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Noor

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Conclusions & Recommendations

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Conclusions:

1. All isolates of *G. xylinus* were cellulose producers with variable degrees.
2. Locally isolated *G. xylinus* N2 harbour only one plasmid and this plasmid may have a regulatory effect on cellulose production.
3. Mutagenesis of *G. xylinus* using UV ray was efficient in obtaining cellulose enhanced producing mutant.
4. Date molasses and yeast extract were optimal carbon and nitrogen sources respectively for cellulose production in concentration of 2% for both, in an initial medium pH 6.5 and incubation at 30°C for one week.

Recommendation:

1. Cloning of cellulose gene (s) in suitable vector and used to transform wild type of *G. xylinus* for cellulose higher production.
2. Studying the effect of other types of physical and chemical mutagens on the ability of *G. xylinus* N2 in cellulose production.
3. Purification of cellulose produced by *G. xylinus* N2_90 for different applications.

1. Introduction and Literatures Review

1.1 Introduction

Cellulose is the most abundant macromolecule on the earth (Brown, 2004), and most cellulose is produced by vascular plants. A substitute to reduce the demand from plants is the production of cellulose using a microbial system (Lynd *et al*., 2002).

Bacterial cellulose produced by *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) could be an interesting alternative for the plant-derived material, especially since the bacterial cellulose is produced in a pure (free from other polymers) it does not have hemicellulose or lignin that need to be removed and can be grown to virtually any shape, crystalline form which makes its recovery relative simple, and it has exceptional physicochemical properties, such as ultrafine reticulated structure, high tensile strength, high hydrophilicity, moldability during formation, and biocompatibility, although its chemical structure is similar to those of cellulose produced by plants and algae (Helenius *et al*., 2006).

The bacterium *G. xylinus* produce pure cellulose. A single cell may polymerize up to 200,000 glucose residues per second into β-1, 4-glucan chains. Advantages of using a bacterial system for production of cellulose is that the bacterium grows rapidly under controlled conditions and produces cellulose from a variety of carbon sources including glucose, ethanol, sucrose, and glycerol (Saxena *et al*., 2000).

Bacterial cellulose have wide applications in various fields such as healthcare, cosmetics and beauty, clothing and shoes, baby care products, audio products like speaker diaphragms. It is also being used in paper industry to

enhance paper strength and for making electronic paper (Shah and Brown, 2005; Czaja *et al*., 2006); in pharmaceutical industry as a filtration membrane and wound dressing, gelling agents and in medicines in artificial skin, duraplasty, nerve anastomosis, artificial blood vessels or barrier to bone defects (Czaja *et al*., 2007); American Chemical Society in Science daily on February 2007 reported that biotechnology's next high-value product could be microbial cellulose (Science Daily, 2007). And it is used in preparation of nanocomposites for biomedical purpose (Panesar *et al*., 2009).

The wide application of bacterial cellulose brings big interests towards the bacterial cellulose production for large commercial scale. Some attempts have been made in the area of optimization of culture conditions (Kouda *et al*., 1997), medium composition (Matsuoka *et al*., 1996), strain improvement (Vandamme *et al*., 1998); Norhayati, (2009) designed a Rotary Discs Reactor (RDR) for enhanced production of microbial cellulose, which gave 86.78% higher production of microbial cellulose compared to static fermentation.

According to the great importance of cellulose in industry, this study was focusing on cellulose production from local isolate of *G. xylinus* and genetic determinants affects cellulose production throughout the following steps:

- Isolation and identification of *Gluconacetobacter xylinus* from different food sources.
- Screening the ability of bacterial isolates in cellulose production and selecting the efficient producer isolate.
- Studying the plasmid profile of the selected isolate.
- Determination the role of plasmid in cellulose production, by curing experiments.

- Enhancing the ability of the selected isolate in cellulose production by random mutagenesis.
- Determination of optimum conditions for cellulose production by *G. xylinus* mutant.

1.2 Literature review

1.2.1 *Gluconacetobacter*

Gluconacetobacter is a genus of acetic acid bacteria (AAB) which it a motile / nonmotile Gram-negative, coccoid or rod-shaped, catalase positive, oxidase negative with optimum growth temperature of 25 – 30° C, obligate aerobic bacteria that have the ability to incompletely oxidize a wide range of carbohydrates, alcohols and sugar alcohols. They are involved in the production of several fermented foods and beverages, either in a beneficial (vinegars, cocoa-based products, Kombucha and nata de coco) or detrimental (spoilage of beer, wine and cider) manner and are also used in the production of commercially important fine chemicals and bacterial cellulose. Some AAB have been described as human pathogens (Greenberg *et al*., 2006; Kersters *et al*., 2006; Tuuminen *et al*., 2007), and some AAB strains specially those of *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* spp could produce various polysaccharides e.g. cellulose, dextran and levan. Some mutated *Acetobacter* strains could release heterogeneous exopolysaccharides combined from glucose, mannose, rhamnose and glucuronic acid (Kadere *et al*., 2008).

Acetic acid bacteria are classified into ten genera comprising 52 recognized species in the family Acetobacteraceae of the class Alphaproteobacteria, i.e. the genera *Acetobacter* (19 species), *Acidomonas* (1 species), *Asaia* (4 species), *Gluconacetobacter* (16 species), *Gluconobacter* (7 species), *Granulibacter* (1

species), *Kozakia* (1 species), *Neoasaia* (1 species), *Saccharibacter* (1 species) and *Swaminathania* (1 species) (Malimas *et al*., 2007, 2008a, b; Cleenwerck *et al*., 2008; Cleenwerck and De Vos, 2008; Yamada and Yukphan, 2008).

Multiple studies have shown that accurate identification of AAB isolates is difficult (Greenberg *et al*., 2006; Tuuminen *et al*., 2006). While identification to the genus level can generally be achieved by 16s rRNA gene sequence analysis and some phenotypic tests. Identification to the species level can be problematic. Species identifications based solely on phenotypic data are not recommended and, for this reason, several authors have advised the use of genotypic data as the basis for species identification of AAB (Kersters *et al*., 2006; Cleenwerck and De Vos, 2008; Yamada and Yukphan, 2008).

1.2.2 *Gluconacetobater xylinus* formerly *Acetobacter xylinum*

Acetobacter xylinum, which is the most efficient producer of cellulose, has been reclassified and included within the novel genus *Gluconacetobacter*, as *Gluconacetobacter xylinus* together with some other species (*G. hansenii, G. europaeus, G. oboediens* and *G. intermedius*) (Yamada *et al*., 1998; Yamada, 2000).

G. xylinus is a Gram-negative bacteria, rod to oval shape, they occur singly, in pairs or in chains, reproduce by binary fission, are motile by flagella, do not form endospores, strictly aerobic bacteria and it produce acetic acid (acetic acid bacteria). The optimum temperature for *G. xylinus* growth is 25–30°C, and optimum pH ranges from 5.4 to 6.2. *G. xylinus* had been applied as a model microorganism for cellulose production studies (Cannon and Anderson, 1991). It usually found on fruits and vegetables, in vinegar, fruit juices and alcoholic beverages. *G. xylinus* produces cellulose on the surface of liquid culture media.

Although synthesis of an extracellular gelatinous pellicles by *G. xylinus* was reported for the first time in 1896 by Brown (Brown and Saxena, 2000), attracted more attention in the second half of the 20th century. Intensive studies on bacterial cellulose synthesis using *G. xylinus* as a model bacteria were started by Hestrin *et al*., (1947); Hestrin and Schramm, (1954), who proved that resting and lyophilized *Gluconacetobacter* cells synthesized cellulose in the presence of glucose and oxygen.

In static culture condition the produced cellulose will be gelatinous, leather-like mats formed on the surface of liquid culture media, called a pellicle. Under agitated culture condition cellulose forms irregular granules stellate and fibrous strands (Bielecki *et al*., 2005). On agar media *G. xylinus* forms colonies with equal or undulate edges, transparent or white, smooth or rough, flat or convex (Yamada, 2000).

G. xylinus strains are prone to spontaneous mutations yielding cellulose non-producing cells, which is one of the major problems facing commercial exploitation of bacterial cellulose biosynthesis. The appearance of cellulose-nonproducing (Cel^-) forms in agitated cultures was first described by Schramm and Hestrin, (1954) who isolated three different types of *G. xylinus* cells distinguished by morphology of colonies and efficiency of cellulose biosynthesis:

— Type I: wild-type, cellulose-producing (Cel^+) cells.
— Type II: cellulose-nonproducing forms (Cel^-) capable of reverting through passages.
— Type III: non-reverting cellulose-nonproducing forms (Cel^-).

The morphology of Cel^+ and Cel^- colonies is different. Colonies of Cel^- cells are rough, flat, slimy with undulate edges, in contrast to those of the Cel^+ type,

which are spherical with smooth edges, gelatinous and convex. The frequency of Cel^{+} to Cel^{-} conversion depends on culture conditions and their changes.

1.2.3 Bacterial cellulose

Cellulose is the most abundant polymer in green plants and can be found in certain fungi, protozoa, and procaryotes (Whistler and Miller, 1997; Taylor, 2008). Cellulose is a long unbranched polymeric chain with molecular formula $(C_6H_{10}O_5)_n$, is composes simply from D-glucose. In the chain the sugar monomers are uniformly linked by β 1-4 glycosidic bonds as shown in figure (1-1).

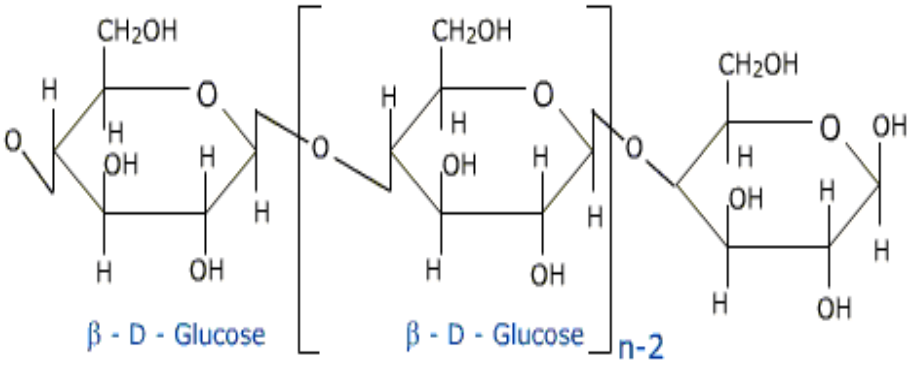

Figure (1-1): Chemical structure of Cellulose (Martin, 2009)

Extensive research on bacterial cellulose show that it is chemically identical to plant cellulose, but it differs in the macromolecular structure and properties (Klemm *et al.*, 2001). One of the most important features of bacterial cellulose is its chemical purity, which distinguished from plant cellulose which usually associated with hemicelluloses, lignocelluloses (Fiedler *et al.*, 1989) and waxy aromatic substance which its removal are very difficult (Ross *et al.*, 1991). Bacterial cellulose can be purified using less energy or chemical-intensive

processes without the hazardous of by-products (Hong *et al*., 2005). Particularly impressive is the fact that the size of bacterial cellulose fibrils is about 100 times smaller than that of plant cellulose as shown in figure (1-2). This unique nano-morphology results in a large surface area that can hold a large amount of water (up to 200 times of its dry mass) the majority of water is not bound with the polymer and can be squeezed out by gentle pressing (Czaja *et al*., 2004).

Bacterial cellulose also have high tensile strength, extremely insoluble and elasticity, durability and shape retention, high crystallinity index due to its reticulated structure, in which numerous ribbon-shaped fibrils, are composed of highly crystalline and highly uniaxially oriented cellulose subfibrils, this structure, not found in the plant originating cellulose, brings about higher crystallinity index (60-70%) of bacterial cellulose (Watanabe *et al*., 1998).

Bacterial cellulose has high mechanical strength due to the inter-fibrils hydrogen bonds, which give stability to the structure (Aase, 2007; Kristoffer, 2008). In drying process of bacterial cellulose, the nano-fibers arrange parallel to each other and form layered sheets. These give the dried cellulose sheets high stability and strength, as there is the formation of more hydrogen bonds among the fibers (Yamanaka, 1989; Jonas and Farah, 1998). Many of other distinguishing features of bacterial cellulose are shown in table (1-1).

Bacterial cellulose can be molded into any shape and size during it synthesized, depending on the fermentation technique and conditions used (Bielecki *et al*., 2002). Highly nano-porous material that allows for the potential transfer of antibiotics or other medicines into wound, while at the same time serving as an efficient physical barrier against any external infection (Czaja *et al*., 2006), for all this properties have awide industrial impotant.

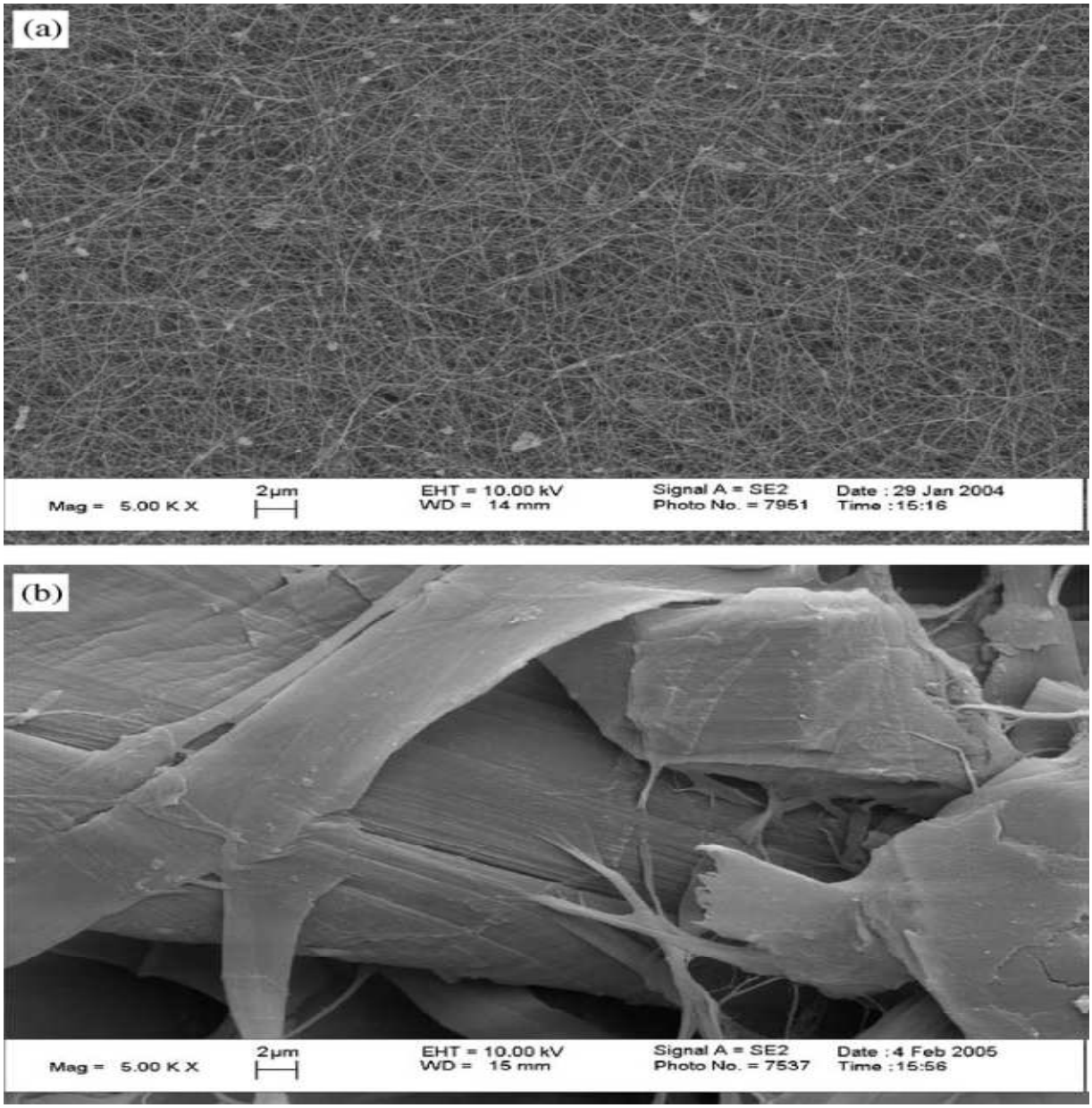

Figure (1-2): A comparison of microfibrillar organization between *Gluconacetobacter* cellulose (a) and wood pulp (b) (bothat 5000x). Ultrafine net of microbial cellulose left has a very smooth network of microfibrils. Newsprint from wood pulp right has similar microfibrils, but they are part of a larger aggregation of the cell wall remains (Wojciech *et al*., 2006)

Table (1-1): Distinguish Features of Bacterial Cellulose (Brown, 2000)

Feature	Characterization
Purity	Cellulose is the only biopolymer synthesized. Absence of lignin or hemicelluloses. Completely biodegradable and recyclable of a renewable resource.
Great Mechanical Strength	Strength crystalline cellulose I. Consistent dimensional stability. High tensile strength. Light weight. Remarkable durability.
Extraordinary Absorbency in the Hydrated State	Remarkable capacity to hold water. Selective porosity. High wet strength. High surface-to-volume carrier capacity.
Direct Membrane Assembly during Biosynthesis	Intermediate steps of paper formation from pulp unnecessary. Intermediate steps of textile assembly from yarn unnecessary. Extremely thin, sub-micron, optically clear membranes can be assembled.

Bacterial cellulose is synthesized by several bacterial genera, *Gluconacetobacter xylinus* which is the most efficient producer of cellulose (Yamada *et al.*, 1998; Yamada, 2000). An overview of bacterial cellulose

producers are presented in table (1-2). Bacterial cellulose are a products of primary metabolism. The polymer structure depends on the organism, although the pathway of biosynthesis and mechanism of its regulation are probably common for the majority of cellulose-producing bacteria (Ross *et al*., 1991; Jonas and Farah, 1998).

Table (1-2): Bacterial cellulose producers (Jonas and Farah, 1998)

Genus	Cellulose structure
Guloconacetobacter	Extra-cellular pellicle
Achromobacter	Fibrils
Aerobacter	Fibrils
Agrobacterium	Short fibrils
Alcaligenes	Fibrils
Pseudomonas	No distinct fibrils
Rhizobium	Short fibrils
Sarcina	Amorphous cellulose
Zoogloea	Not well defined

1.2.4 Application of bacterial cellulose

Because of the unique properties of bacterial cellulose, resulting from the ultrafine reticulated structure, bacterial cellulose have many applications in paper, textile, food industry, cosmetics and medicine (Ring *et al*., 1986), as listed in table (1-3).

Table (1-3): Industrial application of bacterial cellulose (Brown and Sexena 2000)

Industry	Product
Food	Serum cholesterol reduction Base for artificial meat Thickeners (ice cream and salad dressing) Desserts (low calorie ice cream, chips, candies)
Healthcare	Wound care dressings Drug delivery agents, either oral or dermal Artificial skin substrate
Cosmetics and Beauty	Skin creams Base for artificial nails
Environmental	Oil spill cleanup sponge Absorptive base for toxic material removal
Petroleum and Mining	Mineral and oil recovery
Clothing and shoes	Artificial leather products One piece textiles
Public Utilities	Water purification via ultra filters and reverse osmosis membranes
Baby care products	Disposable recyclable diapers
Audio products	Superior audio speaker diaphragms
Forest products	Artificial wood strengthener (plywood laminates) Filler for paper High strength containers

Specialty Papers	Archival document repair Paper base for long-live currency

1.2.5 Bacterial cellulose biosynthesis

Cellulose is exist in a crystalline or non-crystalline state. Two common crystalline forms of cellulose defined as I and II are distinguished by X-ray, nuclear magnetic resonance (NMR) and infrared analysis (Johnson and Neogi, 1989; Brown, 1999). Cellulose I is a parallel 1, 4-β-glucan chains are arranged uniaxially, this type of cellulose are synthesized by the majority of plants and by *G. xylinus* in static culture whereas 1,4-β-glucan chains of cellulose II are mostly anti-parallel and arranged in random manner and linked with a large number of hydrogen bonds which give it a higher thermodynamic stability, this type of cellulose is synthesized by few organisms like some algae, moulds and bacteria such as *Sarcina ventriculi* (Jonas and Farah, 1998; Watanabe *et al*., 1998).

The bacterium *G. xylinus* produces pure cellulose, a single cell may polymerized up to 200,000 glucose residues per second into 1,4-β-glucan chains, another advantages of using a bacterial system for cellulose production is that the bacterium grows rapidly under controlled conditions and produces cellulose from a variety of carbon sources including glucose, ethanol, sucrose and glycerol. Moreover, genetic analysis is aided by isolation of a large number of mutants affected in cellulose biosynthesis (Haigler and Benziman, 1982).

The synthesis of cellulose in *G. xylinus* require two-step process involving polymerization and then crystallization of the individual glucan chains into native cellulose I. The production of cellulose I probably requires a certain topological organization of the biosynthetic apparatus, and it is believed that *in vitro* cellulose

synthesis in extracts from *G. xylinus* results in the production of the crystalline cellulose II (Bureau and Brown, 1987).

G. xylinus produce the cellulose between the outer and the plasma membrane. A single cell of *Gluconacetobacter* has a linear row of pores, these pores are secrete mini-crystals of glucan chains (cellulose-synthesizing complexes or terminal complex TC). In the first step of cellulose formation glucan chain are aggregate to consist approximately 6-8 glucan chain, these subelementary fibrils are assembled in the second step to form microfibrils followed by their tight assembly to form ribbon in the third step as shown in figure (1-3) and (1-4). The ribbon can be observed directly using light microscope (Brown, 1999; Klemm *et al*., 2001).

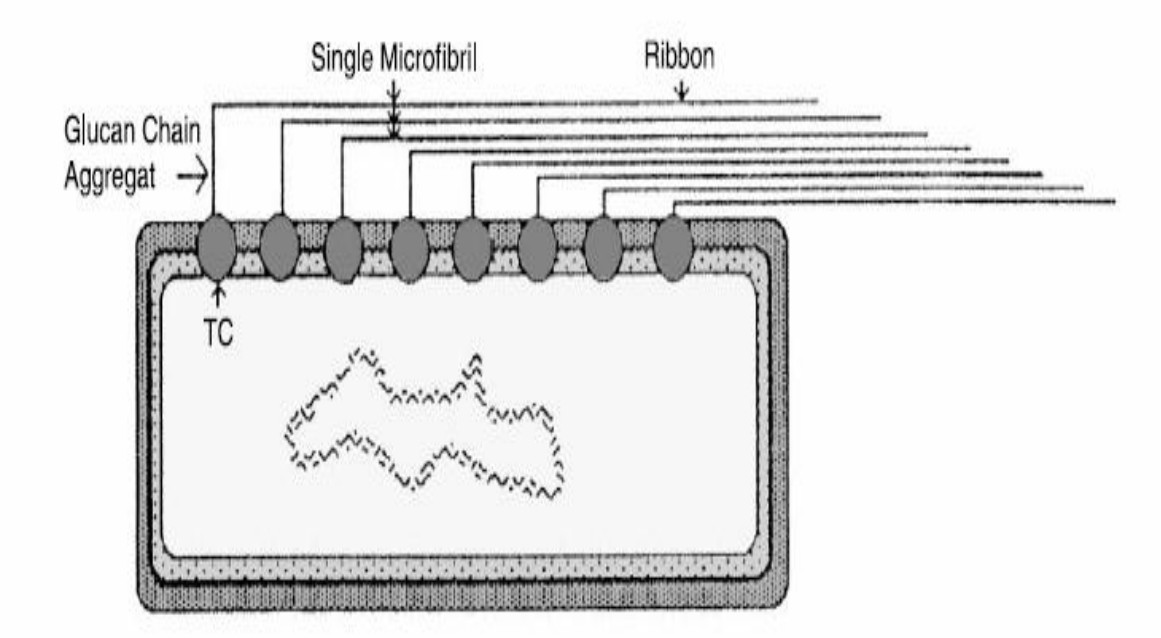

Figure (1-3): Formation of bacterial cellulose (Klemm *et al*., 2001)

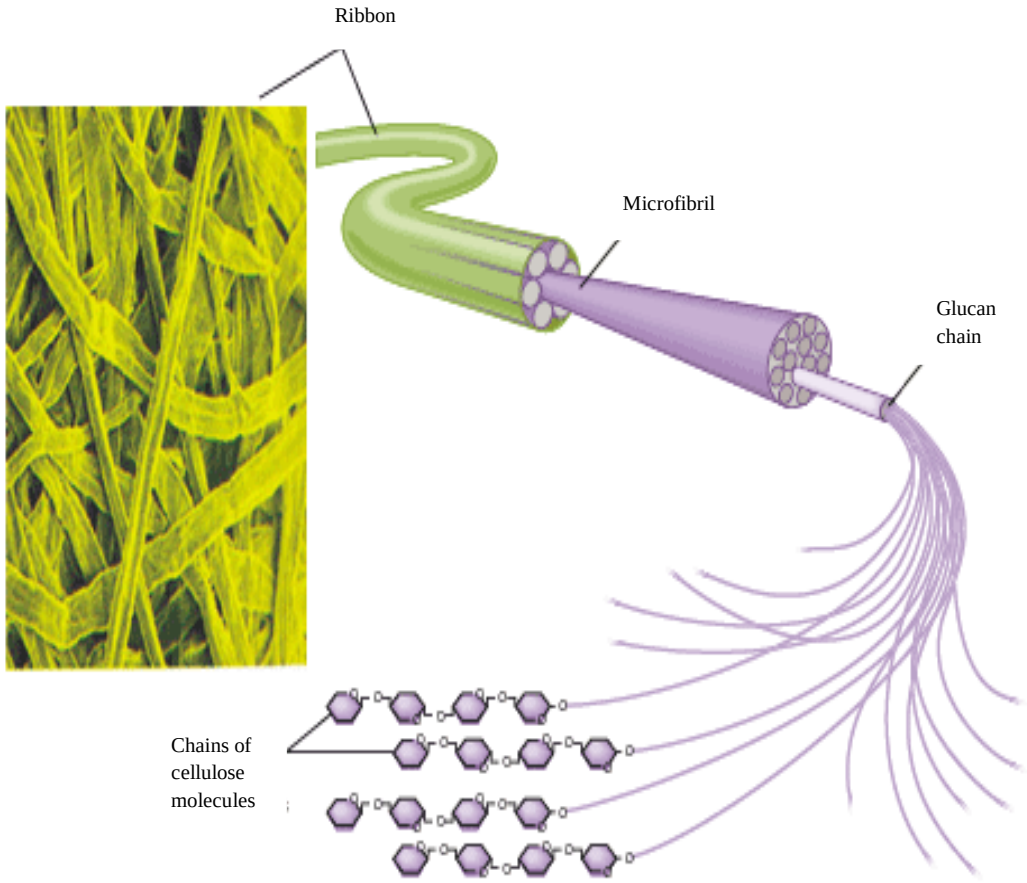

Figure (1-4): The structural binding material in cellulose (Kontturi, 2005)

The biosynthetic pathway for cellulose biosynthesis indicated in figure (1-5) is very well understood in *G. xylinus*. The pathway from the substrate glucose to cellulose involves a number of reactions in which glucose is first converted to glucose-6-phosphate by the enzyme glucokinase. In the second step, glucose-6-phosphate is converted to glucose-1-phosphate by the enzyme phosphoglucomutase. In the next step, glucose-1-phosphate is converted to uridine-diphosphoglucose (UDP-glucose) by the enzyme UDPG pyrophosphorylase.

UDPG pyrophosphorylase (also known as glucose-1-phosphatase) is thought to play an important role in cellulose synthesis because the Cel^{-} forms of *G. xylinus* lack this enzyme. The UDP-glucose produced is used as a substrate by the enzyme

cellulose synthase which convert it to cellulose (Saxena *et al*., 2000).

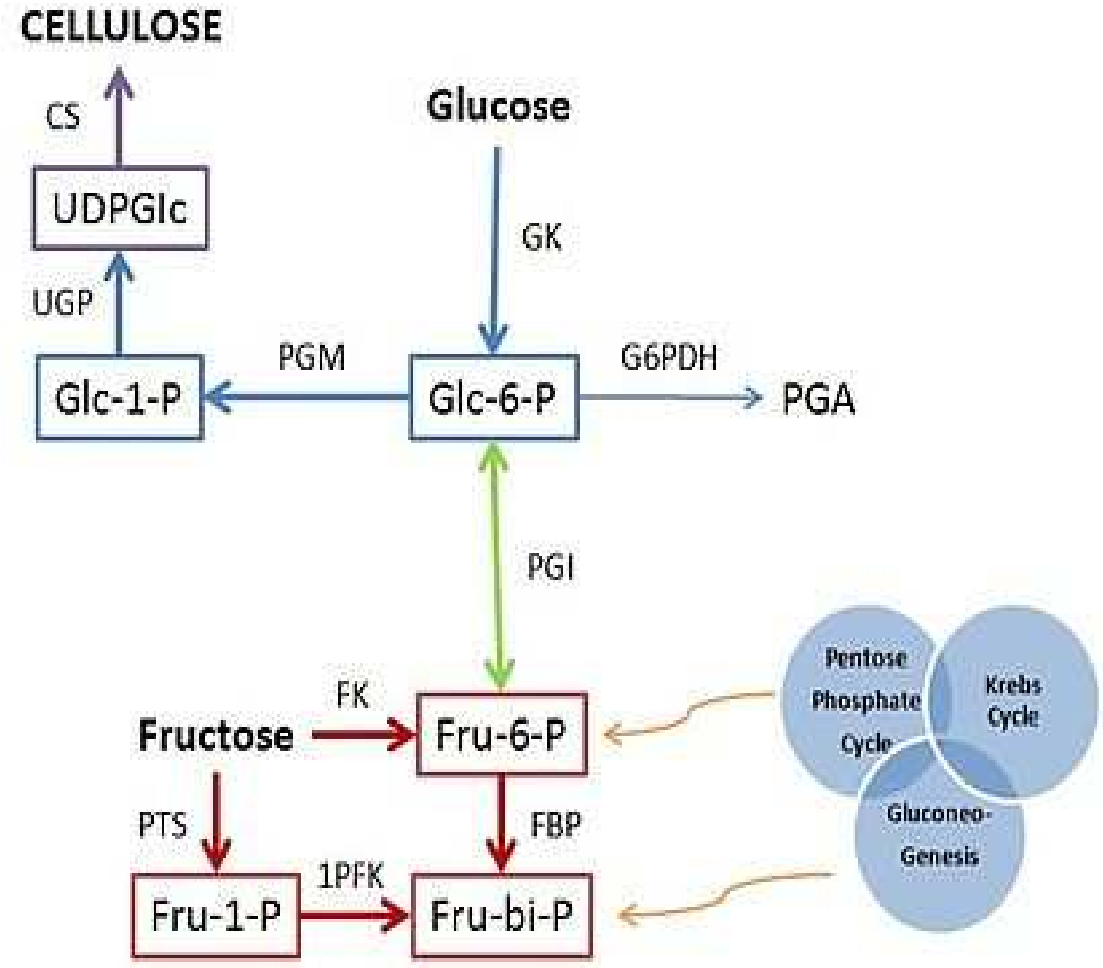

Figure (1-5): Pathways of carbon metabolism in *G. xylinus.* CS, cellulose synthase; FBP, fructose-1,6-biphosphate phosphatase; GK, glucokinase; G6PDH, glucose-6-phosphate dehydrogenase; 1-PFK, fructose-1-phosphofructokinase; PGI, phosphoglucoisomerase; PGM, phosphoglucomutase; PTS, system of phosphotransferases; UGP, pyrophosphorylase UDPGLc; Fru-bi-p, fructose-1-6,bi-phosphate; Fru-6-p,fructose-6-phosphate, Glc-6-p, glucose-6-phosphate; PGA, phosphogluconic acid; UDPGlc, uridine diphosphoglucose (Ross *et al*., 1991; Tonouchi *et al*., 1996).

Cellulose synthase (CS) is considered to be the most important enzyme in this process (Saxena and Brown, 2005). It is subjected to a complicated regulation mechanism, which control the activation and inactivation of the enzyme (Klemm *et al*., 2001).

The rate of cellulose production depended proportionally on the surface-area of the culture medium and was unaffected by the depth and volume of the medium. The optimum pH for cellulose production was 4.0 to 6.0 (Embuscado *et al*., 1994).

Cellulose synthesized by *G. xylinus* are depending on the physiological state of the cell involves either the pentose phosphate cycle or the krebs cycle, coupled with gluconeogenesis (Ross *et al*., 1991; Tonouchi *et al*., 1996). In *G. xylinus*, cellulose synthesis is tightly associated with catabolic processes of oxidation and consumes as much as 10% of energy derived from catabolic reactions. Bacterial cellulose production does not interfere with other anabolic processes, including protein synthesis (Ross *et al*., 1991). *G. xylinus* also can convert various carbon compounds, such as hexoses, glycerol, dihydroxyacetone, pyruvate, and dicarboxylic acids, into cellulose, usually with about 50% efficiency (Cannon and Anderson, 1991; Iannino *et al*., 1998). The latter compounds enter the Krebs cycle and due to oxalacetate decarboxylation to pyrovate undergo conversion to hexoses via gluconogenesis, similarly to glycerol, dihydroxyacetone and intermediates of pentose phosphate cycle as shown in figure (1-5).

1.2.6 Regulation of cellulose biosynthesis

The cellulose biosynthesis are regulated by c-di-GMP (cyclic di Guanine Mono Phosphate). Figure (1-6) illustrated the structure of c-di-GMP.

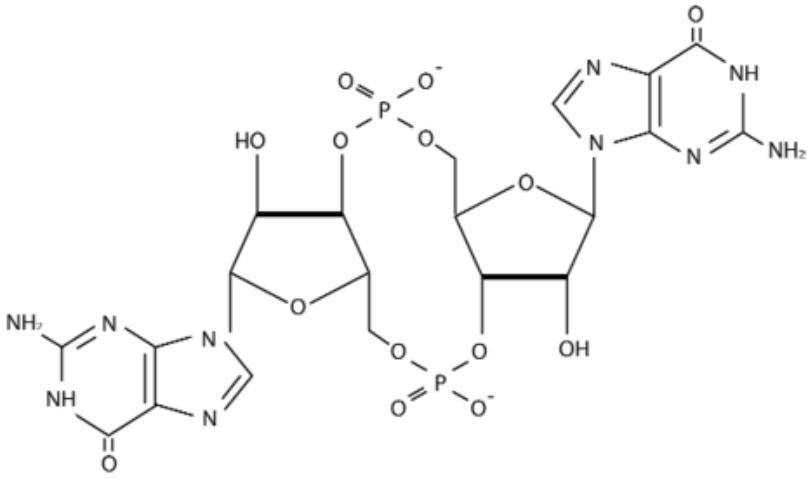

Figure (1-6): Structure of c-di-GMP. G, Guanine (Ross *et al.*, 1987)

In the absence of c-di-GMP, the cellulose synthase will be in low activity or inactivity state. C-di-GMP may be referred to be allosteric effector of cellulose synthase, it meaning that it binds directly to the enzyme in a regulatory site which it distinct from catalytic or substrate binding-sites (Ross *et al.*, 1990).

The c-di-GMP functions as a reversible, allosteric activator of the membrane bound cellulose synthase. Diguanylate cyclase catalyzes the synthesis of c-di-GMP from two molecules of GTP, via the linear dinucleotide triphosphate pppGpG, in two distinct PP_i-releasing steps. Within the cell, pp_i is rapidly cleaved to yield p_i (Benziman and Palgi, 1970). The pathway of c-di-GMP degradation is initiated by c-di-GMP-spesific Ca^{+2}-sensitive phosphodiesterase A, PDE-A, which cleaves a single phosphodiester bond in the cyclic structure, yielding the inactive linear dimer pGpG, which it converted to free 5'-GMP units through the action of second phosphodiesterase B, PDE-B. The Ca^{+2} inhibition of PDE-A represents an additional locus of regulatory control, according to this feature of the system, fluctuations in Ca^{2+} levels may modulate the rate of cellulose synthesis as they

influence the persistence of activator in the system as shown in figure (1-7) (Tal *et al*., 1998).

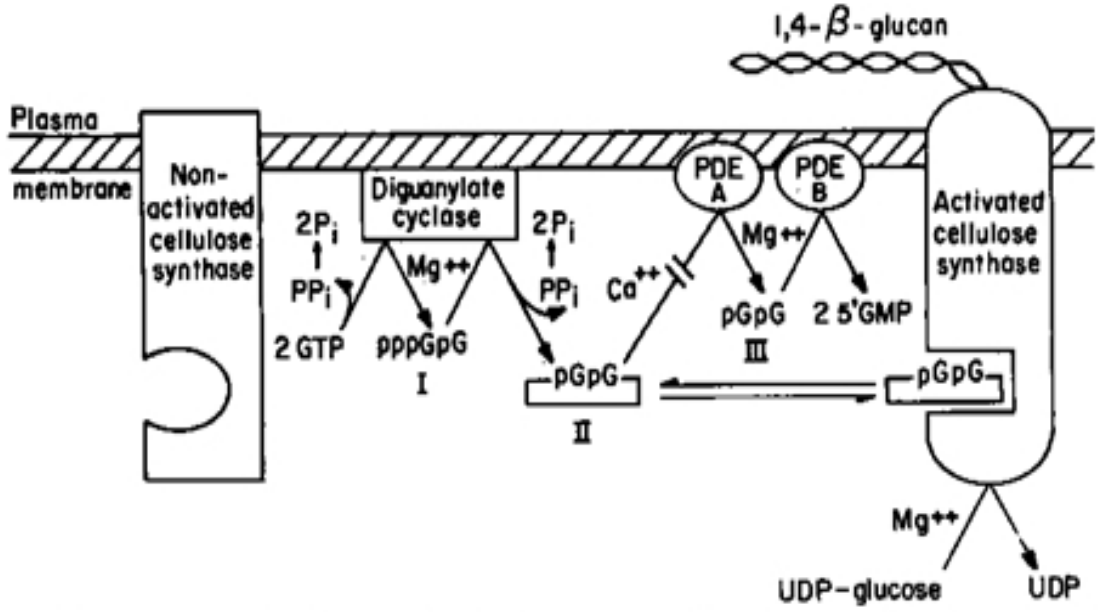

Figure (1-7). Proposed model for regulation of cellulose synthesis in *G. xylinus* (Ross *et al*., 1991)

1.2.7 Cellulose synthase (CS)

Cellulose synthase of *G. xylinus* is a typical anchored membrane protein, having molecular mass of 400-500 kDa and CS is localized on the cytoplasmic side of the cell membrane. Because of this localization, purification of CS was extremely difficult, and isolation of the membrane fraction, before CS solubilization and purification was necessary. Furthermore, *G. xylinus* CS appeared to be a very unstable protein . CS isolation from membranes was carried out using digitonin, or detergents (Triton X-100) and treatment with trypsin (Lin and Brown, 1989; Brown and Saxena, 2000), followed by CS entrapment on cellulose. Mayer *et al*., (1989) mentioned that the purified CS preparation contained three different types of subunits, having

molecular mass of 90, 67 and 54 kDa. Saxena and Brown, (1989) found only two types of polypeptides (83 and 93 kDa).

1.2.8 Physiological functions of bacterial cellulose in bacterial culture

1. To hole the cell in aerobic environment: cells of cellulose-producing bacteria are entrapped in the polymer network and supporting the bacterial population with air and liquid (Williams and Cannon, 1989).
2. To colonized on food and substrate: the polymer matrix takes part adhesion of the cell onto accessible surface and facilitates nutrient supply, since their concentration in the polymer lattice is markedly enhanced due to its adsorptive properties in comparison to the surrounding aqueous environment (Jonas and Farah, 1998; Costeron, 1999). Okamoto *et al*., (1994) elucidated that cellulose synthesized by *G. xylinus* also plays a storage role and can be utilized by the starving microorganisms.
3. Prevent potential competitor: because of the viscosity and hydrophilic properties of the cellulose layer it prevent unfavorable change (decrease in water content, variations in pH, appearance of toxic substances, pathogenic organisms, etc.) during cells growth (Ross *et al*., 1991).
4. Protect from ultraviolet radiation (UV): bacterial cellulose can protect the growing bacteria from the killing effect of UV light (Koo *et al*., 1991). As much as 23% of growing bacteria which covered with the bacterial cellulose are survived for 1 hour treatment with UV. Removal of the cellulose brought about a drastic decrease in bacterial viability (3% only) (Ross *et al*., 1991).

5. Help retain moisture: it may be used as moisturized, because it is very hydrophilic and aid to retain moisture to prevent substrates drying whiles the bacterium growing on them (Ross *et al*., 1991).

1.2.9 Bacterial cellulose production

Cellulose can be produced in several techniques, the choice of cultivation technique is depend on commercial destination considering that cellulose ultrastructure and its physical and mechanical properties are strictly affected by the culture method (Galas *et al*., 1999).

1.2.9.1 Static culture condition

In static culture method, gelatinous membrane of bacterial cellulose is accumulated on the surface of the culture medium and form a pellicle. The productivity of bacterial cellulose is depend on the surface area of the medium, in such system the pellicle is formed on an oxygen-permeable membrane to increase surface area (Yoshino *et al*., 1996), bacterial cellulose yield in static culture is also depending on the surface to volume ratio (s/v). Optimum s/v ratio are protect from either too high (unnecessary) or too low aeration (Krystynowicz *et al*., 1997).

The static culture method have disadvantages such as it cannot be used to produce large-scale industrial production because it need a high labor cost and low productivity because the synthesis of the polymer only in the form of a sheet (Toyosaki *et al*., 1995; Zuo *et al*., 2006), and the control of bacterial cellulose synthesis in this culture method is very difficult since the pellicle limit an access to the liquid medium, one of the important parameter which require continous control is pH.

Vandamme *et al*., (1998) applied an *in situ* pH control via an optimized fermentation medium design, based on introducing acetic acid as an additional substrate for *Gluconacetobacter* sp. LMG 1518. Products of acetic acid catabolism counteracted the pH decrease caused by keto-gluconic formation and provided constant pH of the growth medium, equal to 5.5, throughout the whole process.

1.2.9.2 Agitate culture conditions

Cellulose can be produced in the form of a fibrous suspension, irregular masses, pellets or spheres. The productivity of bacterial cellulose in agitated culture is depend on the growth of the cellulose-producing organisms increasing the cell density is essential for increasing bacterial cellulose productivity, screening on suitable strains and culture medium components are very important (Dudman, 1960). Since the growth of *Gluconacetobacter* (a typical aerobic bacterial cellulose producer) is dependent on oxygen supply, oxygen supply is directly associated with bacterial cellulose productivity, but excessive oxygen supply is reported to decrease in bacterial cellulose productivity because it losing of substrate by direct oxidation (Yamanaka *et al*., 2000). Agitated culture is considered more suitable for the commercial bacterial cellulose production due to the higher production rates that potential can be achieved (Yamanaka *et al*., 1989). Bacterial cellulose production in agitation and aeration encounters many problems such as spontaneous appearance of Cel^- mutans (cellulose non-producers), which contributes to a decline in polymer synthesis (Ross *et al*., 1991) and it often lead to morphological and structural abnormalities of bacterial cellulose due to high shear stress caused by the agitation (Zuo *et al*., 2006).

Studies on the effect of culture medium composition on the efficiency of cellulose synthesis under agitated culture condition revealed that culture media

contain ethanol (2% v/v) gives the highest yield of cellulose polymer (Son *et al*., 2001; Krystynowicz *et al*., 2002). According to Son *et al*. (2001) this phenomenon resulted not from change in metabolism but from the absence of the conversion of Cel^+ cells to Cel^-. Hence, the spontaneous change of *G. xylinus* Cel^+ cells to cellulose- negative phenotype in agitated cultures can be decrease by addition of ethanol to culture media.

1.2.9.3 Horizontal fermentors

Cellulose production in horizontal fermentor is the combination of stationary and submerged cultures. The polymer is deposited on the surface of rollers or discs, rotating around the long axis. The advantages of this method include a large polymer surface, synthesis of cellulose in a form of hollow fibers, different in diameter, as well as good process control, easy scale enlargement, appropriate accessible surface for adhesion of bacteria and product deposition, higher rate of cellulose production (Sattler and Fiedler, 1990).

1.2.9.4 Rotary Discs Reactor (RDR)

This reactor was designed by Norhayati, (2009) for enhanced production of microbial cellulose by *G. xylinus*. One of the major factors that determine the success of fermentation process is aeration during fermentation. This reactor consists of an array of discs that is mounted to a shaft. The shaft is connected to a driven motor so that the rotation of the shaft together with the discs is achievable and controllable. The discs on the shaft are positioned in a horizontally set trough that contains a biological medium in which at least a portion of the contained discs are being submerged. In the preliminary study of discs selection, discs made from

stainless steel fabricated with 0.3cm mesh sizes gave the highest result compared to others.

Also it was found that rotational speed gives significant effect towards microbial cellulose production where fermentation in RDR using 7 rpm gave the highest microbial cellulose production which gave 86.78% higher production of microbial cellulose compared to static fermentation (Norhayati *et a*l., 2011).

1.2.10 Bacterial cellulose purifications

Bacterial cellulose obtained through static or agitated culture is not pure and contains some impurities such as culture broth components and *G. xylinus* cells. Prior to use in medicine, food production or paper industry, all these impurities must be removed. One of the most widely used purification methods is based on treatment of bacterial cellulose with solutions of hydroxides (mainly sodium and potassium), sodium chlorate and hypochlorate, H_2O_2, diluted acids, organic solvent or hot water. The reagents can be used alone or in combinations, bacterial cellulose immersed in the solutions for 14-18 h, in some cases up to 24 h at elevated temperature (55- 65°C), markedly reduces the number of cells and coloration degree. Bacterial cellulose boiled in 2% NaOH solution after preliminary running tap water treatment (Yamanaka *et al*., 1990). Another purification method by immersing bacterial cellulose in 0.1% NaOH at 80°C for 20 minutes and next washed it with distilled water (Watanabe *et al*., 1998), or by washing the bacterial cellulose in running tap water for overnight, followed by boiling in 1% NaOH solution for 2 h, washed its in tap water to accomplish NaOH removal for 24 h, neutralized with 5% acetic acid (Krystynowicz *et al*., 1997).

In medical application of bacterial cellulose requires special processes to remove bacterial cells and toxins, which can cause pyrogenic reaction. One of the

most effective protocols begins with gentle pressing of bacterial cellulose pellicle and immersed in 3% NaOH for 12 h (repeat 3 times) and after that incubated in 3% HCl solution, pressed and thoroughly washed in distilled water. The purified pellicle is sterilized in autoclave or by cobalt-60 radiation. Dilute alkaline solutions are capable of hydrolyzing and removing impurities present in the cellulose pellicle (Supaphol and Spruviell, 2000).

Attalla and Vanderhart (1984) showed that native cellulose is a composite of two distinct crystalline forms mainly comprising 1α and 1ß phases. Sugiyama *et al*., (1990) confirmed the existence of two different crystalline forms co-existing within single cellulose micro fibrils through electron microscopy and diffraction studies. The hydrothermal annealing treatment using alkaline solutions can cause transformation of metastable 1α phase, which occurs predominantly in bacterial cellulose, to more thermally stable 1ß phase.

1.2.11 Factor effecting growth and cellulose production

Panesar *et al.*, (2009) reported that the optimum pH for bacterial cellulose production was at 6.5-7.0, optimum temperature was at 28 °C, also he indicated that with increase in incubation time cellulose production also increased and maximum production of cellulose was observed at 168 hrs, mannitol followed by glucose was the best carbon sources for maximum cellulose production (Similar trend of cellulose production has also been reported by Jonas and Farah, (1998) and methionine, peptone and sodium nitrate were found to be most effective nitrogen source. Matsuoka *et al.*, (1996) reported that vitamins and amino acids played important role for cell growth and cellulose production.

1.2.12 Improvement of bacterial cellulose producing strains

The genetic analysis of cellulose biosynthesis in *G. xylinus* has included the isolation of mutants that effect cellulose production, characterization of indigenous plasmid species, and the cloning of gene in the process. A number of researchers, beginning with Schramm and Hestrin (Schramm and Hestrin, 1954), have described the isolation of cellulose-negative (Cel^-) mutants. Both spontaneous and mutagen-induced variants have been isolated. Many reports have indicated that apparent spontaneous cellulose negative mutants arise at a high rate when wild-type cells are grown in aerated liquid culture (Smith, 1990). The frequency with which these mutants occur also increases with the age of the culture. The spontaneous mutants have a mucous appearance on solid agar like true Cel^- mutants, but the majority reverts back to wild-type when grown statically in broth culture (Cannon and Anderson, 1991).

A variety of mutagens have been used in an effort to induce mutation in *G. xylinus* i.e., N-methyl-N'-nitro-N-nitrosoguanidine (MNNG*).*, nitrous acid, ethyl methanesulfunate (EMS) and ultraviolet light are very effective, Ishigawa *et al*. (1995) used 10 µg/ml MNNG to improve productivity of *G. xylinus* by mutagenesis and produced a strain in which cellulose yields were up to 40% higher than that of the parent strain, where Siripong *et al*., (2012) reported that using 30 µg/ml of MNNG the bacterial cellulose production will increase 54.68% higher than that of parent strain and bacterial cellulose yields of the UV mutant was 50.59% higher than that parent strain. While Hungund and Gupta, (2010) reported that chemical mutants of *G. xylinus* by using EMS was 50% higher than the parent strain and physical mutant by using UV was 30% more than the parent strain.

1.2.13 Genetics of *Gluconacetobacter xylinus*

Hidetaka *et al*., (2011) determined the whole genome sequence of *Gluconacetobacter xylinus* by comparative analysis of genomes of *G. xylinus* NBRC 3288, a cellulose-nonproducing strain, with those of the cellulose-producing strains by the conventional whole-genome shotgun strategy using dye terminator chemistry on an ABI Prism 3730XL sequencer (ABI, Foster City, CA). The genome of *G. xylinus* NBRC 3288 consists of a single circular chromosome of 3,136,818 bp with 60.92% GC content. The chromosome is predicted to contain 3,195 protein-encoding open reading frames (ORFs), five copies of rRNA operons, and 60 genes encoding tRNAs. Of 3,195 predicted protein-coding genes, putative functions were assigned to 73.9% (2,358 genes), while the remaining 26.1% (837 genes) were annotated as hypothetical genes. The analysis of the *G. xylinus* NBRC 3288 genome identified 11 genes related to cellulose synthesis within two operons, cellulose synthase opreon and the operon involved in synthesis of cyclic di-GMP, an allosteric activator of cellulase synthase.

Abbas *et al*., (2005) reported that *G. xylinus* contains a complex system of plasmid DNA molecules. A 44 kilobases (kb) plasmid was isolated in wild type of *G. xylinus,* and by comparisons between wild type and cured cells of *G. xylinus* showed that there is considerably difference in cellulose production.

Valla, *et al*., (1987) found that most strains of *G. xylinus* are containing a complex system of plasmid, this plasmid is often generated either a 49-kb plasmid of medium copy number or a 44-kb plasmid of high copy number. 44-kb plasmid was found to be almost identical to 49-kb plasmid, but the difference in size could not simply be explained by a deletion of 5 kb from the 49-kb plasmid. Coucheron,

(1991) indicated the presence of an additional DNA sequence in the 44-kb plasmid that was lacking in the 49-kb plasmid.

1.2.14 Genetic organization of the cellulose synthase operon in *Gluconacetobacter xylinus*

Formation of bacterial cellulose is catalyzed by the cellulose synthase complexes which are aligned linearly in the *G. xylinus* cytoplasmic membrane. Synthesis of the metastable cellulose in *G. xylinus* including at least two steps the first one is polymerization of glucose molecules to the linear 1,4-ß-glucan, and the second is assembly and crystallization of individual nascent polymer chains into ribbon structures, characteristic for each cellulose-producing organism (Miss, 2007). These protein complexes are coded by cellulose synthase operon, an operon encoding four proteins required for bacterial cellulose biosynthesis (*bcs*) in *G. xylinus*, which was isolated via genetic complementation with strains lacking cellulose synthase activity (Saxena, *et al.*, 1990; Wong, 1990). Nucleotide sequence analysis indicated that the cellulose synthase operon is 9217 base pairs long and consists of four genes. The four genes-*bcsA, bcsB, bcsC, and bcsD* appear to be translationally coupled and transcribed as a polycistronic mRNA with an initiation site 97 bases upstream of the coding region of the first gene (*besA*) in the operon. Results from genetic complementation tests and gene disruption analyses demonstrate that all four genes in the operon are required for maximal bacterial cellulose synthesis in *G. xylinus*. The calculated molecular masses of the proteins encoded by *bcsA, bcsB, bcsC, and bcsD* are 84.4, 85.3, 141.0, and 17.3 kDa, respectively (Smith, 1990).

The domain *A* coded by *bcsA* and attached inner membrane, is thought to be the catalytic domain synthesizing cellulose from UDP-glucose. Another domain *B*

coded by *bcsB* which it the activator-binding subunit that accelerated cellulose synthesis by combined to cyclic-di-GMP. It is proposed that domain *C* and *D* coded by *bcs C* and *D* might relate to the aggregation of each cellulose chain synthesized, and it considered to be located on the outer surface of the membrane and to play a crucial role in the crystallization and/or extrusion of the cellulose immediately after polymerization (Amano *et al.,* 2005), as shown in figure (1-8).

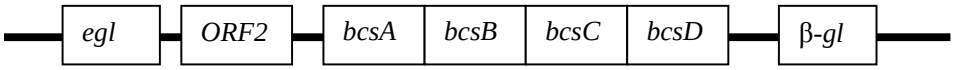

Figure (1-8): Structure of cellulose synthesizing protein gene of *Gluconacetobacter xylinus*. egl: endo- β-1,4-glucanase, ORF2: gene involve in cellulose synthesis, β-gl: β-glucosidase, *bcsA-D*: cellulose synthase operon (Amano *et al.*, 2005)

Another operon was found in the upstream of the *bcs* operon (Standal *et al*., 1994; Nakai *et al*., 1998). This operon consists of two genes encoding a carboxymethylcellulase (CMCase) and an ORF2 polypeptide, A spontaneous mutant, with the ORF2-coding region, was found to lack cellulose-producing ability (Coucheron, 1991), indicating that the ORF2 polypeptide is involved in cellulose synthesis. Tomonori *et al*., (2002) indicated that the ORF2 gene is involved in the production and crystallization of cellulose I microfibrils by this microorganism.

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2. Materials and Methods

2.1 Materials

2.1.1 Apparatus and Equipments

The following equipments and apparatus were used in this study:

Equipments	Company/ Country
Autoclave	Hirayama/ Japan
Balance	Satorius/ Germany
Compound light microscope	Olympus/ Japan
Cooling centrifuge	Hermile/ Germany
Distillator	GFL/ Germany
Electrophoresis Unit	Bio Rad/ Italy
Incubator	Sanyo/ Germany
Laminar air flow hood	Sanyo/ Germany
Millipore filter unit	Millipore corp/ USA
Micropipettes	Ependrof / Germany
Oven	Gallenkamp Sayo/ England
pH-meter	Martini/ Germany
Power supply	LKB/ Sweden
Sensitive balance	Denver/ Germany
U V Transilaminator	Vilber Lourmat/ France
Vortex	Stuart/ England
Water bath	Grant/ England

2.1.2 Chemicals and Biological materials

The following chemicals and biological materials were used in this study:

Material	Company/ Country
Glucose, Fructose, Lactose, Galactose, Xylose	Analar/ U.K.
Ethanol, Sodium hydroxide	Merck/ Germany
Agar, Gelatin	Biolife/ Italy
Peptone, Yeast extract, Urea,	Himedia/ India
HCl, Citric acid, Na_2HPO_4, NaH_2PO_4, Calcium carbonate, Isopropanol, Chloroform, KCl, NaCl, $MgSO_4.7H_2O$, Glycerol, Sucrose, Maltose, Mannose, Mannitol,	BDH/ U.K.
Bromocresol green, Bromocresol purple	Thomas Baker/ India
Boric acid, Bromo phenol blue	Riedel-Dettaen/ Germany
Ethylene diamine tetra-acetic acid, Sodium dodecyl sulphate, Tris-hydrochloride, NaCl, NaOH, Sodium glutamate	Fluka/ Switzerland
Agarose, Ethidium bromide, Acridine orange, Lysozyme	Sigma/ USA

2.1.3 Antibiotics

2.1.3.1 Antibiotic discs

The following antibiotic discs were used in this study:

Antibiotics	Abbreviation	Concentration (µg/ Disc)	Company/ Country
Ampicillin	Amp	25	Al-Razzy/ Iraq
Penicillin G	P	10	Al-Razzy
Cephalexin	CF	30	Al-Razzy
Tetracycline	T	30	Al-Razzy
Chloramphenicol	C	30	Al-Razzy
Erythromycin	E	15	Al-Razzy
Nalidixic acid	NA	30	Al-Razzy
Gentamycin	G	10	Al-Razzy
Bacitracin	BA	10	Al-Razzy

2.1.3.2 Antibiotic powders

The following antibiotic powders were used in this study:

Antibiotics	Abbreviation	Company/Country
Chloramphenicol	C	Oxoid/ England
Erythromycin	E	Oxoid
Nalidixic acid	NA	Oxoid
Cephalexin	CF	Oxoid

2.1.4 Media

2.1.4.1 Ready to made media

The following media were prepared according to the instructions of manufacturer companies and sterilized by autoclaving:

Media	Company/ Country
Brain heart infusion broth	Oxoid/England
Brain heart infusion agar	Oxoid
Simmon citrate agar	Difco/USA
Urea agar base	Difco

2.1.4.2 Laboratory prepared media

2.1.4.2.1 GYC medium (Sowden and Colvin, 1978)

This medium used for the isolation of *Gluconacetobacter xylinus* and was prepared to be consist of the following components:

Component	Weight (g)
Glucose	50
Yeast extract	10
Calicium carbonate	30
Agar	20

All components were dissolved in 900 ml of distilled water, pH was adjusted to 7, then volume was completed to 1000 ml and sterilized by autoclaving.

2.1.4.2.2 Frateur‘s ethanol medium (Frateur, 1950)

This medium used for the isolation of *Gluconacetobacter xylinus* and was prepared to be consist of the following components:

Component	Weight (g)
Yeast extract	10
Calicium carbonate	20
Agar	20

All components were dissolved in 900 ml of distilled water, pH was adjusted to 7, then volume was completed to 980 ml and sterilized by autoclaving. After cooling to 45° C, 20 ml of ethanol was added and mixed gently.

2.1.4.2.3 Modified Carr‘s medium (Sowden and Colvin, 1978)

This medium used for the growth of *Gluconacetobacter xylinus* and was prepared to be consist of the following components:

Component	Weight (g)
Yeast extract	30
Agar	20

All components were dissolved in 900 ml of distilled water, pH was adjusted to 7, then volume was completed to 980 ml and sterilized by autoclaving. After cooling to 45° C, 20 ml of ethanol was added and mixed gently.

2.1.4.2.4 Media for cellulose production Hestrin-Schram medium (HS-medium) (Hwan *et al.*, 2004)

This medium was prepared to be consist of the following components:

Component	Weight (g)
Glucose	20
Yeast extract	5
Peptone	5
Na_2HPO_4	2.75
Citric acid	1.15

All components were dissolved in 900 ml of distilled water, pH was adjusted to 6, then volume was completed to 1000 ml and distributed each 100 ml in 500 ml flask and sterilized by autoclaving.

2.1.4.2.5 Semi- solid agar medium for motility test (KoBy and Ronald, 1974)

This medium was prepared to be consist of the following components:

Component	Weight (g)
Glucose	20
Yeast extract	2
Peptone	3
Agar	5

All components were dissolved in 900 ml of distilled water, pH was adjusted to 7, then volume was completed to 1000 ml, sterilized by autoclaving, and left to solidify in vertical position.

2.1.4.2.6 Pepton water for indole test (Collee *et al*., 1996)

This medium was prepared to be consist of the following components:

Component	Weight (g)
Peptone	20
Sodium chloride	5

Ingredients were dissolved in 900 ml of distilled water, pH was adjusted to 7.0, mixed thoroughly, then volume was completed to 1000 ml and distributed into test tubes and sterilized by autoclaving.

2.1.4.2.7 Gelatin medium for gelatinase test (Baron and Finegold, 1994)

This medium was prepared by dissolving 12 g of gelatin in 100 ml of Brain-Heart infusion broth, then pH was adjusted to 6, and sterilized by autoclaving.

2.1.4.2.8 Urea agar medium for urease test (Collee *et al*., 1996)

It was prepared by adding 24 g of urea agar base to 950 ml of distilled water, pH was adjusted to 6.8-7.0 and sterilized by autoclaving. After cooling to 50 °C, 50 ml of 20% urea solution sterilized by filtration was added, mixed gently then medium was distributed into sterile test tubes and left to solidify in slant position.

2.1.4.2.9 Carbohydrate fermentation (Atlas *et al.*, 1995)

This medium was prepared to be consist of the following components:

Component	Weight (g)
Peptone	20
Yeast extract	5
Bromocresol purple	0.4

All components were dissolved in 900 ml distilled water, pH was adjusted to 7, then volume was completed to 1000 ml and distributed into test tubes (5 ml/tube) and sterilized by autoclaving. After cooling, sugar source (glucose, fructose, sucrose, lactose, maltose, mannose, mannitol, galactose and xylose) sterilized by filtration was added to each tube separately in a final concentration of 1%, then sterilized Durham tubes were placed in each tube in inverted position.

2.1.4.2.10 Alcohol oxidation medium (Gibbs and Shapton, 1968)

This medium was used for alcohol oxidation test and was prepared by dissolving 30g of yeast extract and 20g of agar in 900 ml of distilled water, pH was adjusted to 7, ten ml of 2.2% bromocresol green was added, then volume was completed to 980 ml and sterilized by autoclaving. After cooling to 45° C, twenty ml of ethanol was added and mixed gently.

2.1.4.2.11 Glycerol ketogenesis medium (Gibbs and Shapton, 1968)

This medium was used for glycerol utilization test and was prepared by dissolving 30 g of yeast extract and 20 g of agar in 900 ml of distilled water, then

30 ml of glycerol was added, pH was adjusted to 7, volume was completed to 1000 ml and sterilized by autoclaving.

2.1.4.2.12 Glutamate agar (Andelib and Nuran, 2009)

This medium was used for glutamate utilization test and was prepared to be consist of the following components:

Component	Weight (g)
Glucose	10
Sodium glutamate	5
KH_2PO_4	1
KCl	0.1
$MgSO_4.7H_2O$	0.2
Agar	20

All components were dissolved in 900 ml of distilled water, pH was adjusted to 7.0, mixed thoroughly then volume was completed to 1000 ml and sterilized by autoclaving.

2.1.5 Reagents and Dyes

Reagents and dyes were prepared according to Atlas *et al.*, (1995) and as follows:

2.1.5.1 Catalase reagent

Catalase reagent was prepared to be consist of 3% hydrogen peroxide.

2.1.5.2 Oxidase reagent

Oxidase reagent was freshly prepared in dark bottle by dissolving 1 gm of tetramethyle-*p*-phenylenediamine dihydro chloride in 100 ml distilled water.

2.1.5.3 Kovac's reagent

Kovac's reagent was prepared by dissolving 10 g of p-aminobenzaldehyde in 150 ml of isoamylalcohol and then 50 ml of concentrated hydrochloric acid was slowly added and mixed gently.

2.1.5.4 Gram stain

Its compose of 4 reagents;

- A primary stain- crystal violet
- A mordant- Gram's iodine solution
- A decolorizing agent- an organic solvent (alcohol)
- A secondary stain or counter stain- safranin

2.1.5.5 Benedict's reagent (Robert *et al.,* 2002)

This reagent was prepared by dissolving 10 g of anhydrous sodium carbonate, 17.3 g of sodium citrate and 1.73 g of copper sulfate pentahydrate in 100 ml of distilled water.

2.1.6 Buffers and Solutions

2.1.6.1 Buffers and Solution for extraction of plasmid DNA

Buffers and solution for extraction of plasmid DNA were prepared according to Sambrook and Russell, (2001) and as follows:

2.1.6.1.1 SET buffer (pH=8)

This solution was prepared to be consist of 75 mM NaCl, 25 mM EDTA and 20mM Tris-HCl, pH was adjusted to 8 and sterilized by autoclaving.

2.1.6.1.2 Lysozyme solution (10mg/ml)

It was freshly prepared by dissolving 10 mg of lysozyme in 1ml of sterilized distilled water.

2.1.6.1.3 Sodium dodecyl sulphate solution (10%)

It was freshly prepared by dissolving 10g of SDS in 100 ml of distilled water.

2.1.6.1.4 Sodium chloride solution (5M)

It was prepared by dissolving 29.2 g of NaCl in 80 ml of distilled water, then volume was completed to 100 ml with distilled water and sterilized by autoclaving.

2.1.6.1.5 TE buffer (pH=8)

It was prepared to be consist of 10mM Tris-HCl and 1mM EDTA, pH was adjusted to 8 and sterilized by autoclaving.

2.1.6.2 Gel electrophoresis buffers and solutions

Gel electrophoresis buffers and solutions were prepared according to Sambrook and Russell, (2001) and as follows:

2.1.6.2.1 Tris-Borate-EDTA (TBA) buffer (5X) (pH=8)

Tris-Borate-EDTA (TBA) Buffer (5X) was prepared by dissolving 54g Tris-HCl and 27.5 g boric acid in 900 ml distilled water, then 20 ml of 0.5 M EDTA was added, volume was completed to 1000 ml with distilled water, pH was adjusted to 8 and sterilized by autoclaving.

2.1.6.2.2 Loading buffer (6X)

This solution was prepared by dissolving 0.25 gm of bromophenol blue and 40 g of sucrose in 80 ml of distilled water, then volume was completed to 100 ml with distilled water.

2.1.6.2.3 Ethidium bromide solution (10mg/ml)

It was prepared by dissolving 1 g of ethidium bromide in 100 ml of distilled water and stirred on magnetic stirrer until complete dissolving, then it was filtered through a wattman filter paper No.1 and store in dark bottle at 4° C until use.

2.1.6.3 Acridin orange solution (10 mg/ ml)

Stock solution of acridin orange was prepared by dissolving 1 gm of acridin orange in 100 ml of distilled water.

2.1.6.4 Antibiotic solutions (Sambrook and Russell, 2001)

Penicillin G, chloramphenicol, erythromycin, nalidixic acid and cephalexin were prepared as stock solution (100mg/ml) by dissolving 1 g of each antibiotic in 10 ml distilled water, sterilized by filtration and stored at –20 °C until use.

2.1.6.5 Phosphate buffer solution (Criuckshank, *et al.*, 1975) (pH=7)

Phosphate buffer solution was prepared by dissolving 14.3g of NaH_2PO_4 and 1.14g of Na_2HPO_4 in 900 ml of distilled water, pH was adjusted to 7.0, mixed thoroughly then volume was completed to 1000 ml with distilled water and sterilized by autoclaving.

2.2 Methods

2.2.1 Sterilization methods

2.2.1.1 Moist heat sterilization (Autoclaving)

Media, buffers and solutions were sterilized by autoclaving at 121 °C and 15 Ib/in^2 for 15 minutes, except some heat sensitive solutions.

2.2.1.2 Dry heat sterilization

Electric oven was used to sterilize glassware and some other tools at 180 °C for 2 hours.

2.2.1.3 Membrane sterilization (Filtration)

Millipore filter unit was used to sterilize heat sensitive solutions by using millipore filters (0.45 µm).

2.2.2 Isolation of *Gluconacetobacter xylinus*

2.2.2.1 Samples collection

A total of 120 samples of date vinegar, date syrup vinegar, apple vinegar and rotting fruits (apple, peach and grape) were collected from local markets in Baghdad governorate.

2.2.2.2 Samples preparation

- **Vinegar samples**

One-milliliter of each vinegar sample was added to 9 ml of sterilized peptone water prepared in item (2.1.4.2.6), mixed thoroughly and serial dilutions for each sample were done separately, then 100 µl aliquots from the appropriate dilution were taken and spread on GYC medium prepared in item (2.1.4.2.1) and Frateur's ethanol medium prepared in item (2.1.4.2.2), and incubated at 30°C for 48h. After incubation, colonies which produced a zone of $CaCo_3$ hydrolysis were selected for further investigations.

- **Rotting fruits**

One-gram of each fruit sample was added to 9 ml of sterilized peptone water, mixed thoroughly and serial dilutions for each sample were done separately, then 100 µl aliquots from the appropriate dilution were taken and spread on GYC medium and Frateur's ethanol medium, and incubated at 30°C for 48h. After

incubation, colonies which produced a zone of $CaCo_3$ hydrolysis were selected for further investigations.

2.2.3 Identification of bacterial isolates

Morphological, cultural characteristic and biochemical tests were performed for full identification of bacterial isolates.

2.2.3.1 Morphological and cultural characteristics

Colony shape, color, size and viscous growth of each bacterial isolates were examined on Frateur‘s ethanol medium after incubation at 30°C for 48h.

2.2.3.2 Microscopical examination

A loopfull of each bacterial isolate was fixed on microscopical slide and stained to examine cell shape, grouping, gram reaction and spore formation.

2.2.3.3 Biochemical tests

Biochemical tests were achieved for identification local isolates of *Gluconacetobacter* spp as follows:

2.2.3.3.1 Catalase test (Atlas *et al*., 1995)

Single colony of each bacterial isolate was fixed on a clean glass slide using a sterile wooden stick applicator, then a drop of hydrogen peroxide solution (3%) was placed onto the colony. Production of gaseous bubbles indicates a positive result.

2.2.3.3.2 Oxidase test (Atlas *et al*., 1995)

This test was achieved by moistening filter paper with few drops of freshly prepared solution of tetramethyl-*p*-phenylenediamine dihydro-chloride, aseptically, then single colony of each bacterial isolate was picked up using a sterile wooden stick applicator and smeared on the moistened filter paper. Development of a violet to purple color within 10 seconds indicates a positive result.

2.2.3.3.3 Motility test (Collee *et al*., 1996)

Semi-solid agar medium prepared in item (2.1.4.2.5) was stabbed with fresh culture of bacterial isolates and incubated at 30° C for 48 hr. presence of diffuse, hazy growth indicates a positive result.

2.2.3.3.4 Indol test (Atlas *et al*., 1995)

Peptone water prepared in item (2.1.4.2.6) was inoculated with bacterial isolates and incubated at 30 °C for 48 hr. After incubation 0.05 ml of Kovac's reagent prepared in item (2.1.5.3) was added and mixed gently. Appearance of pink color ring on the surface of medium indicates a positive result.

2.2.3.3.5 Simmon citrate test (Atlas *et al*., 1995)

This test was used to detect the ability of bacterial isolates to utilize citrate as a sole source of carbon and energy. In this test, a colony of each bacterial isolate was inoculated on simmon citrate agar medium and incubated at 30 °C for 48 hr. Appearance of blue color indicates a positive result.

2.2.3.3.6 Gelatinase test (Atlas *et al*., 1995)

Tubes of gelatin medium prepared in item (2.1.4.2.7) were inoculated with fresh culture of each bacterial isolate, and incubated at 30° C for 48 hr, then tubes were kept in refrigerator at 4 °C for two hours. liquification of gelatin medium indicates a positive result compared with the control treatment.

2.2.3.3.7 Urease test (Atlas *et al*., 1995)

Urease test was achieved by streaking Christensen urea agar slants prepared in item (2.1.4.2.8) with single colony of each bacterial isolate, and incubated at 30° C for 48hours. Appearance of red to violet color indicates a positive result.

2.2.3.3.8 Carbohydrate fermentation test (Atlas *et al*., 1995)

This test was used to detect if the bacterial isolates were able to produce acid and/ or carbon dioxide gas (CO_2) from the fermentation of a particular sugar source. This test was achieved by inoculating a set of carbohydrate-supplement tubes prepared in item (2.1.4.2.9) with each bacterial isolate and incubated at 30°C for 48 hr. Changing culture medium to yellow and presence of gas bubbles in durham tubes indicates a positive result.

2.2.3.3.9 Alcohol oxidation test (Gibbs and Shapton, 1968)

This test was achieved to examine the ability of bacterial isolates to oxidize alcohol, using alcohol oxidation medium prepared in item (2.1.4.2.10) by inoculating this medium with each bacterial isolate and incubated at 30°C for 7 days. Changing the color of culture medium from green to yellow indicates a positive result.

2.2.3.3.10 Glycerol ketogenesis test (Gibbs and Shapton, 1968)

This test was achieved by inoculating the surface of glycerol medium prepared in item (2.1.4.2.11) with fresh culure of each bacterial isolate and incubated at 30°C for 48 hr., then production of ketone was examined by transfering bacterial growth with a part of medium to Benedict's solution. Presence of red color precipitate indicates a positive result.

2.2.3.3.11 Growth on glutamate (Andelib and Nuran, 2009)

This test was used to detect the ability of bacterial isolates to grow on glutamate. Glutamate agar medium prepared in item (2.1.4.2.12) was inoculated with fresh culture of each isolate and incubated at 30°C for 48 hr. Presence of heavy growth indicates a positive result.

2.2.4 Screening the ability of bacterial isolates in cellulose production

2.2.4.1 Cellulose production (Son *et al.*, 2001)

Ability of bacterial isolates in cellulose production was examined by inoculating conical flasks containing 100 ml of cellulose production medium (HS-medium) prepared in item (2.1.4.2.4) with 1ml of fresh culture of each bacterial isolate, and incubated at 30°C for one week. Cellulose production was investigated as the appearance of white pellicle of cellulose on the surface of culture medium.

2.2.4.2 Extraction of crude cellulose

Cellulose was extracted from the production medium according to Son *et al.,* (2002) by harvesting of cellulose pellicles by filtration throughout filter paper No.1, then washed with distilled water, and heated in water bath with 0.5% NaOH at 80°C for 15 min. to remove microbial cells and medium components, then washed with distilled water, placed in petri dish and dried in oven at 105°C for 1-2 hr. to determine cellulose dry weight.

2.2.5 Maintenance of bacterial isolates

Maintenance of bacterial isolates was performed according to Maniatis *et al*., (1982) and as follows:

2.2.5.1 Short term storage

Bacterial Isolates were maintained for few weeks by inoculating slants of modified Carr's agar medium, and incubated at 30°C for 48 hr, then tightly wrapped with parafilm, and stored at 4 °C.

2.2.5.2 Medium term storage

Bacterial Isolates were maintained for few months by streaking slants of modified Carr's agar medium with each bacterial isolate in small screw-capped tubes and incubated at 30°C for 48 hours, then stored at 4 °C.

2.2.5.3 Long term storage

Bacteria can be maintained for many years in medium containing 15% glycerol at low temperature without significant loss of viability. This was done by

adding 1.5 ml of glycerol to 8.5 ml of exponential growth of bacterial isolates in small screw-capped bottles and stored at – 20 °C.

2.2.6 Antibiotic sensitivity test (Atlas *et al.*, 1995)

Disc diffusion method was used to investigate antibiotic sensitivity of the bacterial isolates. A sterile cotton swab was dipped into the inoculum (freshly culture, 48 hr) and the entire surface of brain heart infusion agar plates was swabbed three times by rotating the plate approximately 60° between streaking to ensure even distribution of inoculum. The inoculated plates were kept at room temperature for 10 minutes to allow absorption of excess moisture, then discs of antibiotic were fixed using sterile forceps on the surface of plates and incubated at 30°C for 48 hours in an inverted position. After incubation, diameters of inhibition zones (clear area around the disks) were measured.

2.2.7 Extraction of plasmid DNA

Plasmid DNA of locally isolated *G. xylinus* was extracted according to Salting out method described by Pospiech and Neuman, (1995) as follows:

The selected isolated of *G. xylinus* was propagated in modified Carr's agar medium prepared in item (2.1.4.2.3) to minimize cellulose production in culture medium which interfere with biomass harvesting. According to this observation, modified Carr's medium was inoculated with fresh culture of the selected isolate, and incubated at 30°C for 48 hours. Bacterial cells were harvested by centrifugation and resuspended in TE buffer, then recentrifuged for 15 min. at 6000 rpm. Plasmid DNA was extracted as follows:

1. Pelleted cells were washed with 3 ml of SET buffer, then cells were resuspend in 1.6 ml of SET buffer, and 1 ml of freshly prepared lysozyme solution was added and incubated at 37 °C for 30 min.
2. One ml of 10% SDS was added and mixed by inversion, then incubated at room temperature for 30 min.
3. Two ml of 5M NaCl was added and mixed by inversion at room temperature.
4. An equal volume of chloromform was added, mixed by inversion for 15 min. then centrifuged (6000 rpm at 4°C) for 20 min.
5. The aqueous phase was transferred to another sterile epprndorff tube, and 0.6 volume of isopropanol was added mixed by inversion, then kept at room temperature for 5 min.
6. Eppendorff tubes were centrifuged at 13000 rpm for 15 min. at 4 °C, then isopropanol was discarded and the precipitated DNA was dissolved in 100 µl TE buffer and stored at – 20 °C.

2.2.8 Agarose gel electrophoresis (Maniatis *et al*., 1982)

Plasmid profile for the selected isolate was detected on agarose gel (0.8 %). Gel was run horizontally in 1 X TBE buffer prepared in item (2.1.6.2.1). Electrophoretic buffer was added to cover the gel. Samples of DNA were mixed with loading buffer (1:10 v/v) prepared in item (2.1.6.2.2) and loaded into the wells and run for 2-3 hours at 5 V/cm, then agarose gel was stained with ethidium bromide by immersing in distilled water containing the dye at a final concentration of 0.5 µg/ml for 30-45 minutes. DNA bands were visualized by UV transilluminator. Gel was de-stained using distilled water for 30- 60 min. to get rid of background before photographing of DNA bands.

2.2.9 Curing of plasmid DNA (Abbas *et al*., 2005)

Curing experiment was performed on the selected isolate of *G. xylinus* by using acridin orange as a curring agent. Bacterial cells were grown in 10 ml of HS broth to mid log phase. Aliquots (0.1 ml) of fresh culture was used to inoculate test tubes containing series of 10 ml of HS broth medium with different concentrations of acridin orange (25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300 µg/ml), then tubes were incubated at 30°C for 48 hrs.

Growth density of each culture in different tubes was observed visually and compared with control to determine lethal dose of acridin orang. Aliquot (0.1 ml) of growth culture incubated with the sub-lethal dose of acridin orange was taken and diluted appropriately and spread on Carr's agar medium and incubated at 30°C for 48 hours, then growing colonies were replica plated on Carr's agar medium containing antibiotics that the wild-type of bacterial isolate was resist and incubated at 30°C for 48 hours. After incubation, bacterial colonies unable to grow in presence of these antibiotics were taken from master plate and subjected to study their plasmid profile by extraction of plasmid DNA and analyzed on agarose gel as in item (2.2.7) and (2.2.8) respectively.

2.2.10 Mutagenesis of *G. xylinus*

Mutagenesis was achieved according to Siripong *et al*., (2012) in an attempt to improve the ability of the selected isolate in production of cellulose by subjection to UV radiation using the UV- transiluminator. The tray of the irradiation approximately was 15X25 cm exposes sample in glass petri dish, to direct irradiation from four of 15 watts, 254 nm bulbs and the distance between the

UV source and irradiated suspension was 11 cm. The dose rate of UV irradiation was 2.5 J/m^2/s.

Wild-type of the selected isolate was first grown in modified Carr's agar medium at 30°C for 48 hr, then bacteria were harvested in sterile phosphate buffer (pH 7) and mixed with a vortex mixer, followed by centrifuging 10 ml at 4000 rpm for 10 min. The cell pellet was diluted in phosphate buffer and the cell suspension were poured in sterilized petri dishes and exposed to 0, 20, 40, 60, 70, 80 seconds UV irradiation under sterile conditions. Then 0.1 ml of cell suspension was taken after each treatment, diluted to appropriate dilution and plated on modified Carr's agar medium. Plates were then incubated at 30°C for 48 hr. to determine the viable count and survivals of bacterial cells.

According to the UV survival curve, the treatment that led to a survival percentage of approximately 10% as compared with the control was suspected to have the higher mutation rate. From this treatment a number of colonies were picked up randomly and tested for cellulose production.

2.2.10.1 Detection of *G. xylinus* cellulose enhanced producer mutants

One hundred colonies of suspected mutants were selected to examine cellulose production by using the method which previously mentioned in item (2.2.4) and compared with wild type.

2.2.11 Determination of optimal conditions for cellulose production by enhanced producer mutant of *G. xylinus*

Several factors were studied to determine the optimal conditions for cellulose production by the enhanced producer mutant of *G. xylinus*.

2.2.11.1 Effect of carbon source

Different carbon sources (glucose, ethanol, maltose, fructose and dates syrup) were added to each culture medium (HS medium) to determine the optimum for cellulose production, each of these carbon sources was added to the production medium at a concentration of 2% (w/v). Cultures were inoculated and incubated at 30°C for one week, then cellulose dry weight was determined as mentioned in (2.2.4).

2.2.11.2 Concentration of carbon source

Five concentrations (1, 2, 3, 4 and 5% w/v) of the appropriate carbon source were added to production medium to detect the optimum for cellulose production. Cultures were inoculated and incubated at 30°C for one week, then cellulose dry weight was determined as mentioned in (2.2.4).

2.2.11.3 Effect of nitrogen source

Six of different nitrogen sources were used to determine the optimum for cellulose production, these nitrogen sources are peptone, yeast extract, tryptone, malt extract, urea and sodium nitrate, each of these nitrogen sources were added to the production medium (HS medium) at a concentration of 1% (w/v). Production

medium was inoculated and incubated at 30°C for one week, then cellulose dry weight was determined as mentioned in (2.2.4).

2.2.11.4 Concentration of nitrogen source

Five concentrations (0.5, 1, 1.5, 2 and 2.5% w/v) of the optimum nitrogen source were used to determine the optimum concentration for cellulose production. Each of these concentrations was added to production medium (HS medium). Cultures were inoculated and incubated at 30°C for one week, then cellulose dry weight was determined as mentioned in (2.2.4).

2.2.11.5 Effect of pH

In order to determine the optimum medium pH for cellulose production, production medium was adjusted to different pH values (5, 5.5, 6, 6.5, 7 and 7.5), then inoculated and incubated at 30°C for one week. Cellulose dry weight was determined as mentioned in (2.2.4).

2.2.11.6 Effect of temperature

In order to determine the optimum incubation temperature for cellulose production by the selected mutant, production medium was inoculated and incubated at different temperatures (20, 25, 30, 37, 40 and 45°C) for one week, then cellulose dry weight was determined as mentioned in (2.2.4).

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3. Results and Discussion

3.1 Isolation of *Gluconacetobacter xylinus*

A total of 66 vinegar samples including dates vinegar, dates syrup vinegar, and apple vinegar in addition to 54 rotting fruits including apple, peach and grape, were collected from local markets in Baghdad governorate. From these samples a total of 105 bacterial isolates were obtained and examined to identify acetic acid bacteria, as indicated in table (3-1).

Table (3-1): Food samples used for isolation of *Gluconacetobacter* spp

Source	No. of Samples	No. of Isolates	Acetic acid producer colonies
Vinegar	66	72	45
Rotting fruits	54	33	29
Total	120	105	74

From the total isolates, 74 of them were suspected to be *Gluconacetobacter* spp according to the formation of a clear zone around their colonies on Frateur's ethanol medium as a result of acetic acid production that dissolves calicium carbonate in a zone up to 12 mm, which gives an indicator that these isolates were belonged to acetic acid bacteria as described by Anedelib and Nuran (2009). These isolates were further identified according to the cultural, microscopical and biochemical tests.

3.2 Identification of bacterial isolates

3.2.1 Morphological and cultural characteristics

Bacterial isolates which were suspected to be belong to the genus *Gluconacetobacter* were grown on Frateur's ethanol medium at 30°C for 48 hours. Results showed that these bacterial isolates were appeared to be creamy in color, circular, raised or convex and approximately 3 mm in diameter as mentioned by Frateur's, (1950).

3.2.2 Microscopical properties

Results of microscopical examinations of these bacterial isolates showed that twenty of them were gram negative, non-spore forming, rod to oval shape and single, in pairs or in chains. This result referred that these isolates were closely related to *Gluconacetobacter* spp. as mentioned by Son *et al*., (2002).

3.2.3 Biochemical tests

The isolates suspected to be belong to the genus *Gluconacetobacter* were subjected to some biochemical tests. Results indicated in table (3-2) showed that six of these twenty isolates were motile, positive for catalase, glycerol ketogenesis, alcohol oxidation and able to ferment glucose, fructose and mannose, while they were negative for oxidase, urease, gelatinase and indole production. Furthermore, they were negative for citrate and glutamate utilization, unable to ferment lactose and galactose and variable in maltose, mannitol, xylose and sucrose fermentation. These results confirmed that these six isolates were belonged to *Gluconacetobacter xylinus* according to Jozef *et al*., (1986).

Table (3-2): Biochemical tests for identification of locally isolated *Gluconacetobacter xylinus*

Isolate / Biochemical test	N1	N2	N3	N4	N5	N6
Gram stain	-	-	-	-	-	-
Motility	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-
Urease	-	-	-	-	-	-
Indole	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-
Growth on Glutamate	-	-	-	+	-	-
Glycerol ketogenesis	+	+	+	+	+	+
Oxidation of ethanol	+	+	+	+	+	+
Acid production from						
Glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Mannose	+	+	+	+	+	+
Maltose	+	-	+	-	-	-
Mannitol	+	+	-	-	-	-
Lactose	-	-	-	-	-	-
Xylose	+	-	-	+	-	-
Sucrose	+	-	+	-	-	-
Galactose	-	-	-	-	-	-

(+): Positive Result; (-): Negative Result

3.3 Screening of *G. xylinus* local isolates on cellulose production

Ability of locally isolated *G. xylinus* on cellulose production was screened in order to select the efficient one in cellulose production. Ability of these isolates on cellulose production was assayed after culturing in HS broth medium and incubating at 30 °C for one week, then dry weight of crude cellulose produced by each isolate was determined. Results showed in table (3-3) indicated that all of the six isolates of *G. xylinus* were cellulose producers according to the formation of white pellicle on the surface of HS broth medium as shown in figure (3-1). Cellulose dry weight was ranged between 2.7-5.1 g/L. Among these isolates *G. xylinus* N2 was the best in cellulose production which had the maximum cellulose production (5.1 g/L), and this may be attributed to physiological and genetic properties of this isolate. This isolate was selected to study the role of plasmid in cellulose production, and enhancing its ability in cellulose production by mutagenesis.

Table (3-3): Ability of locally isolated *G. xylinus* on cellulose production in HS broth medium after incubation at 30°C for one week

Isolate	Source of isolation	Cellulose weight (g/L)
G. xylinus N1	Rotting apple	2.7
G. xylinus N2	Date vinegar	5.1
G. xylinus N3	Date vinegar	3
G. xylinus N4	Date syrup vinegar	3.8
G. xylinus N5	Apple vinegar	2.9
G. xylinus N6	Apple vineger	4.7

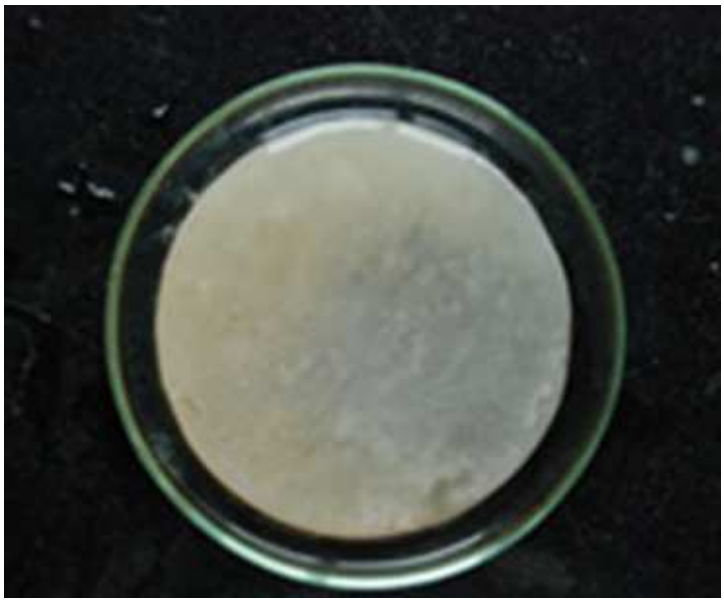

Figure (3-1): Cellulose pellicle produced by locally isolated *G. xylinus* N2 after incubation in HS medium at 30°C for one week

3.4 Antibiotic susceptibility of *G. xylinus* N2

Antibiotic susceptibility of the locally isolated *G. xylinus* N2 against nine antibiotics was studied. Results indicated in table (3-4) showed that this isolate was resistant to five antibiotics (penicillin G, cephalexin, chloamphenicol, erythromycin and nalidixic acid), while it was sensitive to the other four antibiotics (ampicillin, tetracycline, gentamycin and bacitracin). Resistant to these antibiotics may be encoded by chromosomal and/ or plasmid genes.

Resistance of *G. xylinus* N2 to β-lactam antibiotics (penicillin and cephalexin), which inhibits bacterial cell wall formation, may be attributed to the degradation of antibiotics by β-lactamase enzyme which is plasmid or chromosomally encoded enzymes (Shah *et al*., 2004).

Resistance to erythromycin and chloramphenicol, which inhibits of protein synthesis in susceptible organisms, is due to a plasmid encoded enzyme that altering the binding site (ribosome) by methylation of the 23S rRNA, thereby, blocking binding of the drug (King *et al*., 2000; Kenneth *et al*., 2005).

Resistance to nalidixic acid, which block DNA replication by inhibiting subunit of DNA gyrase (topoisomrase) and prevent super coiling of DNA, is due to a plasmid encoded protein that is bind to DNA gyrase protecting it from action of the drug (Morita *et al*., 1998; Robicsek *et al*., 2006).

Table (3-4): Antibiotic susceptibly of the locally isolated *G. xylinus* N2

Antibiotics	Susceptibility
Ampicillin	S
Bacitracin	S
Cephalexin	R
Chloramphenicol	R
Erythromycin	R
Gentamycin	S
Nalidixic acid	R
Penicillin G	R
Tetracycline	S

R: Resistant; S: Sensitive

3.5 Plasmid profile of *G. xylinus* N2

Plasmid profile of locally isolated *G. xylinus* N2 was studied by extraction genomic DNA according to salting-out procedure described by Pospiech and Neuman, (1995).

Result in figure (3-2) showed that locally isolated *G. xylinus* N2 has one plasmid DNA band after electrophoresis on agarose gel. This bacterial plasmid could be has a role in cellulose production and/or antibiotic resistance to pencillin G, chloramphenicol, nalidixic acid, cephalexin and erythromycin.

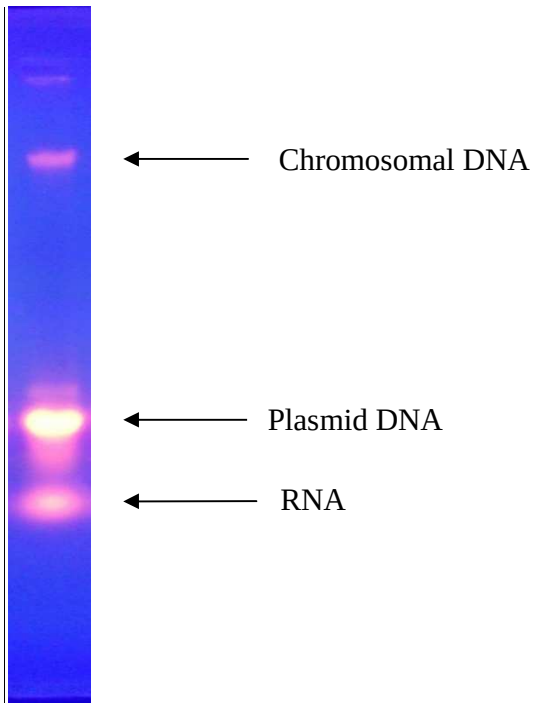

Figure (3-2): Plasmid profile of the locally isolated *G. xylinus* N2 on agaros gel (0.8%) at (5 V/cm) for 2 hrs.

Abbas *et al*., (2005) who extracted a 44 kilobases (kb) plasmid from wild type of *G. xylinus,* and referred that this plasmid can be involved in cellulose biosynthesis. While Valla, *et al*., (1987) mentioned that most strains of *G. xylinus* are containing a complex system of plasmid, these plasmids are often generated either a 49-kb plasmid of medium copy number or a 44-kb plasmid of high copy number.

3.6 Curing of plasmid DNA

Curing experiment was done to determine the role of indigenous plasmid of *G. xylinus* N2 in cellulose production. This was achieved by incubation fresh culture of *G. xyliuns* N2 with gradual concentrations of acridin orange (25-300 µg/ml) in HS medium at 30°C for 48 hours.

Results indicated in table (3-5) Showed that, locally isolated *G. xylinus* N2 was able to grow in the presence of acridin orange till the concentration of 200 µg/ml (a concentration which is regarded as a subinhibitory concentration). From the culture medium containing this concentration of acridin orange (200 µg/ml), aliquot of 100 µl was taken, diluted and spread on modified Carr's agar medium and incubated at 30°C for 48h., then 100 colonies were selected randomly to examine their antibiotic resistance on selective medium containing antibiotic to which the wild type was resist (penicillin G, erythromycin, chloramphenicol, cephalexin and nalidixic acid) in order to detect the cured colonies which were lost their ability to conferring the resistance phenotypes to those antibiotics, then to study the role of indigenous plasmid in the ability of *G. xylinus* N2 in cellulose production.

Table (3-5): Effect of different concentrations of acridin orange on growth of *G. xylinus* N2 after incubation at 30°C for 48hours in HS medium

Concentration (µg/ml)	Bacterial growth
25	+++
50	+++
75	+++
100	++
125	++
150	+
175	+
200	±
225	-
250	-
275	-
300	-

(+++): very good growth; (++): good growth; (+): moderate growth; (±): slightly growth; (-): no growth

Results showed that 22 from the total selected colonies (100 colonies) were unable to grow in the presence of erythromycin, chloramphenicol and nalidixic acid and became sensitive to these antibiotics. One of these colonies was selected randomly and examined for the presence of its own plasmid by extraction of genomic DNA and electrophoresis on agarose gel. Result mentioned in figure (3-3) showed that this cured colony of *G. xylinus* N2 was lost their own plasmid, and this referred that the plasmid is responsible for resistance to erythromycin, chloramphenicol and nalidixic acid in *G. xylinus* N2. In other word, gene(s)

responsible for resistance of these antibiotics are located on plasmid in this bacterium.

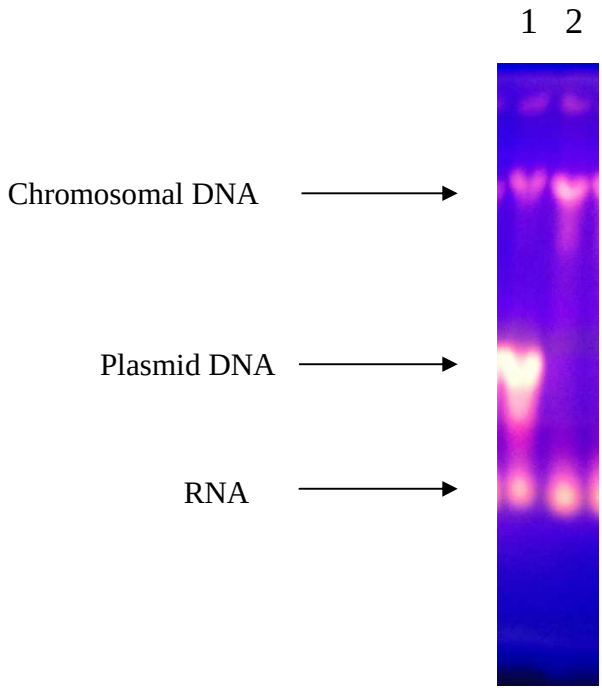

Figure (3-3): Plasmid profile of wild type and cured *G. xylinus* N2 on agarose gel (0.8%) at (5 v/cm) for 2 hrs.

Line (1): wild type isolate

Line (2): cured isolate

Cured *G. xylinus* N2 isolate was examined for their ability to produce cellulose by growing it in HS broth medium at 30°C for one week. Results indicated in table (3-6) showed that cured *G. xylinus* was still able to produce cellulose but with a significant decrease in cellulose production comparing with wild type. Dry weight of cellulose produced by cured *G. xylinus* N2 was decreased to 1.82 g/L in comparison with the productivity of wild type (5.1g/L). This result

declared that plasmid of *G. xylinus* N2 was not responsible for cellulose production, furthermore, this trait is chromosomally located, but the plasmid may have a regulatory effect for cellulose production. This result was agreed with Valla *et al*., (1987) who mentioned that plasmid of *G. xylinus* can be involved in cellulose biosynthesis, and Abbas *et al*., (2005) who found that cured cells of *G. xylinus* showed considerable difference in cellulose production.

Table (3-6): Cellulose dry weight produced by wild type and cured *G. xylinus* N2

Bacteria	Cellulose weight (g/L)
Wild type	5.1
Cured	1.82

Sexena *et al*., (1994) had been characterized the genes responsible for cellulose production controlled by an operon in the chromosome of *Gluconacetobacter xylinus*, and Tomonori *et al*., (2002) also reported that there is a second operon (*ORF2* operon) in the chromosome of *G. xylinus* which involve in the production and crystallization of cellulose.

3.7 Mutagenesis of *G. xylinus* N2

G. xylinus N2 was subjected to random mutagenesis using UV ray to induce mutations in an attempt to increase its ability for cellulose production. Mutagenesis was achieved by subjecting thin layer of fresh bacterial culture in open lid petri dish to direct effect of UV irradiation, then samples of the subjected culture were taken 20 second intervals. Results indicated in figure (3-4) showed the killing effect of UV ray on the suspension of bacterial cells. From this figure it was found

that *G. xylinus* N2 was UV moderate, and was lost its viability with the increase of UV irradiation dose. Survivals were decrease from 100% before irradiation to 1.2% when exposed to UV irradiation for 80 seconds.

Results in figure (3-4) indicated that the survival percentage of *G. xylinus* N2 was 9.6% (killing 90.4%) after subjecting to UV ray for 70 seconds. So this treatment was selected for mutants isolation because many studies mentioned that treatment allowing survival of approximately 10% of cell population generate more mutants (Queener and lively, 1986; Siripong *et al*., 2012).

It is known that mutation in bacteria can be induced by agent such as UV irradiation whose mutagenesis is affected via missrepair of damaged DNA by photoreactivation and SOS repair system and have been termed indirect mutagens (Al-Bakri and Umran, 1994).

Physical mytagenesis using UV ray was used successfully to induce random mutations in genomic DNA of *G. xylinus* for different purposes. It was reported that UV mutants of *G. xylinus* showed higher cellulose yield than the wild strain which was 30% more than the wild strain (Hungund and Gupta, 2010). Also Siripong *et al*., (2012) reported that cellulose production by UV mutant of *G. xylinus* was 39.60% higher than that of the parent strain.

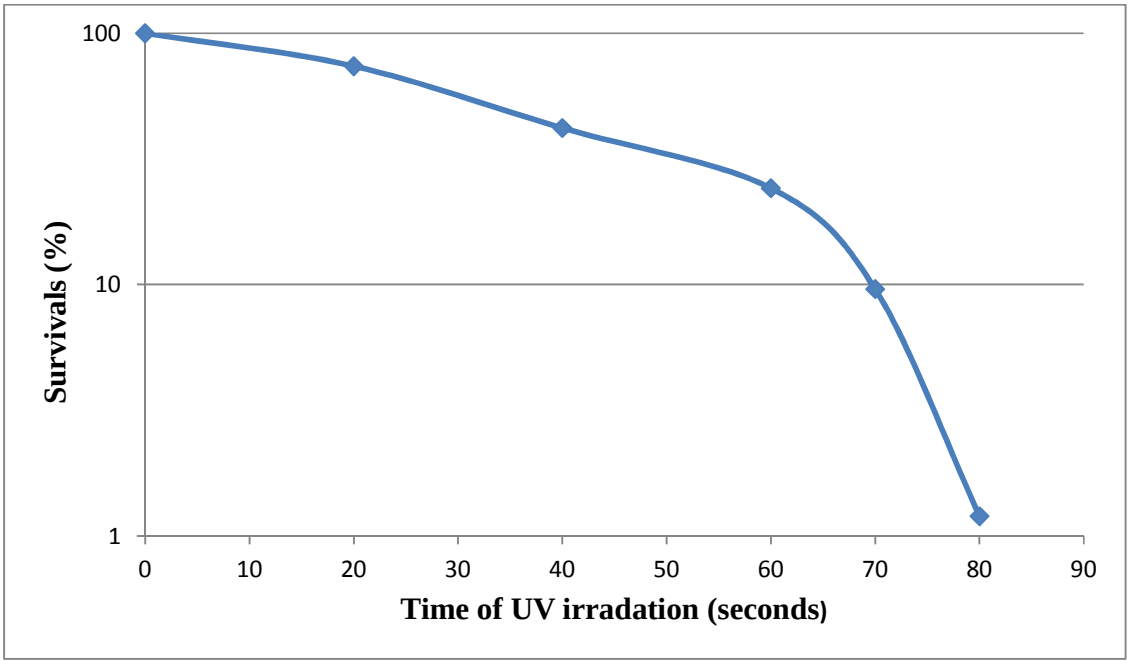

Figure (3-4): Effect of UV irradiation on *G. xylinus* N2 for different periods of time

3.7.1 Ability of *G. xylinus* N2 mutants in cellulose production

In order to isolate cellulose enhanced producer mutants of *G. xylinus* N2, one hundred colonies were selected from mutagenized culture, which exposed to UV irradiation for 70 seconds (treatment of approximately 90% killing).

These colonies were screened for their ability on cellulose production after culturing them in HS-broth medium. Results indicated in table (3-7) showed that ability of cellulose production was varied among these mutants (between 0 g/L and 6.9 g/L). Two of these mutants (N2_87 and N2_90) showed enhanced production of cellulose, which were about 6.3 and 6.9 g/L (26% and 38%) respectively in comparison with the productivity of wild type (5 g/L), 38% of these mutants lost their ability on cellulose production, while 13% of these mutants maintained their

ability on cellulose production, and 47% of these mutants showed a decrease in their ability on cellulose production.

Uv ray can induce random mutation in the genomic DNA, some mutation may occur in cellulose production genes or in regulations genes cause losing or decrease in cellulose production.

These results were agreed with those obtained by Hungund and Gupta, (2010) and Siripong *et al*., (2012) who obtained UV mutants of *G. xylinus* with higher cellulose yield as mentioned above.

Table (3-7): Cellulose dry weight produced by mutants of *G. xylinus* N2

Mutant	Cellulose dry weight (g/L)	Mutant	Cellulose dry weight (g/L)
wild type	5	*G. xylinus* N2_13	0
G. xylinus N2_1	1.6	*G. xylinus* N2_14	0
G. xylinus N2_2	0	*G. xylinus* N2_15	2.85
G. xylinus N2_3	0	*G. xylinus* N2_16	2.8
G. xylinus N2_4	4.88	*G. xylinus* N2_17	1.16
G. xylinus N2_5	1.17	*G. xylinus* N2_18	0
G. xylinus N2_6	0	*G. xylinus* N2_19	2.97
G. xylinus N2_7	1.7	*G. xylinus* N2_20	0
G. xylinus N2_8	2.9	*G. xylinus* N2_21	0
G. xylinus N2_9	3.7	*G. xylinus* N2_22	0
G. xylinus N2_10	2.78	*G. xylinus* N2_23	0
G. xylinus N2_11	4.9	*G. xylinus* N2_24	3.66
G. xylinus N2_12	4.2	*G. xylinus* N2_25	4.67

G. xylinus N2_26	3.1	*G. xylinus* N2_52	1.2
G. xylinus N2_27	4.88	*G. xylinus* N2_53	3.4
G. xylinus N2_28	4.5	*G. xylinus* N2_54	0
G. xylinus N2_29	5.2	*G. xylinus* N2_55	0
G. xylinus N2_30	3.1	*G. xylinus* N2_56	0
G. xylinus N2_31	0	*G. xylinus* N2_57	4.2
G. xylinus N2_32	0	*G. xylinus* N2_58	3.69
G. xylinus N2_33	0	*G. xylinus* N2_59	2.78
G. xylinus N2_34	0	*G. xylinus* N2_60	1.8
G. xylinus N2_35	2.69	*G. xylinus* N2_61	1.96
G. xylinus N2_36	0	*G. xylinus* N2_62	3.3
G. xylinus N2_37	4.5	*G. xylinus* N2_63	0
G. xylinus N2_38	3.7	*G. xylinus* N2_64	0
G. xylinus N2_39	0	*G. xylinus* N2_65	1.7
G. xylinus N2_40	3.1	*G. xylinus* N2_66	1.98
G. xylinus N2_41	0	*G. xylinus* N2_67	0
G. xylinus N2_42	1.16	*G. xylinus* N2_68	2.5
G. xylinus N2_43	1.7	*G. xylinus* N2_69	1.9
G. xylinus N2_44	0	*G. xylinus* N2_70	2.65
G. xylinus N2_45	0	*G. xylinus* N2_71	1.8
G. xylinus N2_46	0	*G. xylinus* N2_72	0
G. xylinus N2_47	0	*G. xylinus* N2_73	0
G. xylinus N2_48	0	*G. xylinus* N2_74	0
G. xylinus N2_49	4.7	*G. xylinus* N2_75	2.9
G. xylinus N2_50	2.4	*G. xylinus* N2_76	3.09
G. xylinus N2_51	2.78	*G. xylinus* N2_77	3.1

G. xylinus N2_78	1.7	*G. xylinus* N2_90	6.9
G. xylinus N2_79	0	*G. xylinus* N2_91	3.9
G. xylinus N2_80	0	*G. xylinus* N2_92	1.15
G. xylinus N2_81	1.3	*G. xylinus* N2_93	4.3
G. xylinus N2_82	0	*G. xylinus* N2_94	3.4
G. xylinus N2_83	2.2	*G. xylinus* N2_95	0
G. xylinus N2_84	3.0	*G. xylinus* N2_96	0
G. xylinus N2_85	4.3	*G. xylinus* N2_97	1.0
G. xylinus N2_86	0	*G. xylinus* N2_98	1.98
G. xylinus N2_87	6.3	*G. xylinus* N2_99	3.45
G. xylinus N2_88	2.15	*G. xylinus* N2_100	1.46
G. xylinus N2_89	0		

3.8 Optimum conditions for cellulose production

Optimum conditions for cellulose production by the mutant *G. xylinus* N2_90 were studied using different growth factors including type and concentration of carbon and nitrogen sources, medium pH and incubation temperature.

3.8.1 Effect of carbon source

In order to examine the effect of carbon source on the ability of the enhanced producer mutant *G. xylinus* N2_90 in cellulose production, HS medium was supplemented with one of the five of different carbon sources includes glucose, fructose, maltose, date syrup and ethanol. These carbon sources were added to culture medium in a concentration of 2%. Results indicated in figure (3-5) showed that the maximum production of cellulose was achieved when date syrup

was used as a sole source of carbon and energy to give higher production of cellulose by *G. xylinus* N2_90. Cellulose dry weight in culture filtrate of this mutant was 7.8 g/L, while productivity of cellulose was less when other carbon sources were used to supplement production medium (glucose, 6.8 g/L; fructose, 4.5 g/L; maltose, 1.8g/L and ethanol, 1.2 g/L).

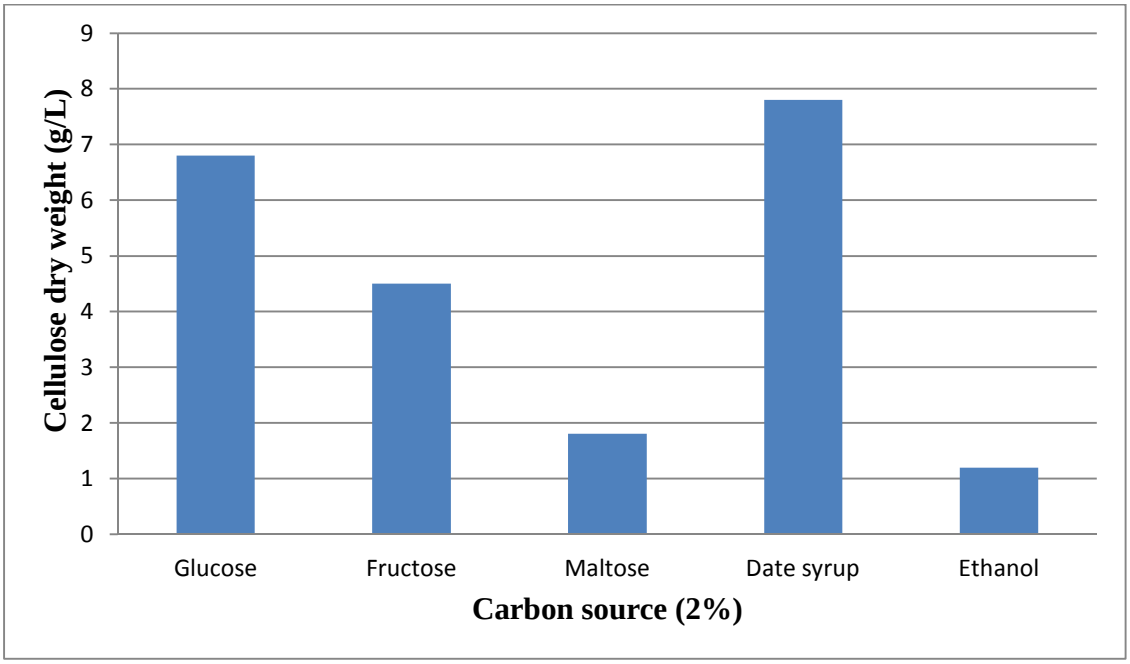

Figure (3-5): Effect of carbon source on cellulose production by *G. xylinus* N2_90 after incubation at 30°C for one week

As mentioned above, the optimal carbon source for cellulose production was date syrup: this may be because it is rich in nutrients, which promote cell growth, and leads to increase cellulose production (Marzieh and Alireza, 2011).

Marzieh and Alireza, (2011) found that yields of cellulose by *G. xylinus* in medium containing date syrup was increased approximately two times more than that in the medium containing sucrose. Sang *et al.*, (2010) also referred that cell

growth and cellulose yield were increased in the date syrup medium more than in the glucose medium.

According to these results, the optimal carbon source (date syrup) was used in the next experiments of optimization for cellulose production.

3.8.2 Effect of date syrup concentration

Different concentrations of the optimal carbon source (date syrup) were used to determine the optimum for cellulose production by mutant *G. xylinus* N2_90. Results indicated in figure (3-6) showed that maximum cellulose production was obtained when date syrup was added to the HS production medium in a concentration of 2 %.

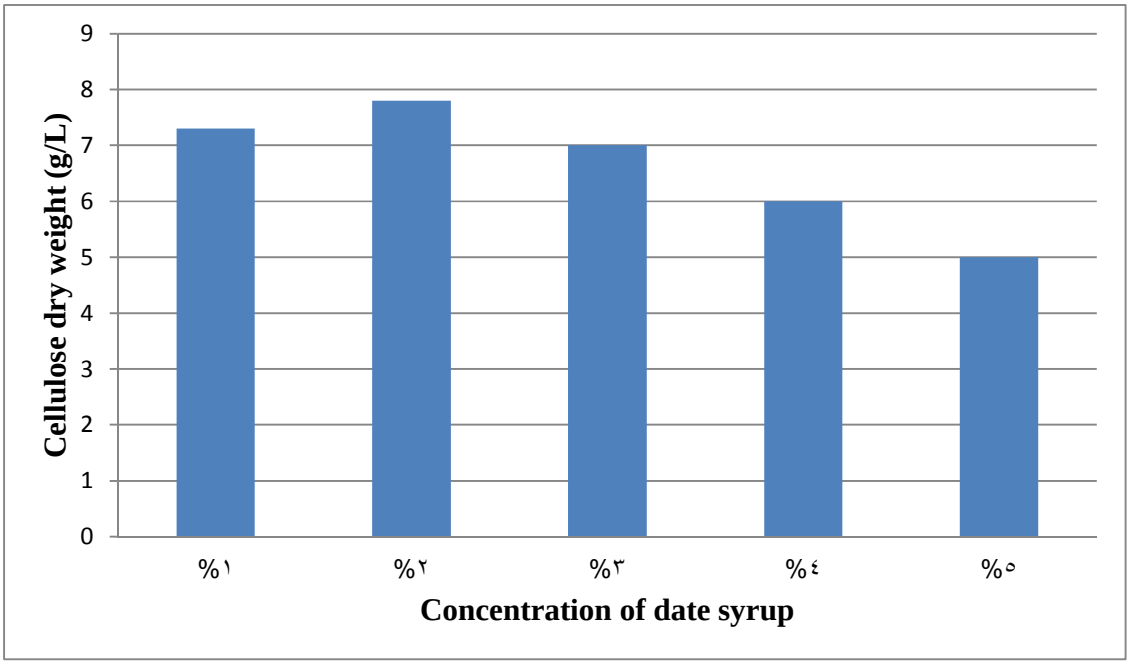

Figure (3-6): Effect of date molasses concentration on cellulose production by *G. xylinus* N2_90 after incubation at 30°C for one week

At this concentration cellulose dry weight was about 7.8 g/L, hence cellulose dry weight was decreased above or less than this concentration (1%, 7.3g/L; 3%, 7 g/L; 4%, 6 g/L and 5%, 5g/L).

Addition of date syrup in high concentration (3-5%) decrease cellulose production by the *G. xylinus* N2_90 and this may be due to the increase of gluconic acid and acetic acid production that decrease medium pH then decrease cellulose production (Prashant *et al*., 2009).

Bae and Shoda, (2005) studied the production of cellulose by *G. xylinus* BPR 2001 using date syrup medium and they concluded that maintaining a lower concentration of the date syrup is essential for efficient cellulose production in jar fermentors, the effect being attributed mainly to the complex nature of date syrup.

According to those results, the optimal concentration of date syrup (2%) was used in the next experiments of optimization for cellulose production.

3.8.3 Effect of nitrogen source

In order to examine the effect of nitrogen source in the ability of the enhanced producer mutant *G. xylinus* N2_90 in production of cellulose, different nitrogen sources were added to the HS medium to determine the optimum for cellulose production, these nitrogen sources included organic sources (Tryptone, peptone, yeast extract and malt extract) and inorganic sources (urea and sodium nitrate). All of them were added to the production medium (HS medium) at a concentration of 1%.

Results indicated in figure (3-7) showed that production of cellulose by *G. xylinus* N2_90 was reached the maximum when production medium was

supplemented with yeast extract, by using this nitrogen source productivity of cellulose was reached to 8.2 g/L. Peptone was also efficient in supplementing production medium because it induce cellulose productivity to 8 g/L, while other organic and inorganic sources were less efficient in enhancing cellulose production.

According to these results, yeast extract was the best among other nitrogen sources, this may be because yeast extract is an excellent stimulator of bacterial growth and nutrition providing nitrogen, amino acid, carbon and vitamins, especially vitamin B complex, that gives the requirements for microorganism for growth and cellulose production (Atlas, 2005).

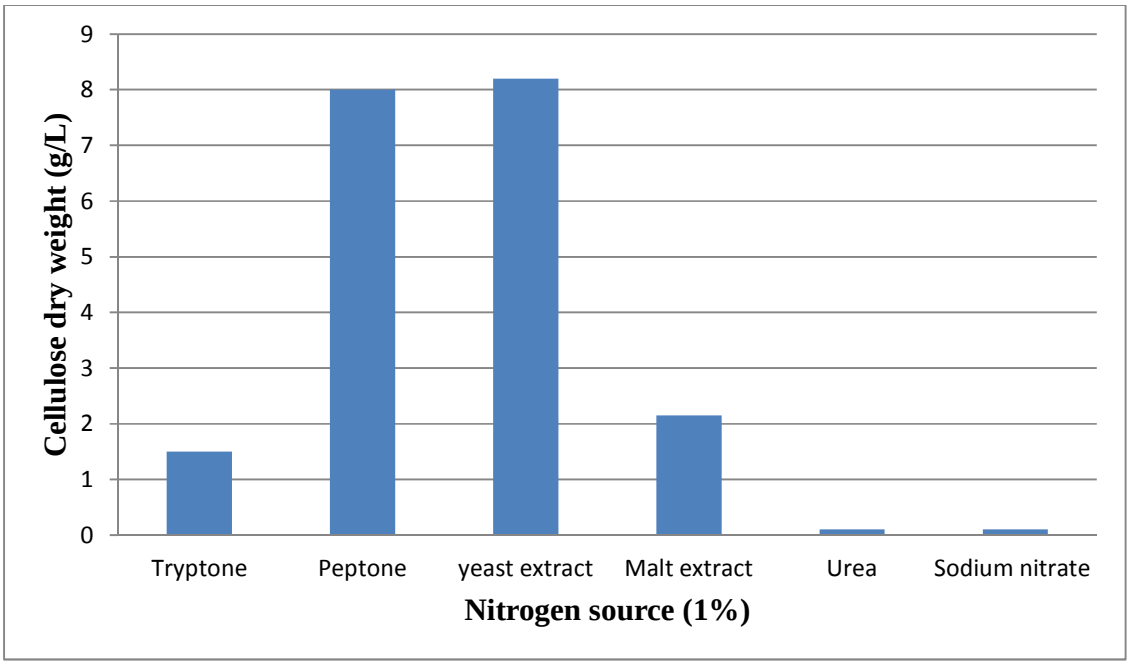

Figure (3-7): Effect of nitrogen source on cellulose production by *G. xylinus* N2_90 after incubation at 30°C for one week

This result was in agreed with Son *et al*. (2001) who reported that optimal nitrogen source for cellulose production by *G. xylinus* was yeast extract then

peptone. Similar observation was found by Al–Shmary, (2007) who noticed that optimal nitrogen source for cellulose production by *G. xylinus* was yeast extract then peptone. From another point of view, Panesar *et al*., (2009) found that peptone was most effective nitrogen source for cellulose production by *G. xylinus*.

According to these results, yeast extract was used as a nitrogen source in the next experiments of optimization for cellulose production.

3.8.4 Effect of nitrogen source concentration

Different concentrations of yeast extract were used to determine the optimum for cellulose production by the mutant *G. xylinus* N2_90. Results indicated in figure (3-8) showed that maximum cellulose production (8.5 g/L) was obtained when yeast extract was added to the production medium (HS medium) in a concentration of 2% w/v.

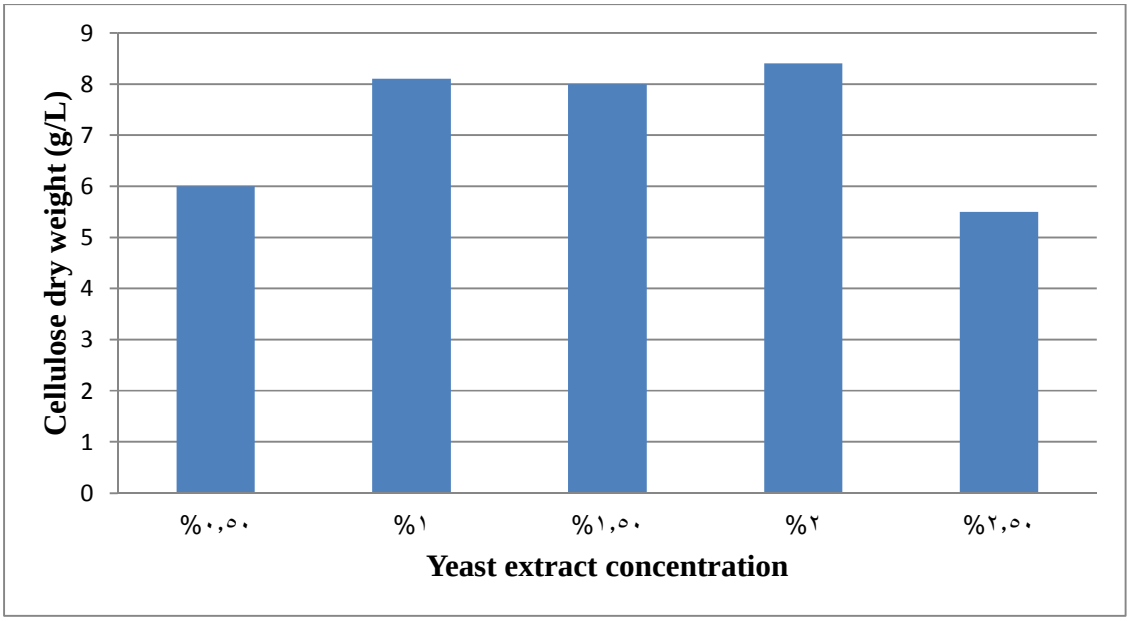

Figure (3-8): Effect of yeast extract concentration on cellulose production by *G. xylinus* N2_90 after incubation at 30°C for one week

Al–Shmary, (2007) reported that optimal yeast extract concentration for cellulose production by *G. xylinus* was 0.8%.

According to these results, yeast extract was used in a concentration of 2% for the next experiments of optimization for cellulose production.

3.8.5 Effect of pH

To investigate the effect of initial medium pH on cellulose production by the mutant *G. xylinus* N2_90, production medium (HS medium) was adjusted to different pH values.

Results indicated in figure (3- 9) showed that maximum cellulose production was obtained when the medium pH value was adjusted to pH 6.5, at this pH cellulose dry weight in culture medium was reached 8.5 g/L, pH 7 was also efficient in cellulose productivity which cellulose dry weight was reached to 8.2 g/L, then decreased above and under the optimum pH value. Also it was found that productivity of cellulose in acidic pH was higher than the productivity in basic pH.

The pH decreases during fermentative production because of the accumulation of gluconic, acetic or lactic acids in the culture broth which drop pH value of the production medium then inhibited cell growth.

This result was in agreement with Al–Shmary, (2007) and Panesar *et al*., (2009) who found that optimal pH value for cellulose production by *G. xylinus* was 6.5-7.0.

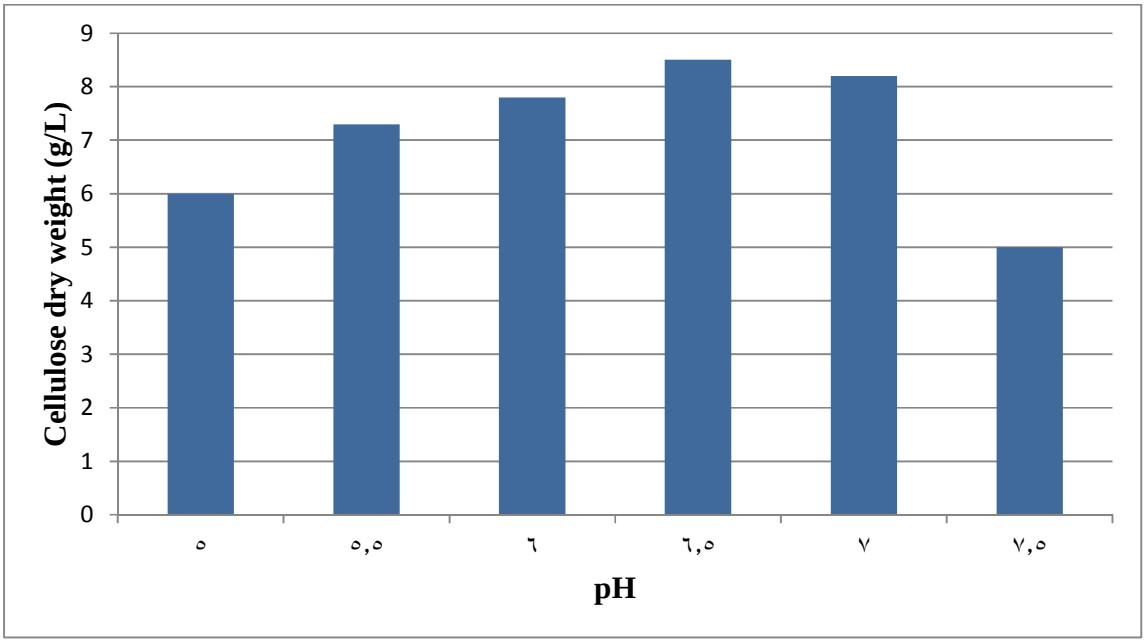

Figure (3-9): Effect of pH on cellulose production by mutant *G. xylinus* N2_90 after incubation at 30°C for one week

According to these results, the production medium (HS medium) was adjusted to pH 6.5 and used in the next experiment of optimization for cellulose production.

3.8.6 Effect of temperature

Different incubation temperatures were used to determine the optimum for cellulose production by the mutant *G. xylinus* N2_90. Results indicated in figure (3-10) showed that the maximum cellulose production was obtained when the cultur medium was incubated at 30 °C for one week. At this temperature, the cellulose production reached its maximum (8.5 g/L), then it was decreased above or under this temperature, while there was no cellulose production when culture medium incubated at 40 and 45°C.

This result is similar to that recorded by Al–Shmary (2007) who found that optimal temperature for cellulose production by *G. xylinus* was at 28°C and 30°C, while Son *et al*., (2001) reported that there was no significant difference in the amount of cellulose production by *G. xylinus* when incubated at 25°C and 30°C with preference the second on the first and a decrease in the productivity of cellulose when raising the temperature to 35°C.

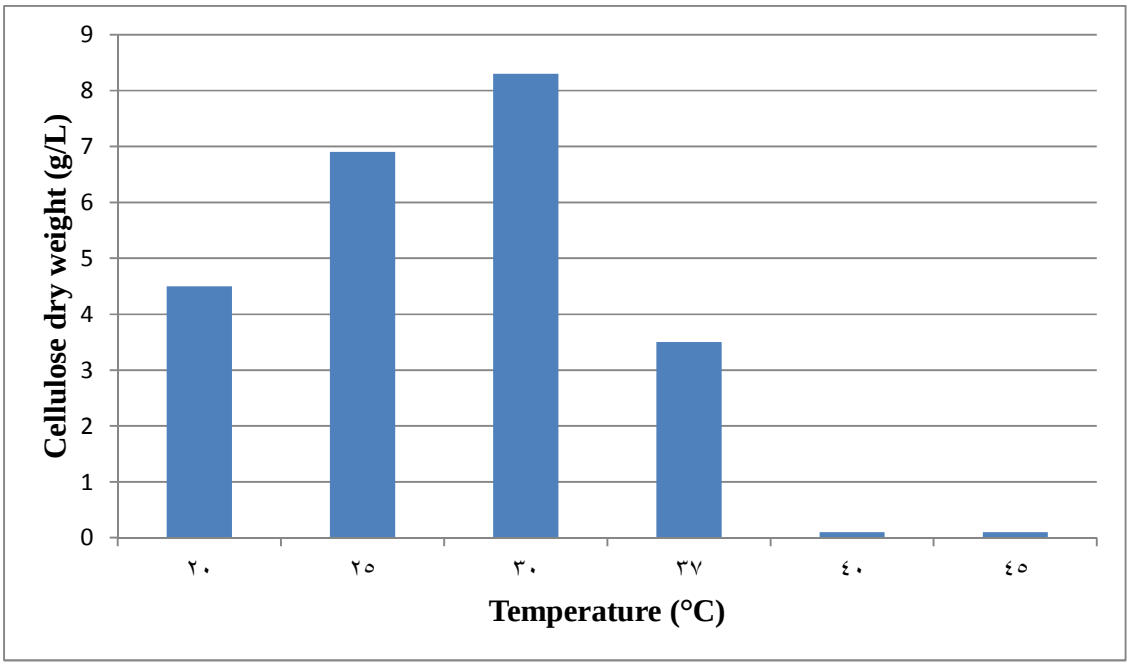

Figure (3-10): Effect of incubation temperature on cellulose production by *G. xylinus* N2_90 for one week

Summary

For the isolation of *Gluconacetobacter xylinus,* different food samples were collected from local markets in Baghdad governorate. These samples includes 66 vinegar samples (dates vinegar, date syrup vinegar and apple vinegar), and 54 rotting fruit samples (apple, peach and grape). From the overall of 120 samples a total of 105 isolates were obtained, seventy four of them were identified as *Gluconacetobater* spp according to their ability in acetic acid production. These isolates were further identified by subjecting them to morphological, microscopical characteristic and biochemical tests. Results showed that six of these isolates were belonged to *Gluconacetobacter xylinus.*

Ability of these isolates on cellulose production was examined by culturing in (Hestrin-Schram (HS) medium) production medium and incubated at 30°C for one week. Results showed that all these six isolates of *G. xylinus* were cellulose producer with variable degrees. Among them, one isolate symbol *G. xylinus* N2 was the most efficient in cellulose production according to high productivity of cellulose (5.1 g/L) in its culture filtrate in comparison with cellulose productivity of the other isolates.

Antibiotic susceptibility of *G. xylinus* N2 was examined against nine antibiotics. Results showed that this isolate was resistant to cephalexin, chloramphenicol, erythromycin, nalidixic acid and penicillin G, while it was sensitive to ampicillin, bacitracin, gentamycin and tetracycline.

Plasmid profile of *G. xylinus* N2 was studied. Results showed that this isolate harbour only one plasmid and this plasmid is not responsible for cellulose production. This was examined throughout curing of plasmid DNA by using

acridin orange as curing agent. The cured isolate was able to produce cellulose, but its ability was decreased to 1.82 g/L in comparison with the productivity of wild type (5.1 g/L), and this may be attributed to the regulatory role of the plasmid of *G. xylinus* N2.

In order to enhance the ability of *G. xylinus* N2 for cellulose production, this isolate was subjected to random mutagenesis by using UV ray. After irradiation, one hundred colonies were selected randomly from the treatment, which gave approximately 90% killing and screened for their ability to produce cellulose. Results showed that only two enhanced producer mutants (*G. xylinus* N2_87 and *G. xylinus* N2_90) were obtained and the productivity of cellulose in their culture filtrate was 6.3 and 6.9 g/L (about 26% and 38%) respectively.

Optimum conditions for cellulose production by the enhanced producer mutant *G. xylinus* N2_90 were studied. Results showed that the optimum conditions for cellulose production are growing this bacteria in production medium (HS medium) containing date syrup (2%) as a sole source for carbon and energy, yeast extract (2%) as a nitrogen source, in an initial pH 6.5 and incubation at 30°C for one week. Under these conditions, cellulose dry weight in culture filtrate of *G. xylinus* N2_90 was 8.5 g/L (about 23%).

Supervisors Certification

We, certify that this thesis entitled "Genetic study on locally isolated *Gluconacetobacter xylinus* and its ability in cellulose production" was prepared by "Noor Dheyaa Hameed" under our supervision at the College of Science/ Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology.

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Scientific Degree: Professor
Date:

Signature:
Name: Dr. Hameed M. Jasim
Scientific Degree: Professor
Date:

In view of the available recommendations, I forward this thesis for debate by the examining committee.

Signature:
Name: Dr. Majed H. Al-Gelawi
Scientific Degree: Professor
Title: Head of Biotechnology Department
Date:

الاهداء

اهدي ثمرة جهدي المتواضع..

إلى من سعى وشقى لأنعم بالراحة والهناء .. الى الذي لم يبخل بشئ من أجل دفعي في طريق النجاح .. الى الذي علمني أن أرتقي سلم الحياة بالحكمة والصبر..

والدي العزيز

إلى ملاكي في الحياة .. إلى معنى الحب وإلى معنى الحنان والتفاني .. إلى بسمة الحياة وسر الوجود إلى من كان دعائها سر نجاحي..

والدتي العزيزة

إلى من بهم أكبر وعليهم أعتمد .. إلى الشمعة المتقدة التي تنير ظلمة حياتي.. إلى من بوجودهم أكتسب قوة ومحبة لا حدود لها..

اخي و أخواتي

الى ملاكنا الصغيرة..

ليان

نور

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

الٓمٓ ﴿١﴾ ذَٰلِكَ الْكِتَٰبُ لَا رَيْبَ ۛ فِيهِ ۛ هُدًى
لِّلْمُتَّقِينَ ﴿٢﴾ الَّذِينَ يُؤْمِنُونَ بِالْغَيْبِ وَيُقِيمُونَ
الصَّلَوٰةَ وَمِمَّا رَزَقْنَٰهُمْ يُنفِقُونَ ﴿٣﴾
وَالَّذِينَ يُؤْمِنُونَ بِمَآ أُنزِلَ إِلَيْكَ وَمَآ أُنزِلَ مِن
قَبْلِكَ وَبِالْءَاخِرَةِ هُمْ يُوقِنُونَ ﴿٤﴾ أُولَٰٓئِكَ عَلَىٰ
هُدًى مِّن رَّبِّهِمْ ۖ وَأُولَٰٓئِكَ هُمُ الْمُفْلِحُونَ ﴿٥﴾

الخلاصة

جمعت ١٢٠ عينة من مصادر غذائية مختلفة شملت ٦٦ عينة من خل التمر، خل الدبس وخل التفاح و٥٤ عينة فواكه تالفة شملت التفاح ، الخوخ و العنب، وقد تم الحصول منها على ١٠٥ عزلة بكتيرية. شخصت ٧٤ عزلة على انها تابعة للجنس *Gluconacetobacter* spp على ضوء قابليتها على انتاج حامض الخليك. واظهرت النتائج ان ٦ من هذه العزلات تعود للنوع *Gluconacetobacter xylinus* اعتمادا على الصفات المظهرية والفسلجية والبايوكيمياوية.

تم غربلة جميع هذه العزلات الستة على اساس قابليتها على انتاج السيليلوز، من خلال زراعتها على وسط الأنتاج (HS medium) وحضنها بدرجة ٣٠°م لمدة اسبوع. اذ اشارت النتائج الى أن جميعها كانت منتجة للسيليلوز. تم انتقاء العزلة الاكفأ في انتاج السيليلوز (*G. xylinus* N2) على اساس اعلى انتاجية للسيليلوز وبلغ الوزن الصافي للسيليلوز المنتج من هذه العزلة (٥,١غم\لتر).

فحصت حساسية العزلة البكتيرية (*G. xylinus* N2) لتسعة انواع من مضادات الحيوية. واظهرت النتائج انها كانت مقاومة لمضادات الحيوية (cephalexin، chloramphenicol، erythromycin ، penicillin G وnalidixic acid) و حساسة لمضادات الحيوية (ampicillin، bacitracin ، gentamycin و Tetracycline).

عند استخلاص الدنا المجيني للعزلة البكتيرية (*G. xylinus* N2) لدراسة النسق البلازميدي، وجد ان هذه العزلة تحتوي على بلازميد واحد، وان هذا البلازميد غير مسؤول على انتاج السيليلوز على ضوء نتائج تحييد الدنا البلازميدي باستخدام مادة الاكردين اورنج كمادة محيدة، اذ لوحظ ان العزلة المحيدة احتفظت بقابليتها على انتاج السليلوز ولكن بكفاءة اقل حيث كان الوزن الصافي للسليلوز المنتج من هذه العزلة المحيدة (١,٨٢ غم\لتر)، مما يشيرالى ان صفة انتاج السيليلوز في العزلة تحت الدراسة هي صفة كروموسومية، والبلازميد له دور تنظيمي في انتاج السيليلوز.

ولاجل الحصول على طافرات ذات انتاجية عالية للسيليلوز، طفرت بكتريا *G. xylinus* N2 باستخدام الاشعة فوق البنفسجية، و درس التاثير القاتل والمطفر للاشعة فوق البنفسجية على هذه البكتريا. اظهرت النتائج ان هذه البكتريا كانت حساسة للتأثير القاتل والمطفر للاشعة فوق البنفسجية؛ انتخبت ١٠٠ مستعمرة من المعاملة التي أدت الى قتل تقريبا ٩٠% من البكتريا، وغربلت لقابليتها على انتاج السيليلوز.

اظهرت النتائج ان هذه الطافرات كانت متغايرة في قابليتها على انتاج السيليلوز، و اظهرت طافرتين (*G. xylinus* N2_ ٨٧ و *G. xylinus* N2_٩٠) قابلية جيدة على انتاج السيليلوز حيث كان الوزن الصافي للسيليلوز المنتج (٦,٣ و ٦,٩ غم\لتر) على التوالي.

استخدمت العزلة الطافرة ذات الانتاجية العالية (*G. xylinus* N2_٩٠) لدراسة الظروف المثلى لانتاج السيليلوز. اظهرت النتائج ان الظروف المثلى لانتاج السيليلوز بفعل هذه العزلة البكتيرية هي بتنميتها في وسط الأنتاج الحاوي على دبس التمر بتركيز (٢%) مصدرا وحيدا للكاربون والطاقة، و خلاصة الخميرة بتركيز (٢%) مصدرا نايتروجينيا وبرقم هيدروجيني ابتدائي ٦,٥ والحضن بدرجة 30°م مدة أسبوع. وقد تم الحصول على اعلى انتاجية للسيليلوز تحت هذه الظروف، اذ بلغ الوزن الصافي للسيليلوز المنتج ٨,٥ غم\لتر.

Committee certification

We, the examining committee certify that we have read this thesis entitled "Genetic study on locally isolated *Gluconacetobacter xylinus* and its ability in cellulose production". And examined the student "Noor Dheyaa Hameed" in its contents and that in our opinion, it is accepted for the Degree of Master of Science in Biotechnology.

(Chairman)
Signature:
Name:
Scientific Degree:
Date:

(Member)
Signature:
Name:
Scientific Degree:
Date:

(Member)
Signature:
Name:
Scientific Degree:
Date:

(Member/ Supervisor)
Signature:
Name:
Scientific Degree:
Date:

(Member/ Supervisor)
Signature:
Name:
Scientific Degree:
Date:

I, have certify upon the decision of the examination of committee.

Signature:
Name: Dr. Kholoud W. Al-Samraay
Scientific Degree: Professor
Title: Dean of College of Science
Date:

Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science

Genetic study on locally isolated *Gluconacetobacter xylinus* and its ability in cellulose production

Athesis

Submitted to the College of Science / Al-Nahrain University in partial Fulfilment of the Requirements for the Degree of Master of Science in Biotechnology

By

Noor Dheyaa Hameed

B. Sc. Biotechnology / College of Science / 2009

Al-Nahrain University

Supervised by

Dr. Majed H. Al-Gelawi
Professor
September- 2012

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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

دراسة وراثية على بكتريا

Gluconacetobacter xylinus

المعزولة محليا وقابليتها على انتاج السيليلوز

رسالة

مقدمة الى كلية العلوم في جامعة النهرين وهي جزء من متطلبات نيل درجة

الماجستير في علوم التقانة الاحيائية

من قبل

نور ضياء حميد

بكالوريوس تقانة احيائية/ كلية العلوم/ ٢٠٠٩

جامعة النهرين

بإشراف

أ.د. ماجد حسين الجيلاوي

شوال-١٤٣٣

أ.د. حميد مجيد جاسم

ايلول- ٢٠١٢