

Immobilization of Selected Microbes at Some Selected Solid Supports for Enhanced Fermentation Process

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Abstract

Immobilization of macro molecules (such as enzymes) and micro-organisms can be generally defined as a procedure leading to their restricted mobility. Advantages of immobilization include easy separation of the enzymes/cells from the product and reuse of the enzymes/cells. In this research coconut tree leaf sheath was used to immobilize selected microbes which were used in fermentation technology. Coconut tree leaf sheath contains cellulose fiber layers which have cross linking between them. *Sacchromyces cerevisiae* was used as the microbial type due to widespread use in fermentation process. Microbes were entrapped within cellulose layers. Coconut tree leaf sheath was found to be an efficient solid support for immobilization. Immobilized microbes can be reused for fresh fermentation media. Immobilization can be carried out utilizing naturally available coconut tree leaf sheath as a solid support, it's usage is very cost-effective and eco-friendly method rather than using synthetic or semi synthetic solid supports.

Keywords: Immobilization; Coconut tree leaf sheath; *Sacchromyces cerevisiae*

Introduction

Immobilization technique is a versatile and economical method that is used in industries [1]. Advantages of immobilization include easy separation of the enzymes/cells from the product and reuse of the enzymes/cells. Further the favorable environment at support allows better colonizing and population increase in micro-organisms which in turn leads in better fermentation. The micro environment in the solid adsorbent protects the microorganisms from unfavorable conditions such as high alcohol concentration; low pH etc. Reuse of enzymes/cells makes the process economically more feasible with higher substrate conversion efficiencies. Immobilization can be carried out in several approaches such as physical adsorption, chemisorptions, entrapment, and cross linkages [2]. In this study, physical adsorption was considered. Physical adsorption can be accomplished in two ways: non-specific adsorption and specific adsorption. Between them, non-specific adsorption is the simplest and easiest way of immobilization. Hence it is economically effective. Generally immobilization is carried out using synthetic resins or semi synthetic resins [3]. Use of novel supports such as mesoporous silicas, hydrogels, and smart polymers, and cross-linked enzyme aggregates (CLEAs) is in trend nowadays [4]. Synthetic resins and semi synthetic resins have several disadvantages over natural solid supports. Some are polymer compounds can be leached out to products and polymer support can be toxic to enzymes due to change of pH or texture. Therefore activity of enzymes can be degraded [5].

In this research naturally available substances were tested for immobilization of microbe which helps in beverage fermentation.

Coconut tree leaf sheath (Figure 1) is consisting of cellulose layers all over it. Hence it's having high tensile strength as well as high surface area [6]. Large numbers of pores are included among cellulose layers. In these pores, cells of microorganism can be entrapped or can form non-specific bonds such as vander-waal bonds and hydrogen bonds with cellulose layers. On the other hand coconut leaf sheath is an eco-friendly, cost effective substance. It is inert which do not show any adverse or toxic effect on micro-organisms. Approximate availability of



Figure 1: Coconut tree leaf sheath.

coconut tree leaf sheath is 9000 tons per year in worldwide [7]. So it is sufficiently available in world wide. Therefore usage of coconut tree leaf sheath is more feasible.

Coconut tree leaf sheath consist of cellulose layers (Figure 2) [8]. Due to -OH functional groups of cellulose, it can be induced inter and intra hydrogen bonding in between cellulose layers.

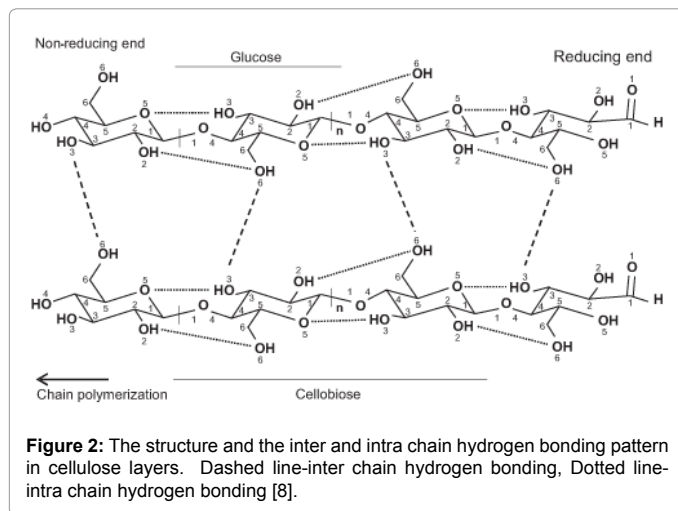
Saccharomyces cerevisiae is traditionally used in many fermentation

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processes. Generally *Saccharomyces cerevisiae* (Bakery Yeast) is used for fermentation in beverage industry. It uses simple sugars as a source of energy such as glucose and fructose or disaccharides such as sucrose and maltose. In anaerobic respiration process, alcohols and some organic acids are generated from sugar substrates as byproducts in neutral or slightly acidic medium [9]. Therefore *Saccharomyces cerevisiae* is used in alcoholic beverage industry around the world.

Cell wall structure of *Saccharomyces cerevisiae* is important for immobilization. *Saccharomyces cerevisiae* cell wall represents 30% of the dry weight of the cell and is composed largely of polysaccharides (85%) and proteins (15%) [10,11]. From that percentage of polysaccharides, glucan is present in 80-90% which is polymerized by D-glucose monomer linking with glycosidic bonds [11]. The cell wall of *Saccharomyces cerevisiae* consists of two types of β -glucans. β -(1,3)-glucan accounts for 50-55%, whereas β -(1,6)-glucan represents 10-15% of the total *Saccharomyces cerevisiae* cell wall polysaccharides [11]. In addition to β -glucans, mannoproteins and N-acetylglucosamine are also present in cell wall of *Saccharomyces cerevisiae* N-acetylglucosamine can be linked through β -(1-4) and mannoprotein residues can be linked to β -(1,6)-glucan through a processed glycosylphosphatidylinositol or to β -(1,3)-glucan through alkali-labile bond [10,12-17]. Based on these analyses, a structure for *Saccharomyces cerevisiae* cell wall has been proposed [12,14,18-20] (Figure 3).

Cell wall mannoproteins (CWP) can be linked to the -1,3-glucan via alkali-sensitive bonds (ASB) or to PIR proteins (PIR) via a disulfide link (SS). GPI Cell Wall Proteins (GPI-CWP) are attached to the -1,6-glucan through a remnant GPI anchor (GPI Rem.). The links between -1,3-glucan and -1,6-glucan or PIR proteins are still uncharacterized [10].

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A lower level of branching and polymerization degree is characterized by better solubility (Figure 4). It is believed that insoluble β -glucans are those whose degree of polymerization (DP) is higher than 100 [21,22]. Insoluble or slightly soluble β -glucans contain very long, multi-branched side chains in the particle (Figure 5).

Instrumentation

The double beam UV- Visible Spectrophotometer model HITACHI (U-2910) at Institute of Chemistry Ceylon was used for the UV- visible absorbance measurements.

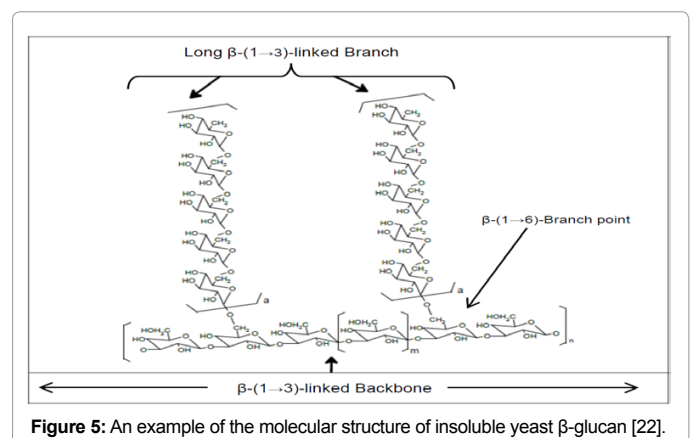
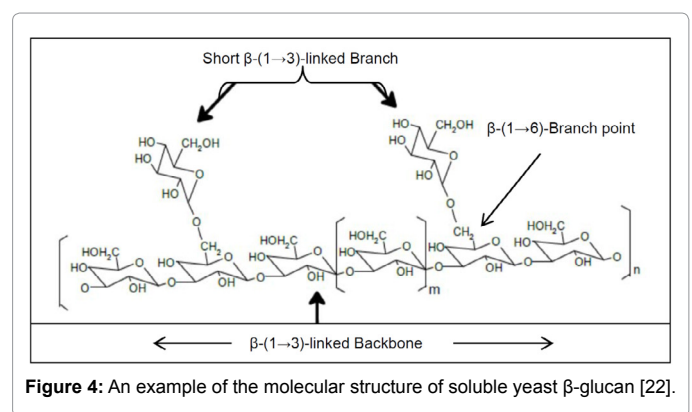
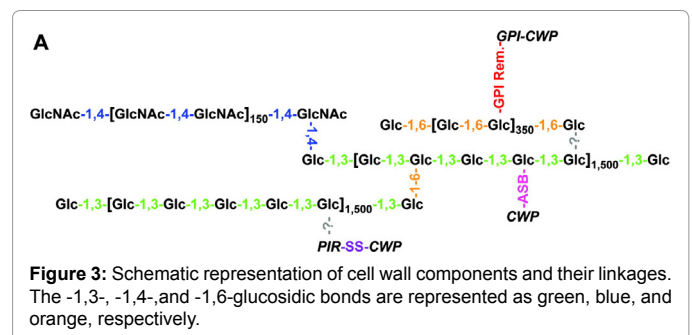
The Gas Chromatograph (GC) model (GC4000/GL SCIENCES) at Institute of Chemistry Ceylon was used for obtaining ethanol concentration values.

The Scanning Electron Microscope model LEO (LEO 1420VP) at Industrial Technology Institute, Colombo, Sri Lanka was used for obtaining scanning electron microscopy images.

Materials and Methodology

As solid supports sterilized coconut tree leaf sheath and silica coated glass were used. *Saccharomyces cerevisiae* was used as microbial type.

Saccharomyces cerevisiae was incubated in 10 ml of standard YPD



medium (Yeast/Dextrose/Peptone). When it reaches log phase in thier growth cycle [23] 1 ml of medium was introduced to sterilized solid support. Solid support with immobilized microbe was washed with cool distilled water and dried for over night under aseptic techniques. Dried immobilized microbes were introduced to 10 ml of growth media. *Saccharomyces cerevisiae* was inoculated in to growth medium without solid support as the positive control. Absorbance was measured at 600 nm for 27 hours for the plotting of growth curve. Absorbance only of growth medium without inoculating *Saccharomyces cerevisiae* and in the absence of solid support was carried out as negative control.

As another method for ensuring the activity of immobilized microbes on the solid support; Ethanol production of microbes was measured. Dried immobilized microbe system was added to streilized sugar solution (10 g/l). For a duration of 10 days ethanol concentration was monitored using gas chromatography. Column temperature was set at 80°C (Isothermal condition) and as the carrier gas H2 (30 ml/min) was used. After 4 days, solid support was washed with sterilized water at room temperature (28°C) and dried for over night under aseptic techniques. Using dynamic conditions, freshly prepared sugar solution (10 g/l) was added to test reusability. Without solid support microbes were inoculated to standard sugar solution as negative controls.

To get the morphological features of immobilized *Saccharomyces cerevisiae*, dried coconut tree leaf sheath with immobilized microbe was subjected to Scanning Electron Microscope (SEM) imaging. SEM was operated at an accelerated voltage 18 kV and at a working distance of a 15 mm. The samples were gold plated by using gold sputter.

Results and Discussion

Obtaining growth curves

Immobilized *Saccharomyces cerevisiae* on coconut tree leaf sheath had lower gradient in log phase. Immobilized *Saccharomyces cerevisiae* had slower growth rate than *Saccharomyces cerevisiae* in culture. In comparison to yeast in culture [◆], the population on solid support does not come to death phase after 25 hours. This suggests that coconut tree leaf sheath provides favorable environment for the microorganism for better growth (Figure 6). However with silica coated glass, no detectable growth was obtained. It was found that the coating also is unstable.

Ethanol production by immobilized microbes

Under static conditions the immobilized population shows a higher efficiency in ethanol production over control without solid support. The immobilized culture can be reused with similar efficiency (Figure 7).

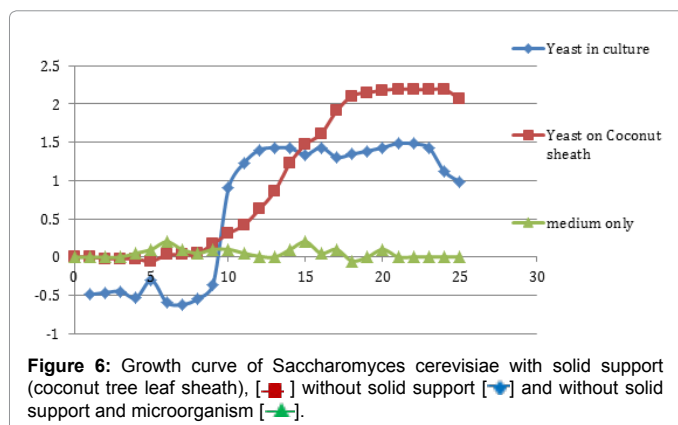


Figure 6: Growth curve of *Saccharomyces cerevisiae* with solid support (coconut tree leaf sheath), [■] without solid support [◆] and without solid support and microorganism [▲].

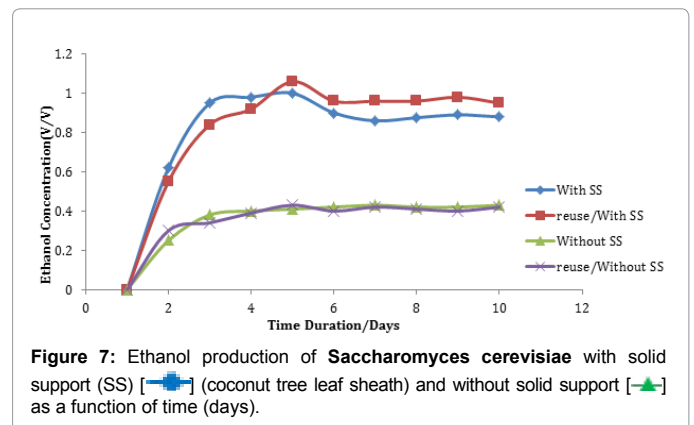


Figure 7: Ethanol production of *Saccharomyces cerevisiae* with solid support (SS) [◆] (coconut tree leaf sheath) and without solid support [▲] as a function of time (days).

Day	Ethanol concentration of immobilized <i>Saccharomyces cerevisiae</i> (V/V)	Ethanol concentration of negative control (V/V)	Percentage of increase in ethanol production over negative control
1	0	0	0
2	0.62	0.25	59.6
3	0.95	0.38	60.0
4	0.98	0.40	59.1
5	1.00	0.41	60.0
6	0.90	0.42	53.3
7	0.86	0.43	50.0
8	0.87	0.42	51.7
9	0.89	0.42	52.8
10	0.88	0.43	51.1

Table 1: Percentage increase of ethanol concentration of immobilized *Saccharomyces cerevisiae* on coconut tree leaf sheath over its negative control.

The immobilized culture produces higher percentage ethanol over unsupported culture under similar conditions which can be shown by the Table 1.

The percentage increase in ethanol yield was calculated by formula given below.

$$\% \text{ Increase} = \frac{\text{Ethanol concentration by immobilized culture} - \text{Ethanol concentration by negative control}}{\text{Ethanol concentration by negative control}}$$

Immobilized microbe was functionally active on coconut leaf sheath.

SEM analysis

According to Figure 8 it shows that coconut tree leaf sheath has large surface area with grooves in structure which can provide safe and favorable micro environments to the microorganism.

Saccharomyces cerevisiae was adsorbed physically and none specifically in to coconut leaf sheath according to Figure 9. This figure shows the efficient colonizing at the solid support. The nutrients which can be absorbed from coconut leaf sheath to microorganism may be an additional advantage towards efficient colonizing.

Coconut tree leaf sheath consist of mostly with cellulose layers (Figure 8). Due to -OH functional groups of cellulose, it can be induced inter and intra hydrogen bonding in between cellulose layers. This hydrogen bonding can be produced with -OH groups of cell wall of *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* can be attached through vanderwalls interaction and hydrogen bonding to coconut tree leaf sheath.

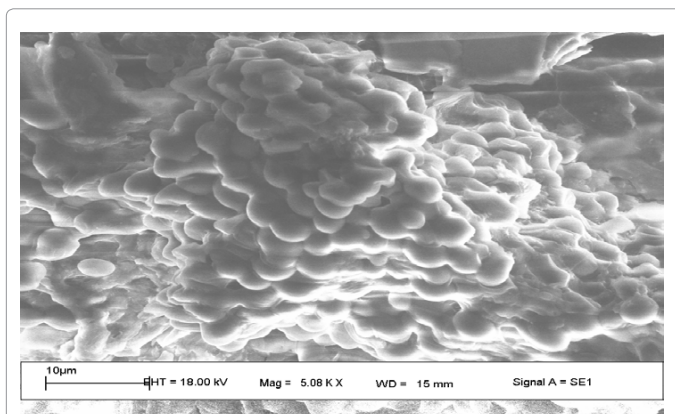


Figure 8: SEM analysis for coconut tree leaf sheath under 101 magnification.

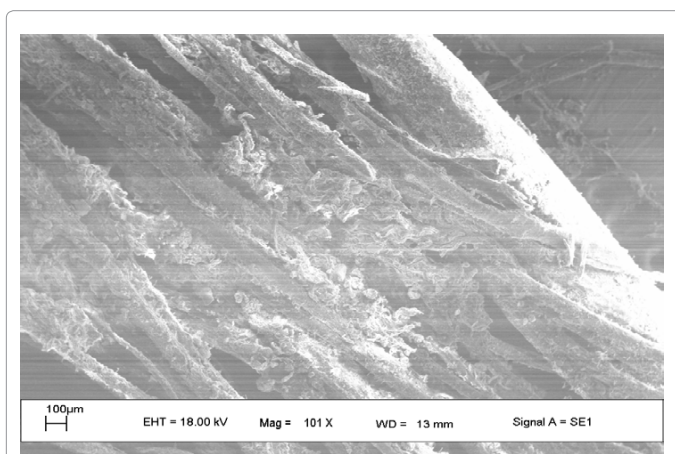


Figure 9: SEM analysis for immobilized *Saccharomyces cerevisiae* on coconut leaf sheath under 5.08×10^3 magnification.

Coconut tree leaf sheath is an efficient solid support for immobilization. Immobilized microbes can be reused in fresh fermentation media. If immobilization can be carried out utilizing naturally available substances as solid supports, it will be very cost-effective and eco-friendly.

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