasic systems or by fixation on supports, the bridge between these two approaches is being built through the use of nanoparticles (NPs) whose activity is very high under mild conditions because of their very large surface area. The NPs are soluble and thus act homogeneously. In view of the catalyst recycling, the NP catalysts often are immobilized or grafted on inorganic or organic polymer supports [71].

Heterogeneous catalysts, which generally consist of metal oxides or hydroxides without organic ligands, are more stable and robust under water oxidation conditions [66]. The heterogeneous processes, include adsorption, desorption as well as several surface reaction steps [67]. The heterogeneous catalysts have an advantage that at the end of reaction the catalyst can be removed by simple filtration. In principle the product is uncontaminated with a transition metal or ligand and allows the catalyst to be recycled into the next reaction [68]. Therefore, the use of heterogeneous instead of homogeneous catalysts in hydrolysis process is an advantage.

1.4.2. Heteropoly acids as homogeneous catalysts for biomass conversion

Solid acid catalysts have various advantages over liquid acid catalysts, such as: simple products separation, possible recycling, and less damage to the reactor [72]. Heteropoly acids (HPAs) are a type of solid acid consisting of transition metal– oxygen anion clusters. HPAs have received much attention due to their fascinating architectures and excellent physicochemical properties, such as Brønsted acidity, high proton mobility and good stability. They dissolve in polar solvents and release H^+, whose acidic strength is stronger than typical mineral acids like sulfuric acid [73].

Lately, HPAs have been widely used as homogeneous catalysts for biomass conversion. Shimizu *et al.* reported HPAs ($H_3PW_{12}O_{40}$, $H_4SiW_{12}O_{40}$) and salts of metal cations (Mn^+) and $PW_{12}O_{40}^{3-}$($M_{3/n}PW_{12}O_{40}$) for selective hydrolysis of cellobiose and cellulose to glucose in an aqueous phase [74]. In that study the glucose yield after 24 h was in the following order: $H_3PW_{12}O_{40}$ (53 wt %)> $H_4SiW_{12}O_{40}$ (51 wt %)> $HClO_4$ (42 wt %)> H_2SO_4 (29 wt %)> H_3PO_4 (12 wt %). This indicates that a stronger Brønsted acid is more favorable for the hydrolysis of a glycosidic bond [74]. Juan *et al.* investigated the potential of $H_3PW_{12}O_{40}$ for the hydrolysis of cellulose to glucose under hydrothermal conditions. A remarkably high yield of glucose (50.5%) and selectivity higher than 90% at 453 K were found after 2 h [75]. Ogasawara *et al.* showed that highly negatively charged polyanion ($H_5BW_{12}O_{40}$) promoted efficiently conversion of crystalline cellulose into water-soluble saccharides in concentrated aqueous solutions (82 % total yield and 77% glucose yield, based on reacting cellulose with a 0.7 M $H_5BW_{12}O_{40}$ solution) [76]. The heteropoly acids could be separated from the homogeneous solution and recycled by extraction with diethyl ether [75], but this process takes a long time, and the extraction is not complete. Some traces of the catalyst remain in the product.

1.4.3. Graphene supported catalysts in biomass conversion

Graphene has received much attention of researchers in the past few years due to its exceptional electronic, mechanical, and optical properties, high surface area, and biocompatibility. It is used in various applications including electrochemical, biosensors [77], transistors for nano-electronics [78], solar cells [79] etc. Because of its excellent adsorptive ability, graphene is also used as support for heterogeneous catalysts. J.M. Yang *et al.* loaded platinum nanoparticles on sulfonated graphene and used it as conductive polymer for fuel-cell applications [80]. *S.M. Choi et al.* synthesized surface-functionalized graphene nanosheets (GNS) with 80 wt % Pt loading with particle size of less than 3 nm. These Pt/GNS catalysts provided improved mass specific activity in alcohol electro oxidation [81]. Polyoxometalate-graphene composites were recently exploited for super capacitor and electrocatalytic (nitrite electro oxidation) applications [82,83].

The graphene supported catalysts have also demonstrated high activity in biomass conversion [72,84.] Supported solid acid catalysts on graphene oxide, and on sulfonated

graphene oxide nanosheets have been prepared by Wei *et al.* [85]. The experimental results indicated that the catalytic activity of the synthesized catalyst was superior to that of other solid acid catalysts and also better than that of H_2SO_4. They assigned the high activity of the catalyst to the formation of hydrophobic cavities on the graphene sheet containing oxygen groups on its surface. Kitano *et al.* reported on the enhancement of the hydrolysis of β-1,4-glucan on graphene-based amorphous carbon bearing SO_3H, COOH, and OH groups. Their results suggested that the synergistic combination of high densities of the functional groups bonded to amorphous carbon causes the efficient hydrolysis of β-1,4-glucan including cellulose [86].

1.5. Sonochemistry

1.5.1. Introduction

Ultrasound is simply sound pitched above human hearing. Ultrasound is the part of the sonic spectrum (Figure 10) which ranges from about 20 kHz to 10 MHz and can be roughly subdivided in three main regions: low frequency, high power ultrasound (20-100 kHz), high frequency, medium power ultrasound (100 kHz-1 MHz), and high frequency, low power ultrasound (1-10 MHz). The range from 20 kHz to around 1 MHz is used in sonochemistry, whereas frequencies far above 1 MHz are used as medical and diagnostic ultrasound [87,88].

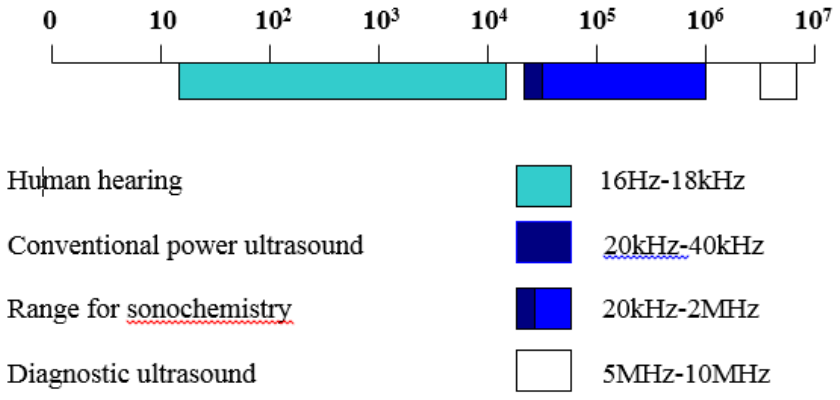

Figure 9: The frequencies of a sonic spectrum [89].

In industry, ultrasound is important for emulsifying cosmetics and foods, welding plastic, cutting alloys, and large-scale cleaning. None of these applications, however, take

advantage of the effects that ultrasound can have on chemical reactivity. In sonochemistry, molecules undergo chemical reaction due to the application of powerful ultrasound radiation (20 kHz to 10 MHz) [90].

The main event in sonochemistry is the creation, growth, and collapse of a bubble that are formed in the liquid. The stage leading to the growth of the bubble occurs through the diffusion of solute vapor into the volume of the bubble. The last stage is the collapse of the bubble, which occurs when the bubble size reaches its maximum value. During the collapse very high temperatures (5000–25,000 K) are obtained and causing the breaking of chemical bonds [87]. Since this collapse occurs in less than a nanosecond, very high cooling rates, in excess of 1011 K/s, are also obtained. This high cooling rate hinders the organization and crystallization of the products. For this reason, in all cases dealing with volatile precursors where gas phase reactions are predominant, amorphous nanoparticles are obtained [91]. The explanations for the formation of nanostructures are that the fast kinetics does not permit the growth of the nuclei, and in each collapsing bubble a few nucleation centers are formed whose growth is limited by the short collapse. If, on the other hand, the precursor is a non-volatile compound, the reaction occurs in a 200 nm ring surrounding the collapsing bubble. The temperature in this ring is in the region as 2093 K, which it lower than the temperature inside the collapsing bubble, but higher than the temperature of the bulk [90].

Sonochemistry is an excellent technique for the synthesis of NPs on the one hand and for coating surfaces by functional NPs on the other hand. These two aspects have been reviewed extensively in the last few years [88,92]. The sonochemical immobilization of the NPs and the strong adherence to the substrate can be the result of either the physical embedding of the particles into the surface layer, or, the NPs could be anchored to the surface by forming chemical bonds or chemical interactions with the substrate and cannot be removed even by washing [90].

1.5.2. Cavitation and “hot spot” theory

The chemical effects of ultrasound fall into three areas: homogeneous sonochemistry of liquids, heterogeneous sonochemistry of liquid-liquid or liquid-solid systems, and sonocatalysis (which overlaps the first two). Applications of ultrasound to

materials chemistry are found in all of these categories. Physical effects of high-intensity ultrasound, which often have chemical consequences, include enhanced mass transport, emulsification, bulk thermal heating, and a variety of effects on solids [90].

The chemical consequences of high-intensity ultrasound do not arise from an interaction of acoustic waves and matter at a molecular or atomic level. Instead, in liquids irradiated with high-intensity ultrasound, acoustic cavitation (the formation, growth, and collapse of bubbles) provides the primary mechanism for sonochemical effects [93]. During cavitation, bubble collapse produces intense local heating, high pressures, and very short lifetimes; these transient, localized hot spots drive high-energy chemical reactions (Figure 11).

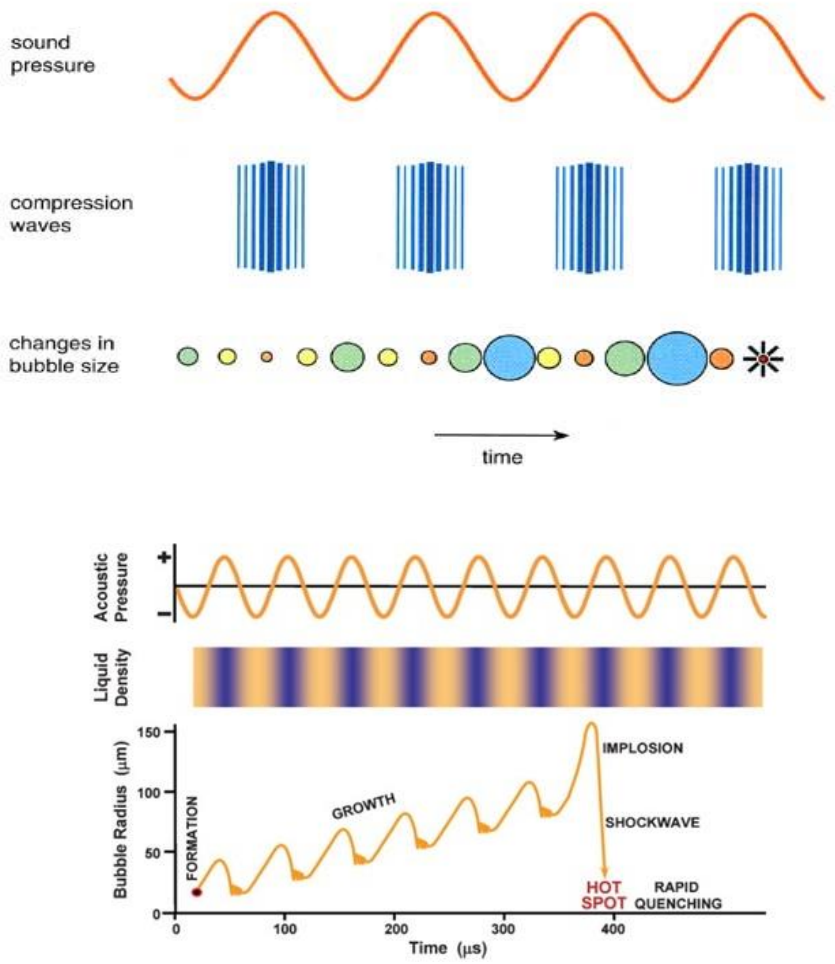

Figure 10: Bubble growth and implosion. Bubble growth and implosion in a liquid irradiated with ultrasound is the physical phenomenon responsible for most sonochemistry. Intense ultrasound waves generate large alternating stresses within a liquid by creating regions of positive pressure (blue color) and negative pressure (orange color). A cavity can form and grow during the episodes

of negative pressure. When the cavity attains a critical size, the cavity implodes, generating intense heat and tremendous pressure.

These hot spots have temperatures of »5000 °C, pressures of about 1000 atm, and heating and cooling rates above 1010 K/s [87,94]. Thus, cavitation serves as a means of concentrating the diffuse energy of sound into a unique set of conditions to produce unusual materials from dissolved (and generally volatile) precursors. Chemical reactions are not generally seen in the ultrasonic irradiation of solids or solid-gas systems. In addition, the interfacial region around cavitation bubbles has very large temperature, pressure, and (possibly) electric field gradients. Liquid motion in this vicinity also generates very large shear and strain gradients; these are caused by the very rapid streaming of solvent molecules around the cavitation bubble, as well as the intense shockwaves emanated on collapse. Ultrasonic cavitation in liquid-solid systems also produces high-energy phenomena. The physical effects primarily responsible for such enhancements include (a) improvement of mass transport from turbulent mixing and acoustic streaming, (b) the generation of surface damage at liquid-solid interfaces by shock waves and microjets, (c) the generation of high-velocity interparticle collisions in slurries, and (d) the fragmentation of friable solids to increase surface area. Cavitation near extended liquid-solid interfaces is very different from cavitation in pure liquids [94]. Near a solid surface, bubble collapse becomes non-spherical, driving high-speed jets of liquid into the surface (Figure 12) and creating shockwave damage to the surface. Because most of the available energy is transferred to the accelerating jet, rather than the bubble wall itself, this jet can reach velocities of hundreds of meters per second. In addition, shockwaves created by cavity collapse in the liquid may also induce surface damage and the fragmentation of brittle materials. The impingement of microjets and shockwaves on the surface creates the localized erosion responsible for ultrasonic cleaning and many of the sonochemical effects on heterogeneous reactions. Finally, during ultrasonic irradiation of liquid-powder slurries, cavitation and the shockwaves it creates can accelerate solid particles to high velocities. As discussed below, the interparticle collisions that result are capable of inducing striking changes in surface morphology, composition, and reactivity [95].

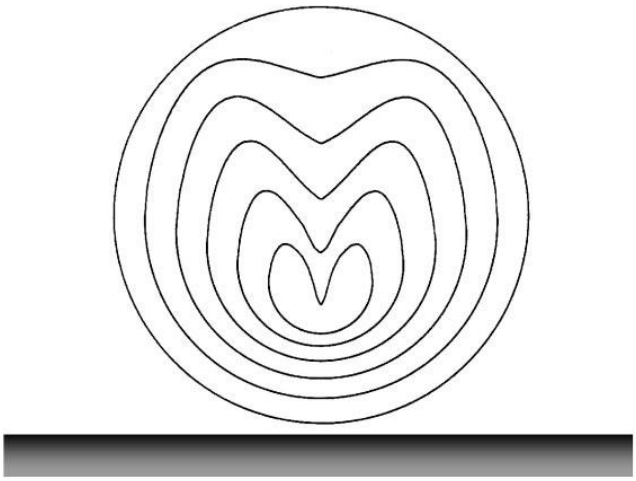

Figure 11: Formation of a liquid microjet during bubble collapse near an extended surface.

A wide range of commercial equipment is now readily available for sonochemical research. High-intensity ultrasonic probes (50 to 500 W/cm^2) of the type used for biological cell disruption are the most reliable and effective source for laboratory-scale sonochemistry and permit easy control over ambient temperature and atmosphere. For larger-scale irradiations, flow reactors with high ultrasonic intensities are commercially available in 20-kW modular units [89].

Sonochemical rates for homogeneous reactions depend on a variety of experimental parameters such as vapor pressure of precursors, solvent vapor pressure, and ambient gas. To achieve high sonochemical yields, the precursors should be relatively volatile, because the primary sonochemical reaction site is the vapor inside the cavitating bubbles [87,96]. In addition, however, the solvent vapor pressure should be low at the sonication temperature, because significant solvent vapor inside the bubble reduces bubble collapse efficiency.

1.5.3. Sonochemistry as a coating technique

The ability of sonochemistry technique to deposit nanomaterials on various substrates in a single step and without the aid of a binder makes it an advantageous technique over other well-known techniques. The additional advantage of this method is the ability to control the particle size of the product by varying the concentration of the precursors in the solution. Nanosized inorganic particles embedded on matrices have various applications. Antibacterial fabrics, for use in medical textiles, such as hospital

uniforms, bedding and wound dressings, may provide a useful weapon in the on-going fight against these infections. Recently, Gedanken group succeeded to sonochemically deposit metal oxides Nps: ZnO, and CuO, onto textile fibers. The aforementioned NPs exhibited potent antibacterial activity even after many washing cycles at Hospital washing machines[97,98]. Ultrasonic waves have been widely used also for the formation of microspheres. Sonochemistry as a coating technique has been exploited for attaching drug-loaded proteinaceous microspheres onto cotton and polyester fabrics [99]. Lately, the sonochemistry technique was applied for the preparation of water-soluble NPs of ionic salts and their deposition on glass slides, and silicon wafers [100].

1.5.4. Application of sonochemistry for bioethanol production

Sonication is widely employed for the pretreatment as well as for the conversion of biomass to biofuels. Most of the work in this field was focused on the delignification of lignocellulose assisted by the application of ultrasonic waves, or the pretreatment of the biomass employing ultrasonic waves. However, a few reports are found describing the direct sonochemical application in the conversion of glucose, or cellulose, or biomass into ethanol [90].

Bamboo (Neosinocalamus affinis) was subjected to successive pretreatments to isolate cellulose rich fractions for further utilization [101]. Ball-milled bamboo underwent ultrasound treatment in ethanol solution at 20 °C for 0-50 min. Then the samples were dissolved with 7 % NaOH/12 % urea solutions at 12 °C, followed by successive extractions with dioxane, ethanol, and dimethyl sulfoxide (DMSO). The yields of the obtained cellulose rich fractions ranged from 75.1 to 77.7 % [101].

Sulaiman *et al.* used ultrasonic waves in the fermentation process [102]. The authors reported production of ethanol from lactose by fermentation with the yeast Kluyveromyces marxianus (ATCC 46537) under various sonication conditions???. Batch fermentations were carried out at low-intensity sonication (11.8 W cm^{-2} sonication intensity at the sonotrode tip) using 10, 20 and 40 % duty cycles. Fermentation reaction was carried out in a 7.5 L (3 L working volume) stirred bioreactor. The sonotrode was mounted in an external chamber and the fermentation broth was continuously recirculated between the bioreactor and the sonication chamber. All duty cycles tested improved

ethanol production relative to no sonication. The final ethanol concentration of 5.2 g L^{-1} was obtained [102].

Ultrasound radiation was used as a pretreatment tool for the production of sugar monomers from sugar cane bagasse (SCB) in a sonoassisted acid hydrolysis [103]. The SCB was subjected to sonoassisted alkaline pretreatment. The cellulose and hemicellulose recovery were 99 and 78.95 %, respectively, and lignin removal was about 75.44 %. The solid content obtained was subjected to sonoassisted acid hydrolysis. Under optimized conditions, the maximum hexose and pentose yield observed were 69.06 and 81.35 % of theoretical yield, respectively [103].

Gedanken *et al.*, further extended the methodology for the conversion of marine biomass (Ulva regida) to bioethanol in a single step simultaneous saccharification and fermentation. The sonication provides a faster way for the simultaneous release of glucose from Ulva regida and its conversion into bioethanol. In a short duration of 30 min the yield of ethanol under mild sonication (4.3 wt % of dry biomass) is significantly high compared to the value (1.0 wt % of dry biomass) under incubation. They suggested that sonication provides more effective stirring then regular stirring. Relative to a conventional stirred reaction, mild sonication accelerated the glucose fermentation by 2.3 times [90].

1.6. Microwave Dielectric Heating

1.6.1. Introduction

In the past 30 years, the microwave oven has become an essential appliance in most kitchens. Faster cooking times and energy savings over conventional cooking methods are the primary benefits [104].

The microwave irradiation also produces efficient internal heating for most chemical reactions, delivering energy exactly where it is needed, even under exothermic conditions. Another valuable advantage of using controlled microwave dielectric heating for chemical synthesis is the dramatic reduction in reaction times: from days and hours to minutes and seconds. These two properties are sufficient motivation to promote the use of microwaves in “greener chemical processes" [105].

In conventional thermal processing, energy is transferred to the material through convection, conduction, and radiation of heat from the surfaces of the material. In contrast, microwave energy is delivered directly to materials through molecular interaction with the electromagnetic field. In heat transfer, energy is transferred due to thermal gradients, but microwave heating is the transfer of electromagnetic energy to thermal energy and is energy conversion, rather than heat transfer. This difference in the way energy is delivered can result in many potential advantages to using microwaves for processing of materials [104]. Microwave heating is able to heat the target compounds without heating the entire furnace or oil bath, which saves time and energy. It is also able to heat sufficiently thin objects throughout their volume (rather than through then outer surface), in theory, producing more uniform heating. However, due to the design of most microwave ovens and due to uneven absorption by the object being heated, the microwave field is usually non-uniform and localized superheating occurs [90,104].

The effect of microwave (MW) dielectric heating is attributed to the ability of some liquids and solids to transform electromagnetic radiation into heat and thereby drive chemical reactions. MW will generally heat any material containing mobile electric charges, such as polar molecules in a solvent or conducting ions in a solid. Polar solvents are heated as their component molecules are forced to rotate with the field and lose energy in collisions [104,106]. Semiconducting and conducting samples heat when ions or electrons within them form an electric current and energy is lost due to the electrical resistance of the material [83–86] .

Not as in conventional joining of ceramics or polymers, that considerable time and energy is wasted in heating up the interface by conduction through the substrates. With microwaves, the joint interface can be heated in-situ by incorporating a higher loss material at the interface. Microwaves may also be able to initiate chemical reactions not possible in conventional processing through selective heating of reactants. Thus, new materials may be created [104].

Since the beginning of the 80s, industrial applications of MW heating in activating physical or chemical reactions such as drying, defrosting, food lyophilization, or devulcanization of rubber are developed. There is a wide scope for the startup

plants????? in industries related to food, rubber, wood, processing of wastes and general drying [105].

1.6.2. Microwaves

Microwaves belong to the portion of the electromagnetic spectrum with wavelengths from 1 mm to 1 m with corresponding frequencies between 300 MHz and 300 GHz [104,106]. Within this portion of the electromagnetic spectrum there are frequencies that are used for cellular phones, radar, and television satellite communications. For microwave heating, two frequencies, reserved by the Federal communications commission for industrial, scientific, and medical purposes are commonly used (0.915 and 2.45 GHz) [104].

1.6.3. Microwave-materials interaction

Energy is transferred to materials by interaction of the electromagnetic fields at the molecular level, and the dielectric properties ultimately determine the effect of the electromagnetic field on the material. Thus, the physics of the microwave/materials interaction is of primary importance in microwave processing. The interaction of microwaves with molecular dipoles results in rotation of the dipoles, and energy is dissipated as heat from internal resistance to the rotation [104].

There were attempts to attribute the MW dielectric heating to the existence of hot spots originating from the specific exciting molecules, or functional groups within molecules. However, the time within which thermal energy is repartitioned from such moieties is much shorter than the period of a microwave wave, thus precluding the presence of such ‘molecular hot spots’ under ordinary laboratory conditions. Processes with solid phases behave somewhat differently. In this case much higher heat transfer resistances are involved, and the possibility of the stationary presence of hot-spots should be contemplated [90].

1.6.4. Use of Microwave for Biomass Conversion to Bioethanol

Microwave (0.3–300 GHz or 1–1,000 mm or 3×10^{8} to 3×10^{11} cycles/s) irradiation is a potential for acceleration of the vital reactions involved in biomass conversion.

Localized heating of the lignocellulosic components upon microwave irradiation is responsible for the degradation of the biomass structure. Upon interaction of the microwaves with the organic matter, enormous heat is generated within the material owing to the absorption of microwaves by water, carbohydrates and other components in the biomass. This leads to the disruption of the biomass structure and makes the carbohydrates accessible to the attack by catalytic species resulting in the acceleration of hydrolysis process [108]. Unlike conventional heating (conduction or convection), during microwave heating, there is direct interaction between the target and the electromagnetic field. As a result, the process of heating is rapid as well as volumetric. Such a rapid heating might cause explosive effect among the target particles resulting in the degradation of the highly recalcitrant structure like lignin. In addition to degrading the lignin and hemicellulose, microwave irradiation alters the cellulosic structure at the nanoscale [18].

Microwave irradiation technique has been extensively exploited for accelerating biomass hydrolysis process leading to fast release of fermentable sugars from polysaccharides [90]. Gedanken *et al.*, have exploited the potential of microwave irradiation for the conversion of a variety of renewable biopolymers in a fast acid catalyzed process using microwave irradiation.

Potato starch was chemically hydrolyzed to glucose using silicotungstic acid as catalyst under microwave irradiation in a short duration time of 5 min. Potato starch was completely converted to glucose, levulinic acid and formic acid. The methodology was further used for the conversion of potato peel waste to glucose. A glucose yield of 79.1 wt % was obtained upon the microwave irradiation (15 min) of potato peel waste. The combined use of solid acid catalyst and microwave irradiation offered a fast and green process for glucose production from starch based waste materials [90].

The type of bonding that links the glucose units in starch is quite different from that present in cellulose. The presence of extensive inter and intra molecular hydrogen as well as the type of linking of the D-anhydro-glucopyranose bonding present in the cellulose structure makes the hydrolysis of cellulose nearly 100 times tougher than starch hydrolysis [90]. Such a rigid polymer like cellulose could be successfully depolymerized in a fast process with the aid of microwave irradiation and sonication [30]. Under optimal

irradiation conditions (7 min, 70 % power, 2.38 M HCl) cellulose could be selectively hydrolyzed to glucose with a glucose yield of 67 wt %. The glucose thus produced from Avicel® PH-101 (commercial cellulose) is demonstrated to be a potential feedstock for bioethanol production [30].

The methodology of the use of microwave irradiation as a potential technique for the production of fermentable sugars further extrapolated for the conversion of renewable and abundant biomass, i.e., pine cones from *pinus radiata*. The experimental stages involved in their conversion to bioethanol include: delignification, hydrolysis of holocellulose and fermentation of holocellulose (Figure 13) [90].

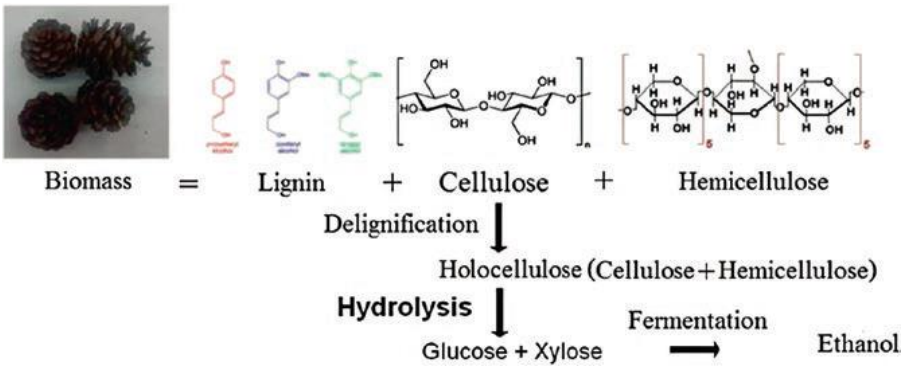

Figure 12: Production of ethanol from pine cones [90]

Pine cones from *Pinus radiata* were found to be a potential feedstock for the production of bioethanol. Alkaline (NaOH) pretreatment was carried out to delignify the lignocellulosic material and generate holocellulose (72 wt % yield). The pretreated biomass was hydrolysed using HCl as catalyst under microwave irradiation and hydrothermal conditions. The hydrolyzate was fermented to ethanol using *Saccharomyces cerevisiae*. 8.3 g of ethanol produced from 100 g of pine cones [90].

2. Research Objectives

2.1. Research Importance

Current energy systems are based mainly on fossil resources such as coal, petroleum and natural gas. However, the rapid consumption of natural resources has prompted research on finding renewable energy sources such as bioethanol to meet increasing demand. Biomass is a sustainable source of organic carbon, which is considered as part of the solution to producing bioethanol. Biomass is obtainable world-wide in the form of organic materials such as grass, wood, agricultural crops and glycogen.

The typical challenges in the economically feasible production of bioethanol include: (1) identifying appropriate feedstock; (2) developing suitable pretreatment methods; and (3) developing fast and efficient methods of hydrolysis for the release of glucose from carbohydrate polymers (starch, cellulose and glycogen) and the subsequent fermentation of the sugars.

Glycogen, a biopolymer, is a rich and abundant source of glucose. Glycogen is an appropriate, more viable and advantageous feedstock for glucose production rather than lignocellulosic biomass, as it needs no additional pretreatment. To the best of our knowledge, the information on the conversion of glycogen to biofuel has not been reported in the literature. Thus, the focus of the current investigation is to develop an efficient method for the production of glucose from glycogen.

Although the process of acidic hydrolysis of biomass to glucose is well known, this work is the first attempt on the hydrolysis of glycogen using microwave irradiation, sonochemistry and hydrothermal heating. In addition, various catalyst developments (especially supported catalysts) which can be recycled after the reaction make the process fast, green, safe and economical.

2.2. Research goals

2.2.1. Major goal

The purpose of this study is to develop a new process, simple and fast for hydrolysis of glycogen to glucose and afterward fermentation to bioethanol. This process should provide a solution to the economic and ecological aspects of biofuels industry. The

process is based on a combination of different energy source (include microwave, sonication and hydrothermal heating) and catalysts. This combination allows the glycogen hydrolysis quickly and efficiently.

2.2.2. Minor goals

1. Production of glucose from glycogen using HCl as catalyst under microwave irradiation. (i) Finding the optimal reaction conditions: HCl concentration, reaction time, and glycogen-catalyst wt ratio (ii) comparison of the reaction products using domestic microwave with reaction products under the same conditions using a conventional method by heating bath, (iii) Determination of the glucose content in the product.
2. , The goal of the research is to use a solid acid catalyst that is environmentally friendly and reusable in order to replace the HCl being corrosive and environmentally unfriendly. HPA catalysts ($H_4SiW_{12}O_{40}$ in H_2O (silico tungstic acid) and $H_3PW_{12}O_{40}$ in H_2O (tungsto phosphoric acid)) were used for this purpose.
3. To employ novel techniques like microwave irradiation as well as sonication and hydrothermal heating for the hydrolysis of glycogen to make the process fast.
4. The promising results obtained with commercial glycogen were checked for reproducibility employing biological glycogen produced using cyanobacteria fed on CO_2.
5. Development of a new supported catalyst (silicotungstic acid (HSiW) supported on graphene (HSiW/G)). The catalyst preparation was performed by a sonochemical method which has been proven to be an effective technique for the synthesis of supported catalysts. This supported catalyst is less acidic and could be easily separated from the reaction mixture for reused.
6. Characterization of the physical and chemical properties of the HSiW/G catalyst and studying the catalytic activity of that in the hydrolysis of glycogen.
7. Evaluation the feasibility of bioethanol production from the leaves of ficus religiosa. This is the first study on this biomass.

2.3. Novelty of the research

1. Acidic hydrolysis (HCl) of glycogen to glucose under microwave irradiation was performed successfully for the first time.
2. The novelty of the research does not only involve the use of glycogen as feedstock for the production of glucose, but also the use of novel techniques like microwave irradiation and sonication to bring about the hydrolysis reaction. To the best of our knowledge, the conversion of biomass like glycogen to glucose under sonochemical irradiation has not so far been investigated.
3. The hydrolysis process is considered ecologically friendly owing to the use of environmentally friendly and reusable solid acid catalysts like HPA's, HSiW/graphene. The novel catalyst, silicotungstic acid (HSiW) supported on graphene (HSiW/G), was developed. The catalyst preparation was performed by a sonochemical method, which has been proven to be an effective technique for the synthesis of supported catalysts. The catalytic activity of HSiW/G was studied for the hydrolysis of glycogen and cellulose for the first time.
4. The feasibility of utilizing glycogen from cyanobacteria for the production of glucose has been demonstrated. The acidic hydrolysis of biological glycogen (synthesized from cyanobacteria) leads to the complete conversion of glycogen under microwave heating. This attempt with biological glycogen was made for the first time. Thus, glycogen from cyanobacteria is a renewable feedstock for the production of glucose.
5. The waste leaves of *ficus religiosa* were utilized as a potential feedstock for the production of bioethanol for the first time.

3. Experimental System

The research includes development of an efficient method for the production of glucose from glycogen and from *ficus religiosa* leaves. A variety of acid catalysts and methods

for the hydrolysis were evaluated to produce glucose in a fast and environmentally friendly manner.

3.1. Materials

Glycogen from bovine liver was purchased from Sigma-Aldrich Co., (St. Louis, MO, USA). In addition, glycogen synthesized from cyanobacteria was used for comparative studies. Dry leaves of *ficus religiosa* fallen from the trees were collected on the grounds of Bar Ilan University, Israel.

Cellulose powder (Avicel® PH-101; ~50 μm particle size), α−D anhydrous glucose 96%, tungstosilicic acid hydrate ($H_4SiW_{12}O_{40}$ nH_2O, HSiW, 99%), and sodium hydroxide 98% were purchased from Sigma-Aldrich Co., (St. Louis, MO, USA). Hydrochloric acid (about 32%) was purchased from Frutarom Ltd., Haifa, Israel. Phosphotungstic acid ($H_3PW_{12}O_{40}$ nH_2O, HPW) and graphene nanoplatelets (6–8 nm thick ×15 microns wide) were purchased from STREM Chemicals (Newburyport, MA USA).

Deionized water and acetonitrile (HPLC grade) were purchased from Merck KGaA, Darmstadt, Germany. The water used was double distilled. Baker's yeast (Saccharomyces cerevisiae) was purchased from a local producer.

3.2. Biomass hydrolysis

3.2.1. Glycogen hydrolysis under microwave irradiation with HCl

A typical glycogen hydrolysis process comprises of subjecting 0.4 g of glycogen in a 20 mL HCl solution to microwave irradiation. The hydrolysis process was carried out in a domestic microwave modified so as to have provision for a distillation column passing through the microwave (MW) oven (for enhanced operation safety). The MW system also contained a stirring facility that operated during the reaction [109,110]. The microwave oven operated at 2.45 GHz in a batch mode under air at atmospheric pressure. The output of the applied microwave reactor was 1100 W. Reaction parameters such as the acid concentration and microwave irradiation time were varied to optimize the hydrolysis

process. After the hydrolysis process, the reaction products were filtered through a filter paper.

To evaluate the effect of temperature as well as microwave heating on the glycogen hydrolysis reaction, the reaction was also carried out in a commercial microwave oven (MARS5, CEM Corporation, Matthews, USA) where there is a provision for temperature control.

3.2.2. Glycogen hydrolysis with heteropoly acids

For glycogen hydrothermal hydrolysis process a stainless steel homemade autoclave of 3 mL volume was used. Typically, 50 mg glycogen and different amounts of HPW were dissolved in 2 mL water and put into the autoclave under ambient conditions. The mass fraction of the catalyst (%) is interpreted as the mass of catalyst (in g) per 100 g of system (reactant, catalyst and water). The autoclave was then placed in a regular air oven for the hydrothermal treatment of glycogen. Reaction parameters such as time of the hydrothermal treatment, temperature of heating, weight ratio of HPW and glycogen were varied to optimize the hydrolysis process.

The glycogen hydrolysis reaction was also carried out under microwave irradiation. For this purpose a domestic MW oven was used. Reaction parameters such as the weight ratio of HPW and glycogen, MW irradiation time were varied to optimize the hydrolysis process.

The glycogen hydrolysis reaction was also carried out under sonication (Sonics and Materials, VC-600, 20 kHz, 1.27 cm Ti horn at 30 % efficiency). In a typical reaction, 200 mg glycogen and different amounts of HPW dissolved in 10 mL water were taken in a sonication flask and sonicated for 1 h to 6 h using 180 W power. To prevent the overheating of the reaction, the sonication flask was immersed in a water bath (at 298 K) for cooling. The reaction temperature during the sonication was controlled using a thermocouple at 353 K.

The best reaction conditions identified for glycogen hydrolysis using HPW catalyst were further used for the reactions with HSiW.

The reusability of HPW was investigated. At the end of the reaction, the HPW was recovered from the solution by extraction using diethyl ether, followed by its

evaporation. The HPW thus regenerated was used for a second run under identical hydrothermal reaction conditions. The reusability of the catalyst was tested twice. The concentration of phosphorous before and after the hydrolysis reaction was determined by inductive coupled plasma (ICP) analysis using ULTIMA JY 2501 instrument. ^{31}P NMR spectra of the fresh and regenerated catalysts in solution state were recorded on a Bruker Avance DPX 400 instrument using D_2O as a solvent.

3.2.3. Glycogen hydrolysis under conventional reflux conditions

For comparison with the microwave irradiation or sonication, the glycogen hydrolysis reaction was also carried out by conventional reflux conditions under an oil bath at 80 °C with HCl or HPW as catalyst.

3.2.4. Glycogen and cellulose hydrolysis with HSiW/G catalyst

For the glycogen hydrolysis with HSiW/G catalyst a stainless steel homemade autoclave of 50 mL volume was used. Typically, 1 g glycogen and 1 g catalyst were dispersed in 20 mL water and placed in the autoclave. The autoclave was then placed in a regular air oven for the hydrothermal treatment of glycogen. Reaction parameters such as time, temperature of heating, weight ratio of the catalyst and glycogen were varied to optimize the hydrolysis process. In addition to glycogen, cellulose was also tested as carbohydrate feedstock for glucose production.

The reusability of the catalyst was investigated. After the reaction, the catalyst was separated from the solution by centrifugation and dried under vacuum overnight. The catalyst was used for a second run without regeneration under identical hydrothermal reaction conditions. The process was repeated 3 times.

3.2.5. *Ficus religiosa* leaves hydrolysis

The dry leaves of *ficus religiosa* were subjected to grinding in a mechanical blunder. The obtained powder material was hydrolyzed under either hydrothermal or microwave irradiation conditions using HCl as catalyst.

Microwave irradiation was carried out in a domestic microwave oven. Typical hydrolysis in microwave oven involved irradiating of leaves in the powder form with 20

mL of HCl for different periods of time. The temperature at the end of the microwave reaction is 85 °C, measured by employing a pyrometer (Fluke, 65 Infrared thermometer).

The hydrothermal hydrolysis was done in stainless steel autoclave by treating 1.0 g of leaves with 20 mL of 1 M HCl. Reaction conditions, namely, the time of heating and temperature were optimized.

3.3. Identification and estimation of hydrolysis products

The progress of the biomass (glycogen, cellulose and *ficus religiosa* leaves) hydrolysis reaction was monitored using ^{13}C NMR spectroscopic analysis on a Bruker Avance DPX 300 instrument. The conversion of biomass as well as glucose formation was deduced from ^{13}C NMR spectra. D_2O is used as a solvent.

The content of glucose produced under microwave irradiation using HCl as catalyst was determined by HPLC analysis. For the HPLC analysis the reaction products of glycogen hydrolysis were neutralized with dilute NaOH and lyophilized for 24 h. The solid mass (0.5 g) was diluted with 2 mL of HPLC grade water and analyzed with an HPLC device (Young Lin Clarity 9100 with 9160 PDA UV detector). UV detection was carried out at 195 nm. The flow rate was set to 1.0 mL min^{-1} and injections of 100 µL were made. A Luna Phenomenex 5µ NH_2 column 100 A column was used (250 mm × 4.6 mm). The ratio of acetonitrile and deionized water used was 80% to 20% [111]. A guard column was attached to the inlet of the column to prevent clogging. The content of the glucose in the hydrolyzate was calculated according to the weight of the solid mass obtained after the hydrolysis reaction and lyophilization. In order to have an idea of the exact amount of solid mass obtained from the hydrolyzate through lyophilization, the residual amount of water was deduced by thermogravimetric analysis (TGA). TGA analysis was carried out using a Mettler TGA/STDA 851 device. In the TGA experiment, the sample powders were heated from 25 to 600 °C at a heating rate of 10 °C min-1 under Ar atmosphere.

The water-soluble products of hydrolysis reactions with HSiW/G catalyst were analyzed by HPLC (Merck-Hitachi LaChrom System L-7000 equipped with L-7455 Diode Array Detector and Schambeck SFD RI 2000 Refractive Index Detector, Bad Honnef, Germany). The analyses were carried out using a 300 × 7.8 mm Rezex-ROA ion

exclusion chromatography column (Torrance, CA, USA) equipped with a matching guard column. The mobile phase used was 0.005 N H_2SO_4 under isocratic elution for 45 min at a flow rate of 0.5 mL min^{-1} at ambient temperature, with an injection volume of 10 μL. EZ Chrom Elite v. 3.1.7 software was used for data acquisition and processing, with the RI signal acquired using an external analog input. The content of the glucose in the hydrolyzate was calculated using a calibration curve obtained from a series of external glucose standards.

The residual solid mass of *ficus religiosa* leaves was separated from the hydrolyzate by filtration through a filter paper (Whatman® 150 mmΦ), washed with excess distilled water and dried in air oven at 120 °C overnight. The residual solid mass calculated from difference in the weights of the initial and final amounts of the dry leaves [112].

The amount of glucose in the hydrolyzate generated from *ficus religiosa* leaves is determined by using non enzymatic method based on insitu generation of carbon nanoparticles from the glucose in the analyte [113]. Briefly, the pH of the hydrolyzate was adjusted to 7. Then, 10 wt % urea and water was added. The analytes obtained treated at 120 °C for 20 min in an autoclave (Tuttnauer cat 2007). The urea under these conditions convert to ammonia and the pH of the solution becomes basic. As a result, carbon nanoparticles of glucose are obtained and the solution turns to pale yellow color owning to the unique absorbing properties of the hydrophilic carbon nanoparticles. The UV absorbance measurements were done using a spectrophotometer (Varian Cary 100 scan UV / vis spectrophotometer) at 275 nm. For calibration plot, standard solutions containing known amount of glucose were prepared and subjected to the hydrothermal treatment along with the analytes.

3.4. Fermentation of hydrolyzate produced from *ficus religiosa* leaves and bioethanol estimation

Fermentation of the hydrolyzate was carried out using Baker's yeast. Typical fermentation process involves 55 mL of neutral hydrolyzate (from the hydrolysis of 3 g leaves) taken in a 250 mL Erlenmayer flask and adding 1.0 g yeast. The contents were

incubated at 30 °C under shaking at 150 rpm. Aliquots of samples from the fermentation broth were collected at regular intervals of time.

The ethanol amount in the broth was determined by ^{1}H NMR using D_2O as solvent and HCOONa as an internal standard. The ^{1}H-NMR spectra was recorded on a Bruker Avance DPX 200.

3.5. HSiW/G catalyst

3.5.1. HSiW/G preparation

An ultrasound-assisted method was used to prepare the supported HSiW/G catalysts. Typically, the following procedure was followed: 2 g HSiW was first dissolved in 80 mL ethanol, followed by the addition of 2 g graphene. Owing to easy evaporation, ethanol is used as the solvent. The synthesis was performed in a glass beaker reactor of 120 mL volume. The mixture was sonochemically irradiated with 1 cm^2 titanium horn immersed 1 cm into the solution for 1 h with an efficiency of 40% (amplitude) with no external cooling (Sonicator 20 kHz, Sonics & Materials, VCX-750). After 1 h the temperature of the reaction slurry was 60 °C. The product was then collected by centrifugation and dried in a vacuum overnight.

In addition to the sonochemical route, the conventional impregnation method was also used for the preparation of the HSiW/G catalyst. The catalyst preparation process in the conventional impregnation method comprises of dissolving 2 g HSiW in 80 mL ethanol, followed by the addition of 2 g graphene. The mixture was kept for 4 h of stirring. Then the solvent was evaporated using a water bath followed by drying of the catalyst under vacuum overnight.

3.5.2. HSiW/G characterization

Here you place the characterization instrument you have used. That is Ok . But why does it appear under HSiW/G characterization? Aren't they general and used for various other parts???

3.5.2.1. XRD (X-Ray-Diffraction Analysis)

X-rays are electromagnetic radiation of wavelength about 1 Å (10-10 m), which is about the same size as an atom. X-ray diffraction has been in use in two main areas, for the fingerprint characterization of crystalline materials and the determination of their

structure. Each crystalline solid has its unique characteristic X-ray powder pattern which may be used as a "fingerprint" for its identification. Amorphous substances do not show diffraction peaks and XRD can't be used for sample identification. By the measurement, the crystalline atoms cause a beam of incident X-rays to diffract into many specific directions. By measuring the angles and intensities of these diffracted beams, a three-dimensional picture of the density of electrons within the crystal can produced. From this electron density, the mean positions of the atoms in the crystal can be determined, as well as their chemical bonds, their disorder and various other information.

The XRD analysis of the HSiW/G was performed on a Bruker D8 diffractometer with Cu Kα – 1.541 Å radiation.

3.5.2.2. Electron microscopy

The morphology and texture of the HSiW on the graphene were studied by transmission electron microscopy (TEM) JEOL JEM-1400 (120 kV) and high resolution scanning electron microscopy (HR-SEM), FEI Company TM MAGELLAN 500L (Holland).

Transmission electron microscopy (TEM) is an imaging technique in which a beam of electrons is focused onto a specimen causing an enlarged image of the sample to appear on a fluorescent screen or on a photographic film, or to be detected by a CCD camera. TEM works like a slide projector. Instead of a light bulb, an electron source is used. This is either a glowing tungsten tip, or a field emitter. The ejected electrons are accelerated to approximately 2/3 of the velocity of light, fast enough to cross samples which are less than 300 nm thick, (approximately 100 atomic layers). The image of the sample is projected using magnetic lenses onto the screen of the detector. Chemical microanalysis on a scale of less than 1 nm has been demonstrated with sensitivity less than 10 atoms. The electrons can be focused onto the sample providing a resolution far better than what is possible with light microscopes.

An environmental-scanning electron microscope (ESEM) is an electron microscope capable of producing high-resolution images of surfaces down to 1.2 nm. The high resolution electron microscope (HR-SEM) is used for morphology and topography analysis at resolutions down to 0.6 nm.

3.5.2.3. Dynamic light scattering (DLS)

Dynamic light scattering (DLS) is a non-invasive, well established technique for measuring the size and size distribution of molecules and particles typically in the submicron region, and with the latest technology lower than 1nm. Typical application of dynamic light scattering is the characterization of particles which have been dispersed or dissolved in a liquid. The Brownian motion of particles or molecules in suspension causes laser light to be scattered at different intensities. Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size using the Stokes-Einstein relationship.

The particle size distribution of HSiW (dispersed in ethanol) was estimated by the DLS method using a Malvern Zetasizer Nanoseries device (Malvern Instruments, Malvern, UK).

3.5.2.4. Inductively coupled plasma (ICP)

ICP is an analytical atomic spectrometer. It can identify most of the elements and quantify their concentration in ppm units (mg/L). The analysis is performed in aqueous homogeneous media.

The amount of HSiW deposited on graphene by sonication was determined by the ICP method on the ULTIMA JY 2501 instrument.

3.5.2.5. BET (Brunauer–Emmett–Teller)

Brunauer–Emmett–Teller (BET) analysis provides precise specific surface area evaluation of materials by nitrogen multilayer adsorption measured as a function of relative pressure using an automated analyzer. The technique encompasses external area and pore area evaluations to determine the total specific surface area in m^2/g yielding important information in studying the effects of surface porosity and particle size in many applications.

Barrett–Joyner–Halenda (BJH) analysis employed to determine pore area and specific pore volume using adsorption and desorption techniques. This technique characterizes pore size distribution independent of external area due to particle size of the sample.

Surface area, pore volume and pore size distribution of the catalyst were measured using a Nova 3200e Quantachrome analyzer. The surface area was calculated from the linear part of the BET plot. The pore size distribution was estimated using the (BJH) model with the Halsey equation, and the pore volume was measured at the P/P0 0.9947 signal point.

3.5.2.6. Fourier transform infrared (FT-IR)

Infrared spectroscopy is an important method for identifying the presence of certain functional groups in a molecule. In order to characterize the functional groups and confirm the presence of HSiW on graphene surface, Fourier transform infrared (FT-IR) spectra were recorded using a FT-IR spectrometer (Bruker Tensor 27).

3.5.2.7. Raman spectroscopy

When monochromatic light is scattered by molecules, a small fraction of the scattered light is observed to have a different frequency from that of the irradiating light. This is known as the Raman Effect, which is an important method for the elucidation of molecular structure, for locating various functional groups or chemical bonds in molecules, and for the quantitative analysis of complex mixtures. Although Raman spectra are related to infrared absorption spectra, a Raman spectrum arises in a quite different manner and thus provides complementary information. Vibrations that are active in Raman may be inactive in infrared, and vice versa. A unique feature of Raman scattering is that each line has a characteristic polarization, and the polarization data provide additional information related to molecular structure.

The Raman spectra of HSiW, graphene and HSiW/G were recorded using a Renishaw InVia Raman microscope equipped with a Leica DM2500 M analysis microscope (Leica Microsystems), sample focusing was done by a 50× (N.A. 0.75) lens. All the samples were irradiated by a 514 nm excitation wavelength with a constant power of ~0.25 mW. Please put just one Chapter for Charaterization methods

3.6. Characterization of *ficus religiosa* leaves

3.6.1. XRD (X-Ray-Diffraction Analysis)

The degree of crystallinity of the cellulose component in the powdered leaves was determined by X-ray diffraction (XRD) studies. The XRD patterns were collected using a Bruker AXS Advance powder X-ray diffractometer (Cu Kα radiation, λ = 1.5418 Å).

3.6.2. Thermal analysis

Thermo gravimetric analysis (TGA) is a technique for measuring the change in the weight of a substance as a function of temperature due to dehydration or decomposition. Changes in weight are a result of the rupture and/or formation of various physical and chemical bonds at elevated temperatures that lead to the evolution of volatile products or the formation of heavier reaction products. From such curves, data are obtained concerning the thermodynamics of the various chemical reactions, reaction mechanisms, and the intermediate and final reaction products.

The thermal stability of the leaves is deduced from a thermogravimetric analysis (TGA). TGA analysis was carried out in a temperature range of 25 to 600 ºC at a heating rate of 10 ºC/min under Ar atmosphere using a Mettler TGA/STDA 851 device.

3.6.3. Elemental analysis

The elemental analysis of the leaves (C, H, N and O) was carried out on C, H, N, O analyzer, Thermo Electron Corporation, Flash EA 1112 series (Italy).

The sample is weighed in tin capsules, placed inside the autosampler at a preset time, and then dropped into an oxidation / reduction reactor kept at a temperature of 900 – 1000 °C. The exact amount of oxygen required for optimum combustion of the sample is delivered into the combustion reactor at a precise time. The reaction of oxygen with the tin capsule at elevated temperature generates an exothermic reaction, which raises the temperature to 1800 °C for a few seconds. At this high temperature both organic and inorganic substances are converted into elemental gases which, after further reduction, are separated in a chromatographic column and finally detected by a highly sensitive thermal conductivity detector (TCD). The oxygen determination is achieved through an

oxygen-specific pyrolysis reactor heated at a temperature slightly above 1060 °C. This allows a complete pyrolysis of the sample in an oxidant-free environment.

4. Integration of the Research Articles

To the best of our knowledge, the information about the conversion of glycogen to biofuel has not been reported in the literature. Thus, this Ph.D. thesis work is mainly focused on an attempt to exploit glycogen as a potential feedstock for the production of glucose. Variety of acid catalysts and methods for the hydrolysis of glycogen were evaluated to produce glucose in a fast and environmentally friendly manner. Glucose is a precursor to variety of fine chemicals and fuels. In addition, those new methods were checked for different biomass hydrolysis including biological glycogen produced by using cyanobacteria fed on CO_2, cellulose and *ficus religiosa* leaves. Such glucose obtained from the leaves was also been exploited for the production of ethanol.

This Ph.D. thesis work resulted in four publications:

4.1 List of articles:

1. Klein, M., Pulidindi, I. N., Perkas, N., Meltzer-Mats, E., Gruzman, A., & Gedanken, A. (2012). **Direct production of glucose from glycogen under microwave irradiation**. RSC Advances, 2(18), 7262. doi:10.1039/c2ra21066e. (Impact factor: 3.708).

2. Klein, M., Pulidindi, I. N., Perkas, N., & Gedanken, A. **Heteropoly acid catalyzed hydrolysis of glycogen to glucose**. Biomass and Bioenergy. Doi: 10.1016/j.biombioe.2015.02.036. (Impact factor: 3.411).

3. Klein, M., Varvak, A., Segal, E., Markovsky, B., Pulidindi, I. N., Perkas, N., & Gedanken, A. (2015). **Sonochemical synthesis of HSiW/graphene catalysts for enhanced biomass hydrolysis.** Green Chem. doi:10.1039/C4GC02519A. (Impact factor: 6.852).

4. Klein, M., Griess, O., Pulidindi, I. N., Perkas, N., & Gedanken, A. **Bioethanol production from *Ficus religiosa* leaves**, Renewable Energy. Under review. (Impact factor: 3.361).

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4.2. Integration of the research articles

Our early research, which presented on article 1, focused on the development of an efficient method for the production of glucose from glycogen using hydrochloric acid and exposure to microwave irradiation. After the hydrolysis process, hydrolyzates were analyzed using ^{13}C NMR for identifying the reaction products, and HPLC for estimating glucose yield. The optimal reaction conditions (time, concentration, etc.) for achieving the highest yield of glucose with hydrolysis reaction were identified.

We have found that the best reaction conditions for the achievement of highest glucose yield (62 wt %) were: 10 min of exposure to microwave irradiation and concentration of 1 M acid (HCl). In addition, we have seen that microwave irradiation has dramatically reduced the reaction time from more than 6 h at conventional conditions (80 °C under an oil bath) to 10 min.

However, as HCl is corrosive, its separation is energy intensive and the process generates large amounts of acidic waste water, the aim of article 2 is to find an environmentally benign alternative to the mineral acid for glycogen hydrolysis.

The heteropoly acids (HPA's) are known as effective homogeneous and heterogeneous **solid** acid catalysts with industrial applications. Unique features of heteropoly acids include: stronger acidity compared to many mineral acids i.e. phosphoric acid, non-corrosive, easy to separate, are reusable, have fewer side products and less waste generating, if any, are non-toxic, easy to handle, and greener than conventional acid catalysts. Article 2 is focused on the development of an environmentally friendly and fast process for glycogen hydrolysis to produce glucose using solid acid catalysts.

In addition to the use of HPA as a catalyst, in this study we were investigating other novel heating methods for the hydrolysis reaction such as hydrothermal heating (autoclave) and sonication. Under optimized hydrothermal conditions (100 °C, 2 h reaction time) complete conversion of glycogen to glucose was achieved by using 2.4 % mass fraction of catalyst. Sonication of an aqueous solution of glycogen in the presence of HPW and HSiW also yielded glucose.

After the hydrolysis reaction, HPW was recovered from the hydrolyzate by extraction using diethyl ether and the reusability of HPW was investigated. It was found that the regenerated catalyst also led to the formation of glucose. Therefore, heteropoly acids are efficient, environmentally friendly and reusable catalysts for the conversion of glycogen to glucose.

Even though heteropoly acids can be separated from homogeneous solution and recycled by extraction with diethyl ether, this process is time consuming, and the extraction is not complete. Some traces of catalysts yet remain among products. Therefore, article 3 was focused on the development of a new supported catalyst that its separation from the reaction products is considerably faster, is efficient and does not require additional environmentally unfriendly materials. In this article, we report on a new silicotungstic acid (HSiW) catalyst supported on graphene (HSiW/G). The catalyst preparation was performed by a sonochemical method. This supported catalyst could be easily separated from the reaction mixture by centrifugation. The physical and chemical properties of the catalysts were studied and it was found that HSiW is homogeneously distributed on the surface of graphene and strongly anchored to the support.

The hydrolysis of glycogen was performed with an HSiW/G catalyst by hydrothermal treatment. The yield of glucose (66 wt %) obtained was dramaticly higher in comparison with the same amount of bare HSiW under same conditions. We attribute this enhancement of catalytic activity to the special structure of HSiW/G that enables higher accessibility of protons for the cleavage of the glycosidic bond in glycogen. In addition, the reusability of the catalyst was investigated. Glucose yield after three reaction cycles was not altered significantly. Moreover, the catalyst usefulness was tested for the hydrolysis of cellulose. A glucose yield value of 33 wt % was obtained, indicating the activity of the catalyst (HSiW/G) for cellulose hydrolysis. Thus, developed HSiW/G catalyst is a reusable, economically viable and a green catalyst for biomass hydrolysis.

In continuance to successful glycogen hydrolysis with different novel methods, we have tried to explore additional sources for glucose production. *Ficus religiosa* leaves that fall from the tree are generally a waste whose disposal is an issue from both environmental and economic viewpoints. Moreover, those leaves are a rich source of holocellulose. Hence, the objective of article 4 is to evaluate glucose production

feasibility from *ficus religiosa* leaves. In addition, in this study, we have conducted bioethanol production by using yeast for the fermentation process. We have shown that under hydrolysis reaction (8 min microwave irradiation, 1 M HCl), 10.1 wt % glucose yield could be obtained, and 3 wt % (dry wt. basis) of ethanol can be produced.

These results indicate the feasibility and efficiency of using various catalysts (hydrochloric acid, heteropoly acid, HSiW/G) along with novel methods (microwave irradiation, sonication and hydrothermal heating) in biomass hydrolysis reaction for glucose production, followed by bioethanol as an environmentally friendly alternative to fossil fuels.

4.3. Article abstracts

4.3.1. Article 1

Direct production of glucose from glycogen under microwave irradiation

Miri Klein, Indra Neel Pulidindi, Nina Perkas, Ella Meltzer-Mats, Arie Gruzman and Aharon Gedanken

RSC Adv. **2012**, *2*, 7262−7267

The production of fermentable sugars from renewable sources is a challenge. An attempt was made to exploit glycogen as a potential feedstock for the production of glucose. The microwave-assisted acidic hydrolysis was applied for glycogen decomposition for the first time. The optimal conditions for the hydrolysis reaction (yield of glucose – 62 wt %) were identified: microwave irradiation time – 10 min and concentration of acid – 1 M HCl. Microwave irradiation has dramatically reduced the reaction time from more than 6 h (at 80 °C under an oil bath) to 10 min. ^{13}C NMR spectroscopy was employed to monitor the progress of the hydrolysis reaction. HPLC analysis was employed to evaluate the yield of glucose. Thus, the viability of the use of glycogen as an economically and environmentally benign precursor to the production of glucose has been demonstrated.

4.3.2. Article 2

Heteropoly acid catalyzed hydrolysis of glycogen to glucose

Miri Klein, Indra Neel Pulidindi, Nina Perkas, Aharon Gedanken

Biomass and Bioenergy **2015**, DOI: 10.1016/j.biombioe.2015.02.036

Complete conversion of glycogen to glucose is achieved by using $H_3PW_{12}O_{40}\cdot nH_2O$ (HPW) and $H_4SiW_{12}O_{45}\cdot nH_2O$ (HSiW) as catalysts for the hydrolysis under optimized hydrothermal conditions (mass fraction of catalyst 2.4 % , 373 K and 2 h reaction time). The reusability of the catalyst (HPW) was demonstrated. In addition to carrying out the glycogen hydrolysis in an autoclave, other novel methods such as microwave irradiation and sonication have also been investigated. At higher mass fraction of the heteropoly acids (10.5 %), glycogen could be completely converted to glucose under microwave irradiation. Sonication of an aqueous solution of glycogen in the presence of HPW and HSiW also yielded glucose. Thus, heteropoly acids are efficient, environmentally friendly and reusable catalysts for the conversion of glycogen to glucose.

4.3.3. Article 3

Sonochemical synthesis of HSiW/graphene catalysts for enhanced biomass hydrolysis

Miri Klein, Alexander Varvak, Elad Segal, Boris Markovsky, Indra Neel Pulidindi, Nina Perkas and Aharon Gedanken

Green Chemistry **2015**, DOI: 10.1039/c4gc02519a

Hydrolysis of biomass for the production of glucose was studied. Silicotungstic acid (HSiW) was deposited on graphene by an ultrasound-assisted procedure. The catalyst (HSiW/G) was characterized using a variety of physico-chemical methods. Homogeneous distribution of HSiW on the surface of graphene was demonstrated. The hydrolysis of glycogen was performed with a HSiW/G catalyst by hydrothermal treatment. The yield of glucose (66 wt %) obtained was about 8 times higher than that obtained with the same amount of bare HSiW. Stability of the HSiW/graphene even after 3 repeated uses was confirmed. The mechanism of the enhancement of catalytic activity was discussed in terms of a special interaction between the graphene support and HSiW and also the appearance of hydrophobic cavities on the surface of graphene. The formation of these cavities facilitates the anchoring of glycogen to the catalyst surface and promotes the attack of protons that leads to selective, rapid, and efficient hydrolysis.

4.3.4. Article 4

Bioethanol production from *Ficus religiosa* leaves

Miri Klein, Ofir Griess, Indra Neel Pulidindi, Nina Perkas, Aharon Gedanken

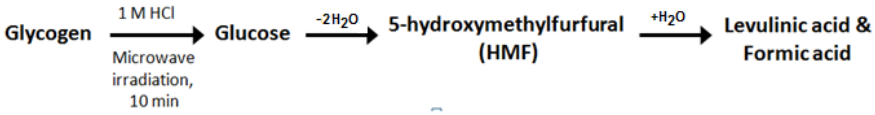
Renewable energy, **2015** (under review)

The demand for alternate transportation fuels is continuously increasing due to the limited and diminishing supply of fossil based fuels. Biomass is an attractive alternative feedstock for the production of biofuels. The objective of the current work is to develop a feasible process for the production of bioethanol from *ficus religiosa* leaves. Under the process conditions (8 min microwave irradiation, 1 M HCl), 10.1 wt % glucose yield could be obtained from the leaves. Microwave based hydrolysis process yielded higher glucose content compared to the conventional hydrothermal process (4.1 wt %). 3 wt % (dry wt. basis) of ethanol could be produced from the leaves of *ficus religiosa.*

5. Discussion

The objective of the current study was to exploit glycogen as a potential feedstock for the production of glucose. Glucose is a precursor to a variety of fine chemicals and bioethanol.

The schematic representation of the conversion of glycogen to glucose and levulinic acid and formic acid is depicted in Scheme 1.



Scheme 1: Schematic representation of hydrolysis of glycogen

To find the best reaction conditions for hydrolysis process, different concentrations of HCl (no acid, 0.5, 1, 3 and 5 M) were tested. 1M HCl was found to be the optimum concentration where complete conversion of glycogen is obtained upon microwave irradiation for a period of 10 min. In the absence of HCl, even with microwave irradiation, glycogen hydrolysis reaction did not proceed. A steady increase in the yield of glucose with irradiation time from 2 (32 wt %) to 10 (62 wt %) min is observed. The optimum time of microwave irradiation where highest yield (62%) of glucose is obtained is 10 min. In the case of heating with an oil bath, even after 6 h, the traces of glycogen are observed. It indicates that conventional heating at 80 ºC with an oil bath requires more than 6 h, whereas the same process is completed in 10 min under microwave irradiation. The yield of glucose, as deduced from HPLC analysis, after 22 h of glycogen hydrolysis under reflux conditions is 39 wt %. The use of microwave irradiation has dramatically reduced the reaction time from more than 6 h to 10 min with 1 M HCl. This difference is due to the superheating achieved under the microwave irradiation of the reaction mixture, rather than in the conventional reflux. As glycogen can be produced from abundant and renewable chemical feedstock like CO_2 [116], the use of

glycogen as a precursor for glucose generation is an economically and environmentally benign alternative to cellulose.

The potential of glycogen as a feasible feedstock for the production of glucose is demonstrated. The accelerating effect of microwave irradiation on the hydrolysis process is elucidated. A cheap catalyst, HCl is identified for glucose production. But the use of HCl at industrial scale production of glucose could be corrosive. Acid concentration and recycling will also add to the process cost, making the process economically unfeasible. So identification of a reusable and non-corrosive catalyst for the bulk production of glucose is necessary which formed the second objective of the study.

The aim of this study is to attempt to use heteropoly acids (HPA's) as an environmentally benign alternative to the mineral acid for glycogen hydrolysis. For glycogen hydrolysis process, a stainless steel home-made autoclave of 3 mL volume was used. Typically, 0.05 g glycogen and different amounts HPW ($H_3PW_{12}O_{40}$ nH_2O) were dissolved in 2 mL water and put into the autoclave under ambient conditions. The autoclave was then placed in a regular air oven at a predetermined temperature. Reaction parameters such as time of the hydrothermal treatment, temperature of heating, weight ratio of HPW and glycogen were varied to optimize the hydrolysis process.

To evaluate the effect of time of hydrothermal treatment, the reaction was performed for various reaction times, namely, 90, 120 and 180 min and at a constant temperature of 100 ºC with the weight ratio of reactant (glycogen, 0.05 g) to catalyst (HPW, 0.05 g) of 1:1. 120 min was found to be the optimal time where a complete conversion of glycogen exclusively to glucose was observed. No by-products [29] like HMF, levulinic acid and formic acid were observed. The effect of reaction temperature (80 to 120 ºC) was also studied. The optimum temperature was found to be 100 ºC at which the complete conversion of glycogen to glucose was obtained. Thus the optimum reaction conditions were found to be 100 ºC and 120 min for the hydrothermal treatment.

A mass ratio of 1:1 (catalyst: reactant) was found to be optimum for the complete conversion of glycogen to glucose. The up-scaling process was studied with 1 g of glycogen with 1 g of HPW and with 40 mL water. Complete conversion of glycogen to glucose was observed when the hydrolysis process was carried out for 4 h at 120 ºC.

The reusability of HPW was also investigated. ^{31}P NMR spectra of the catalyst before and after the hydrolysis showed identical peak positions for the ^{31}P peak. No shift in the phosphorus peak position is noticed. These indicate that the structural integrity of the catalyst is unaltered after the hydrolysis process.

The reaction was also carried out under microwave irradiation. The influence of time of irradiation on the microwave-assisted hydrolysis of glycogen was tested. The hydrolyzate obtained after 15 min exhibited peaks typical of glucose. In addition unreacted glycogen was also observed. As a further increase in the time of irradiation for achieving a complete conversion of glycogen is energy intensive, the option of increasing the catalyst amount was preferred. In the next stage, the amount of catalyst was optimized so as to obtain the highest yield of glucose from glycogen. Complete conversion of glycogen could not be achieved with catalyst mass fraction lower than 10.5 %. The mass fraction of HPW is indeed crucial for the complete conversion of glycogen. Under the aforementioned experimental conditions, in addition to glucose, by-products such as levulinic and formic acids were formed through the formation and decomposition of HMF. The high temperature developed in the microwave oven (hot spots) enable the decomposition of the glucose to the by-products.

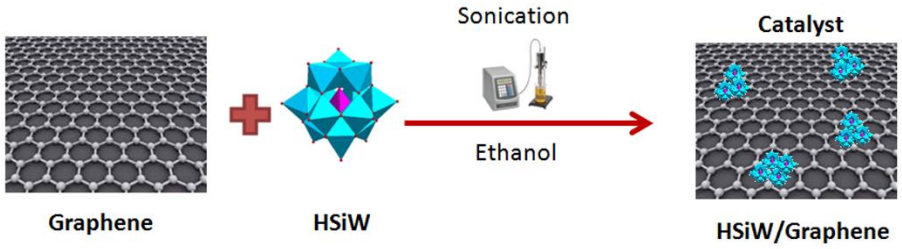
The glycogen hydrolysis reaction was also carried out under sonication. To the best of our knowledge, the conversion of biomass like glycogen to glucose under sonochemical irradiation was not investigated until now. Initially, solutions containing different amounts of HPW (0.25 g – 1.5 g) and 0.25 g of glycogen in 10 mL of water were subjected to sonochemical irradiation for 3 h. No complete conversion was observed by ^{13}C NMR even after 3 h of sonication. So as to attain complete conversion of glycogen, the reaction time was increased from 3 to 4 and 6 h. After 6 h, with the same reaction conditions, only a trace amount of unreacted glycogen is observed in the hydrolyzate and intense peaks typical of glucose were observed. Apart from glucose, no by-products were observed, indicating the specificity of this reaction. The reason this chemistry is occurring under ultrasonic waves is due to collapse of the acoustic bubble. This collapse leads to a local high temperature formed upon the collapse of the bubbles.

In summary, complete conversion of glycogen to glucose is achieved using heteropoly acid (HPW) as catalyst under optimal hydrothermal, microwave irradiation

and sonication conditions. The glycogen hydrolysis process developed is selective, fast and green.

As the heteropoly acid is water soluble, separation of the same after the hydrolysis is time consuming in the solvent extraction method. So as to make the process of separation of the catalyst from the product easier, we have developed a supported heteropoly acid catalyst HSiW/Graphene. The amount of HSiW on graphene was found to be 2.5 wt % by ICP analysis.

The sonochemical deposition of HSiW on the graphene substrate is presented in Scheme 2.



Scheme 2: Synthesis of the HSiW/G catalyst by the sonochemical

XRD results of HSiW/G are similar to those of graphene, which indicates that the HSiW adsorption process does not change the lattice constants of graphene and it adsorbed amorphously on graphene surface. The catalyst was further characterized by BET sorptometry, FTIR and Raman analysis.

The catalyst that prepared (HSiW/G) was used for the hydrolysis of glycogen under hydrothermal method. The activity of bare HSiW was found to be about 8 times lower than HSiW deposited on graphene. The increase of the catalytic activity of the supported HSiW compared to bare HSiW could be due to the effective and uniform distribution of the heteropoly acid on the surface of graphene. In addition, the observed enhanced activity of HSiW/G could be due to the strong binding of the heteropoly anions to the aromatic π-cloud on the graphene surface (similar to π–π interactions) leading to higher accessibility of the protons for the cleavage of the glycosidic bond in glycogen.

The stability of HSiW/G in the have been studied. After the first run under optimal hydrothermal conditions, the HSiW/G was separated from the aqueous product and reused for the second and third runs under identical reaction conditions.

The glucose yield after three reaction cycles was still high (63.3 wt %) indicating that HSiW can be reused without loss in catalytic activity. This indicates that the catalyst is supported system wherein the active component (HSiW) is strongly adhered to the support (graphene).

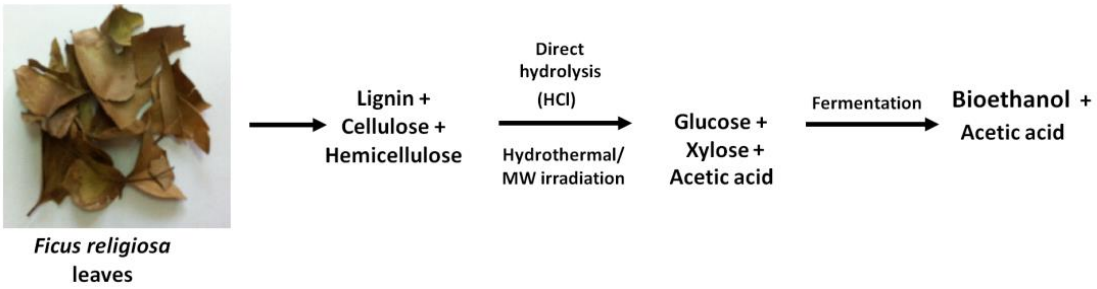
We suppose that the mechanism of interaction between HSiW/G and glycogen include: first, the hydrophobic graphene sheets of HSiW/G provide hydrophobic cavities to the glycogen on the surface of the catalyst. Then the hydrophobic parts (carbon rings) of the glycogen are encapsulated by the graphene, and the hydrophilic parts (OH and O) form hydrogen bonds with the heteropoly acid on the graphene surface. A combination of hydrophilic and hydrophobic regions on the catalyst surface leads to an increased concentration of glycogen on the catalyst surface and thereby contributes to the enhancement of hydrolysis rate and selectivity. Finally, the glycosidic bond is broken owing to the hydrolysis of the reactant.

Moreover, the usefulness of the catalyst was tested for the hydrolysis of cellulose. A glucose yield value of 33 wt % was obtained, indicating the activity of the catalyst (HSiW/G) for cellulose hydrolysis. Therefore, the catalyst designed could be used for the hydrolysis of cellulosic biomass.

The hydrolysis process with HSiW/G is selective, fast and green with a high yield of glucose. The sonochemical method provided strong anchoring of HSiW to graphene and high stability of the catalyst. The results presenting that HSiW/G is a reusable, economically viable and green catalyst.

In addition to glycogen, we have investigated the feasibility of using *ficus religiosa* leaves for the hydrolysis reaction and afterwards production of bioethanol.

Experimental methodology developed for the production of bioethanol from *ficus religiosa* leaves is depicted in Scheme 3.



Scheme 3: Process for the production of bioethanol from *ficus religiosa* leaves

5.5 Conclusions

Glycogen from bovine liver as well as from cyanobacteria has been converted to glucose in an acid catalyzed hydrolysis process. Microwave irradiation, sonication and hydrothermal pathways for glucose production were examined.

The microwave irradiation of glycogen under acid conditions (1 M HCl) yielded a complete conversion with the highest yield of glucose (62 wt %) in a time period as short as 10 min. When the hydrolysis reaction was carried out under conventional heating (oil bath), more than 6 h were needed for the complete conversion of glycogen. Thus, the accelerating effect of microwave energy for the production of fermentable sugar from glycogen has been demonstrated.

However, as HCl is corrosive, its separation is energy intensive and the process generates large amounts of acidic wastewater, identification of a reusable and non-corrosive catalyst for the bulk production of glucose is necessary. Heteropoly acids were found to be green and reusable solid acid catalysts for glycogen hydrolysis. As the heteropoly acid is water soluble, separation of the same after the hydrolysis is time consuming in the solvent extraction method. So as to make the process of separation of the catalyst from the product easier, we have developed supported heteropoly acid catalyst (HSiW) on graphene surface by the sonochemical method.

Physico-chemical characterization of the HSiW/G catalyst was done which demonstrated a homogeneous distribution of HSiW on the surface of graphene supports. The catalytic performance of the synthesized catalyst was evaluated for the hydrolysis of the glycosidic bond of biomass to glucose. The following conclusions have been drawn: the catalytic activity of HSiW/G is significantly higher than that of the bare HSiW. The hydrolysis process with HSiW/G is selective, fast and green with a high yield of glucose

(66 wt %). The sonochemical method provided strong anchoring of HSiW to graphene and high stability of the catalyst. The reuse of the HSiW/G catalyst in 3 cycles was demonstrated. The glucose yield after three reaction cycles was not altered significantly, showing that HSiW/G is a reusable, economically viable and green catalyst.

In addition to glycogen, we have investigated the feasibility of using *ficus religiosa* leaves for the production of bioethanol. The studies demonstrated that the waste leaves of *ficus religiosa* could be a potential feedstock for the production of bioethanol because of the abundance and wide global distribution. Such a process will be both environmentally friendly, in terms of waste management, regulations of CO_2 emissions and also an economically viable energy alternative. It was elucidated that the waste leaves of *ficus religiosa* could yield 3 wt % of ethanol without any pretreatment or delignification. Further studies in this direction, could develop a potential process for the sustainable ethanol production for transportation applications.

Taken together, our study further highlights the potential of glycogen as a feedstock for glucose production. The glycogen hydrolysis process developed is selective, fast and green. As glycogen can be produced from abundant and renewable chemical feedstock like CO_2, the use of glycogen as a precursor for glucose generation is an economically and environmentally benign alternative to cellulose. Thus, glycogen is a potential feedstock for the production of glucose. In addition, the catalysts and the methods used for glycogen hydrolysis could be used for the hydrolysis of cellulosic biomass as well.

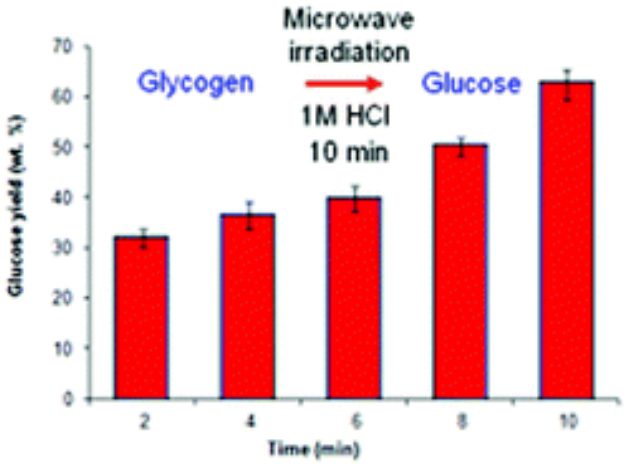
6. Articles Incorporation

6.1. Article 1

Direct production of glucose from glycogen under microwave irradiation

Miri Klein, Indra Neel Pulidindi, Nina Perkas, Ella Meltzer-Mats, Arie Gruzman and Aharon Gedanken

RSC Adv. **2012**, *2*, 7262−7267



6.2. Article 2

Heteropoly acid catalyzed hydrolysis of glycogen to glucose

Miri Klein, Indra Neel Pulidindi, Nina Perkas, Aharon Gedanken

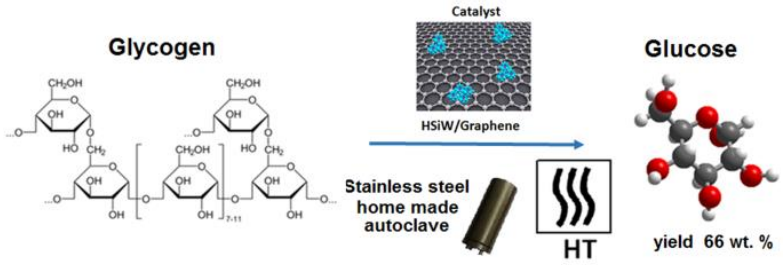
Biomass and Bioenergy **2015**, DOI: 10.1016/j.biombioe.2015.02.036

6.3. Article 3

Sonochemical synthesis of HSiW/graphene catalysts for enhanced biomass hydrolysis

Miri Klein, Alexander Varvak, Elad Segal, Boris Markovsky, Indra Neel Pulidindi, Nina Perkas and Aharon Gedanken

Green Chemistry **2015**, DOI: 10.1039/c4gc02519a



Graphene supported silico-tungstic acid catalyst was synthezied sonochemically. Glucose yield was dramatically increased by reusable HSiW/G in the biomass hydrolysis.

6.4. Article 4

Bioethanol production from *Ficus religiosa* leaves

Miri Klein, Ofir Griess, Indra Neel Pulidindi, Nina Perkas, Aharon Gedanken

Renewable energy, **2015** (under review)

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