LOWERING COSTS FOR MICROBIAL CELLULOSE

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ABSTRACT

We have been conducting research with *Acetobacter xylinium* for microbial conversion of sugars to cellulose. A rotating disk biological contactor should lower costs considerably because its production rates are greater than for the usual method of surface culture. Another major cost saving comes from replacing expensive sugars in the medium with sugars derived from wastes. Extracts of spent grapes from wastes of Portuguese wine factories supply suitable sugars for good production of microbial cellulose.

INTRODUCTION

Microbial cellulose has long been used in foods in Asian countries, but its different properties from wood-derived cellulose open new industrial applications. Microbial cellulose possesses high crystallinity, high degree of polymerization, high tensile strength and tear resistance, and high hydrophilicity that distinguish it from other forms of cellulose. One current application reported by Sony Corp. is acoustic diaphragms for stereo headphones. Various potential applications aside from food uses include adsorbent pads, artificial skin, filter membranes, ultra strength paper, and paper additives (Brown, 1992, Cannon and Anderson, 1991, Okiyama, et al., 1993, Shibazaki, et al., 1993, White and Brown, 1989). Thousands of small producers in the Republic of the Philippines used coconut milk and open static-tray cultures to make microbial cellulose for sale to Japanese companies. The traditional process for making the cellulosic food product Nata in the Philippines has been a back-yard operation characterized as inefficient, labor-intensive and time consuming with minimal technical supervision. Although most of the production has shifted to Japan, no single large-scale fermentation has been reported mainly because scale up is affected by the biology of the producing organism, *Acetobacter xylinium*.

Only at the interface with air does *Acetobacter xylininum* grow rapidly. In static culture, this bacterium synthesizes long intertwined strands of cellulose, presumably to float it to the surface. Remarkably, other materials can be converted to these strands; carboxymethyl cellulose and other compounds support the formation of filaments. *A. xylinum* is an obligate aerobe,

chemotrophic, ellipsoidal to rod shaped, straight or slightly curved organism belonging to the family of Acetobacteraccae. It can occur single, in pairs or as a chain. *A. xylinum* is gram negative and in a few cases gram variable (e.g., old cells). The vast majority of cells in a static culture lie immobilized within the tough cellulose pellicle that covers the surface of the liquid and offers a competitive advantage to *A. xylinum* as it constitutes an effective barrier for oxygen for other organisms living in the culture. Since *A. xylinum* is primarily found on decaying fruits, the pellicle also helps to retain the water for the biochemical degradation of substrate for nutrition and offers protection against the lethal effect of UV light. The optimal temperature is 25 to 30 C and the optimal pH is in the range 5.4 to 6.3 (Bergey's Manual). An important aspect of *A. xylinum* is its conversion of glucose to gluconic acid. This leads to a significant pH decrease of 1 to 2 units depending on the initial pH. The doubling time for *A. xylinum* in static cultures is in the range of 8 to 10 hours and 4 to 6 hours in agitated and aerated systems (Cannon et al, 1991). Valla et al., (1982) on the other hand reported doubling times in shaken cultures as low as 2 hours.

Microbial processes based on surface culture are not popular in industry. There are a few very old installations for making citric acid with surface cultures of the mold Aspergillus, but stirred tank reactors tend to be much more economical. However, stirred processes for microbial cellulose perform poorly because agitation discourages production of the pellicle (Schramm and Hestrin, 1954). A deep tank fermenter employing a patented Acetobacter strain that retains its cellulose synthesizing ability in suspended culture (European Patent 86308092) produces a slurry of partially dried microbial cellulose known as "Cellulon". However, current uses of this non-pellicle product are rather limited.

In our search for a bioreactor system more suitable for scale up, we tried ways of supporting the pellicle. Waste treatment with a rotating biological contactor (RBC) has a 20-year history (Grady and Lim, 1980). A typical installation is 5 to 20 meters long with a rotating shaft with many disks of over a meter in diameter on about one centimeter centers. These dip into a semi-circular trough and carry the film of organisms (Figure 1). As with the trickling filter, the concept is alternate dousing the culture with nutrient medium and exposing the film to air. The volumetric efficiency of the RBC is high because much surface is available, and power requirements are low because the disks rotate slowly with only a minor amount of friction and liquid resistance to overcome.

We are using the concept of the RBC to produce microbial cellulose (Serafica, Bungay, and Mormino, (1997). By 3 days a disk with film on each side is about 3 cm. thick and can be harvested. Stainless steel as fine mesh is not a satisfactory support because it bends badly when heavily loaded with cellulose. We have drilled thin Plexiglass to make excellent disks but have found that the plastic mesh from fabric stores is ideal. No drilling is required, and the coarse mesh allows growth through it to hold the cellulose tightly. It supports a thick load of gum with little bending.

SUGARS FROM WASTES

The Portuguese wine industry must dispose of 100,000 to 200,000 ton/year of grape pomace, the residue after pressing to obtain the juice. There is sufficient sugar remaining to serve as the carbonaceous substrate for a commercial fermentation such as the production of microbial cellulose. We tried several method of extraction and tested the extracts for full or partial replacement of ingredients currently used for cellulose production by *Acetobacter xylinum.*

MATERIALS AND METHODS:

Conditions for extracting sugars from Bagaço (Grape pomace):

White and red grape pomace from the Douro valley in Portugal were generously provided by *Adega Cooperativa de Alijó*. Extracts prepared by homogenizing 15 grams of white grape pomace with 135 ml of deionized water using a hand mixer were placed in a water bath for 0.5 to 3 hours at 75 °C, 60 °C , or 40 °C. After filtering through Whatman No. 1 paper, portions of each extract were centrifuged. The supernatant was filtered through a 0.45 m Membra-fil membrane filter before analysis by HPLC.

The liquid chromatography system (Spectra physics) was equipped with a refractive index (RI) detector. Glucose and fructose were separated on a 5 µm 25 x 4.5 cm NH2 column at room temperature with an isocratic elution with acetonitrile:water (80:20). The flow rate was 2ml/m. Research grades of glucose and fructose (Sigma) were used to calibrate the HPLC.

Determination of amino acids and proteins in the extracts:

Extract of grape pomace from a 60 °C run was centrifuged in Eppendorf tubes at 14000 rpm/m for 5 minutes. The supernatant was filtered through a 0.45 µm Membra-fil membrane filter before injection in the amino acids analyzer using a ninhydrin detection system (model plus-2 from Pharmacia LKB).The instrument used a gradient of five lithium citrate buffers of increasing pH and ionic strengh and a cation exchange column (Ultropac 7). Comercial standards were used for calibration.

Proteins in grape pomace extract were determined using the SDS-PAGE method (16).
Samples werelyophilize for concentration. Concentrated extract was mixed with 750 µl of PSS (B-Mercaptoethanol, glycerol, SDS 20%, 0.2 M tris/ 0.02 EDTA and bromophenol blue) and agitated forl minute. Subsequent steps were heating at 95 °C for 10 minutes, cooling in ice, and centrifuging. Supernatants were applied to the gel for runs of 7 hours. Albumin was the reference.

Organism:

A strain of *Acetobacter xylinum* (Nata organism) was obtained from the National Science Research Institute (NSRI), University of the Philipines, Diliman, quezon City, Philippines, by way of Dr. Gonzalo Serafica who used it in his research at Rensselaer Polytechnic Institute. The culture was mantained at 5 °C in test tubes containing 5% wt. glucose, 0.5% bactopeptone, 0.5% yeast extract, 0.27 sodium phosphate monobasic and 0.12% citric acid. Bacteria for inoculation were centrifuged for 10 minutes and resuspended in autoclaved extract of grape pomace.

Medium preparation:

Thirty grams of white grape pomace are homogenized with 270 ml of deionized water using a handhold mixer and placed in a water bath at 60 °C for 2 hours. The extract is then filtered through Whatman paper n° 1 and the pH is adjusted to pH 5 with 1 N NaOH.
About 100 ml of this extract was then placed in a 250 ml Erlenmeyer flask before autoclaving at 121 °C for 15 minutes .After that the flask is cooled at room temperature.

Determination of weight of cellulose pellicles.

The cellulose pellicles is washed with water to remove any residual component of the medium. The film is then boiled in 2 % NaOH solution for 10 minutes to release cells from the cellulose matrix.

The cellulose is washed with deionized water until the remaining base is removed and the wet weight is measured by taking the water-soaked pellicle and draining for five minutes prior to weighing.(9)

Cellulose experiments.

Because of the importance of the air surface , different flask were used for this experiment.

A) 100 ml in 500 ml Erlenmeyer flask of:

1. Extract of white grape pomace
2. Extract of white grape pomace + Yeast extract (0.5%)

B) 100 ml in 250 ml Erlenmeyer flask of:

1. Extract of white grape pomace.
2. Extract of white grape pomace + Yeast extract (0.5%)

C) 100 ml in 250 ml Erlenmeyer flask of:

1. Extract of white grape pomace.
2. Extract of white grape pomace + Yeast extract (0.5%)

All those flasks were autoclaved at 121 °C for 15 minutes.After cool it at room temperature they were inoculated with the bacteria resuspendated in extract of grape pomace, and left incubating at 30 °C for three weeks.

Yeast extract experiment.

Five mediums from white grape pomace were prepared using different concentration of Yeast extract.(0.1-0.5%).

All those flask were autoclaved and inoculated with *Acetobacter xylinum*, and incubated at 30 °C.

Nitrogen experiment.

Four different mediums were used in this experiment.

1. 200 ml of extract of white grape pomace.
2. 200 ml of extract of red grape pomace.
3. 200 ml of extract of a mixture of white and red grape pomace.(70:30).
4. 200 ml of extract of a mixture of white and red grape pomace (30:70).

Every medium was divided in two 250 ml Erlenmeyer flask. In one of this medium previusly autoclaved was added Ammonium sulfate (1 g/l).The Ammonium sulfate was previusly esterilizated by microfiltration,(0,22 m) .

The rest 100 ml were supplemented with Yeast extract (0.5%), and autoclaved.

All the flasks were inoculated with *Acetobacter xylinum* and incubated at 30 °C.

Cocktail experiment.

Four different mediums were prepared for this experiment.

1. 100 ml of extract of white grape pomace.

2. 100 ml of extract of white grape pomace + Yeast extract (0.5%).
3. 100 ml of extract of white grape pomace + cocktail of vitamins, amino acids and amino bases.
4. 100 ml of extract of white grape pomace + Yeast extract (0.5%) + cocktail.

The cocktail contained ,Folic acid , Nicotinic acid, Pantotenic acid, Biotine, Cianocobalamine, Riboflavine, Piridoxine, Tiamine, Adenine, Timine, Uracil and Citosin, L-glutamic acid, L-alanin, L-arginin, L-asparragin, L-cistein, L-cistin, L-phenilalanin, L-hystidin, L-isoleucin, L-leucin, L-lysin, L-methionin, L-prolin, L-serin, L-tyrosin, L-threonin, L-tryptophan and L-valin
The cocktail was sterilizated by microfiltration, (0,22 m), to avoid the lost of some of the components by autoclaving.
The final concentration in the culture medium was 40 g / l for vitamins and amino bases and it was 3,75 mg / l for amino acids.(15)
All the mediums were incubated at 30 °C.

Results and discussion:

Glucose and fructose (simple sugars) are used by *Acetobacter xylinum* as carbon source for the cellulose production.
The HPLC analysis showed the concentration of glucose and fructose in the different extracts fom grape pomace.
The result showed the best sugars extraction conditions are 60 °C for 2 hours.
For white grape pomace in those conditions the highest concentration for glucose was 4.71 g / l, (Fig. 1) ; and for fructose it was 6.45 g / l.(Fig. 2) Total sugar concentration was 1.1 %, (Fig. 3)
For red grape pomace the concentration of sugars were very low, and it is because of the fermantation of grapes in the winemaking process for red wine.

After three weeks of incubation of the flasks of the Cellulose experiments, only those flasks with yeast extract presented a cellulose pellicle at the surface.(Fig. 4)
It coul be due to the Nitrogen source.
The Yeast extract experiment was done trying to know the minimal amount of yeast extract necessary for the cellulose production by *Acetobacter xylinum.*
After 17 days at 30 °C only the flasks with 0.3 % or more of yeast extract , showed cellulose at the surface, (Fig.4)
It means that 0.3 % of Yeast extract contains the minimal nitrogen required by *Acetobacter xylinum.*
To see if the Nitrogen source necessary is an organic or inorganic source, the results from the Nitrogen experiment were analized.Yeast extract was used as the organic nitrogen source and Ammonium sulfate was used as the inorganic nitrogen source.
After three weeks of incubation surface cellulose appeared in the flask with Yeast extract.(Fig. 5 and table 1) So, an inorganic nitrogen source is not enough for the cellulose production.
From this result we can deduce the best medium for the cellulose production by *Acetobacter xylinum* from grape pomace is made from a mixture of white and red grape pomace (70:30).The white grape pomace contain the adecuate amount of sugar for the cellulose production.We can suspect , that red grape pomace contains some other compounds , (amino acid or amino acids

combination, ...) , that are not presented in white grape pomace, and it can improve the cellulose production by *Acetobacter xylinum.*

The gel from the SDS-PAGE did not show any band from the samples of grape pomace. It means that there are not important proteins in those samples, so , the nitrogen source for cellulose production by *Acetobacter xylinum*, even being an organic source, has not a proteinic origin.

The amino acids analises showed the differents amino acids composition between extract of white and red grape pomace and a standar medium.(Table 2)

Taurine, sarcosine, amino adipic acid, citroline, -amino butyric acid, -alanine, -amino isobutyric acid, tryptophan , and hydroxyproline were not found in any of the samples.

Others amino acids such as L- Methionin, were found only in the standard medium, which can explain the higher cellulose production from this medium.

L-Methionin were reported before as a esential amino acid for the cellulose production by *Acetobacter xylinum.*(8)

With the results from the Cocktail experiment,(not finished at the moment), we will can see if there is a higher cellulose production from a extract of grape pomace plus yeast extract because of the amino acids or vitamins that there are in Yeast extract.

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