COCONUT WATER AS A NOVEL CULTURE MEDIUM FOR THE BIOTECHNOLOGICAL PRODUCTION OF SCHIZOPHYLLAN

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ABSTRACT - In our search for a more practical, appropriate and economical medium for the biotechnological production of schizophyllan from *S. commune* which is suitable for developing countries, we have investigated the possibility of utilizing coconut water as an appropriate medium. Coconut being an ubiquitous crop in tropical areas of the world produced water as a waste product. Thus in this investigation, water from matured coconut was evaluated for schizophyllan production. *Schizophyllum commune* ATCC 38548 was used as the test strain. The procedure of Rau (1999) in the production of schizophyllan was adopted. The performance of *S. commune* grown in coconut water was determined in parallel with its performance in basal semi-synthetic medium consisting of glucose, 30g; yeast extract, 3g, KH_2PO_4, 1g and $MgSO_4.7H_2O$, 0.5g in 1000 ml deionized water and a triple sugar enriched medium containing 10.3g glucose, 7g fructose, 9g sucrose, 3g yeast extract, 1g KH_2PO_4 and 0.5g $MgSO_4 \cdot 7H_20$ dissolved in 1000 ml deionized water. Results of our investigation showed that coconut water could stimulate the growth of *S. commune* with subsequent production of schizophyllan at 7.71g/1000 ml 4 days after incubation which is a day earlier than in the two semi-synthetic media. The basal semi-synthetic and the triple sugar- enriched media yielded 6.69g and 3.99g of schizophyllan per 1000 ml of the medium, 5 days after incubation, respectively.

Keywords: **fungal polysaccharide, glucan, schizophyllan, *Schizophyllum commune***

INTRODUCTION

Schizophyllum commune Fries is a higher fungus which belongs to Family Schizophyllaceae, Order Aphyllophorales, Phylum Basidiomycota of the Kingdom Fungi (Reyes et al 2003). It is known to produce exopolysaccharides called schizophyllan. Schizophyllan is a jelly – like slimy material which is soluble in water. Previous researchers have reported the industrial value of this metabolite. This mushroom is of great importance in the pharmaceutical and food industries since it produces metabolites which are essential in the production of industrial products. For instance, it is utilized in the preparation of skin care products like lotion and creams which acts as viscosifier and as anti-aging, depigmenting and healing agent of the skin (Kim et al 1999, 2000). Its active ingredient called schizophyllan is responsible for increased skin cell proliferation, collagen biosynthesis and enhancing the recovery from sunburn and is used as an immunonotherapeutic agent for cancer treatment in Japan (Rau 1999). It was also reported to have immune-stimulating activities, was claimed to have anti-HIV activity (Shigero et al 1989; Hagiwara and Kikuchi, 1992) and is an immune effect enhancer for vaccines (Honma, 1994). Aside from schizophyllan, *S. commune* was also reported to produce schizostatin (Tanimoto et la 1995), a potent inhibitor of squalene synthase. Squalene synthase is the enzyme involved in the biochemical pathway of cholesterol and triglycerides biosynthesis (Hiyoshi et al 2003). Recently, *S. commune* was used as starter for the production of cheese due to its ability to produce lactose dehydrogenase and milk - clotting enzyme (Matsui et al 2001).

Due to its nutriceutical and industrial significance, several researchers have done a number of investigation that lead to the successful production of schizophyllan. For instance, Rau of the Federal Republic of Germany (1999) and his colleagues (Rau and Brandt, 1994; Gura and Rau, 1993; Rau et al, 1992; 1990) did thorough investigation on the upstream and downstream processing of schizophyllan by growing *S. commune* ATCC 38548 strain in semi – synthetic medium composed of 30g glucose, 3g yeast extract, 1g KH_2PO_4 and 0.5g $MgSO_4 \cdot 7H_20$ dissolved in 1000 ml deionized water under submerged condition. Steiner et al (1987) on the other hand, were able to produce schizophyllan, xylanase and cellulase from a wild strain of *S. commune* from Bangladesh which was cultured in a semi-synthetic medium containing 4% Avicel, 3.5% peptone and 0.5%

$Ca(NO_3)_2 \cdot 4\ H_2O$ dissolved in 1000 ml distilled water. However, trials of growing *S. commune* in natural substrates for the production of schizophyllan have not been fully explored. Rau (personal communication) mentioned that growing this fungus on molasses derived from sugar beet for the production of schizophyllan was successfully tried. The difficulty lies only on the downstream processing of the product due to the presence of impurities in molasses. Further downstream processing of the product entails additional cost which eventually made the end product more expensive. In this investigation, the possibility of utilizing coconut water as a medium for schizophyllan production was explored. Fresh coconut water is clean and nutritious. Being a liquid endosperm of the coconut fruit, coconut water contains minerals as well as other nutrients that can support the growth of fungi. In the Philippines, it was successfully used as a medium for the mycelial culture of edible mushrooms and other microscopic fungi (Bulseco et al, 2005; Garcia et al 2004; Tayamen et al 2004; Dagdag and Reyes, 1991; Reyes et al 1992, 1993). Coconut water is an abundant waste resource in the coconut industry especially in the tropical regions of the world. In the Philippines for instance, coconut water is ubiquitous considering that the country is the largest producer of coconut in the world (Sakakibara 1994) and a quarter (3 million hectares) of the country's agricultural land is planted to coconut (Tiglao, 1999). When coconut is cracked and its meat is taken for oil production, its water becomes waste and oftentimes disposed. In the Philippines, housewives utilized this renewable resource as fermentation substrate for the home – made production of nata de coco and vinegar. With its nutritional attributes (http://coconutboard.nic.in/tendnutr.htm) and abundance as well as with the excellent performance of *S. commune* in this medium as we report in this paper, it can be used as a suitable and economical medium for the production of schizophyllan in submerged condition.

MATERIALS AND METHODS

Revival of stock culture. Stock culture of *S. commune* ATCC 38548 was revived in a basal semi-synthetic broth medium (pH 5.1, unadjusted) consisting of glucose monohydrate, 33g/1000 ml; yeast extract, 3g/1000 ml, KH_2PO_4 (Merck KGaA, Germany), 1g/1000 ml and $MgSO_4.7H_2O$ (Fluka Chemika AG CH-9471), 0.5g/1000 ml. For stocking purposes, approximately 10 mm mycelial block from the old stock culture was aseptically sliced and inoculated at the center of a newly prepared potato dextrose agar slant using a sterile inoculating needle. To prepare the submerged culture, 10 ml of the inoculum was aseptically siphoned out and inoculated into a 500 ml capacity Erlenmeyer flask with baffles containing 100 ml of the semi-synthetic broth medium. Inoculated media were then incubated at 27^oC under dark condition.

Experimental Lay-out. To determine the influence of coconut water on the mycelial biomass, schizophyllan production and glucose concentration in the medium, the following treatments with two replicates were set-up:

T_1 – glucose – based basal semi synthetic broth (pH 5.1, unadjusted)

T_2 - coconut water (pH 5.7, unadjusted)

T_3 – triple sugar enriched basal semi synthetic broth (pH 4.9, unadjusted)

Two - 2000 ml capacity flasks with baffles were dispensed with 1000 ml of the basal semi synthetic broth medium with each flask containing 500 ml. Similarly, two flasks of the same capacity as in the first two flasks were filled with 500 ml each of coconut water which was derived from newly cracked matured coconut,. The two flasks for the last treatment (T_3) containing 10.3 g glucose, 7 g fructose (Merck), 9 g sucrose (Fluka BioChemika), 3 g yeast extract, 1 g KH_2PO_4 and 0.5g $MgSO_4 \cdot 7H_20$ dissolved in 1000 ml deionized water was also prepared as in the first treatment. The 6 flasks were plugged with cotton, covered with aluminum foil and sterilized by autoclaving at 121^oC, 15 pounds per square inch for 20 minutes.

Preparation of Inocula. The 4-day old submerged culture of *S. commune* was homogenized using an Ultra – Turrax T25 (Janke & Kunkel IKA®-Labortechnik) homogenizer to disintegrate the mycelial pellets. Ten ml (i.e. 2% of the total volume of the medium in the flask) of the homogenized culture was aseptically inoculated into the previously sterilized culture media and subsequently incubated in shaken condition at 27^oC without illumination.

Analysis of Cultural Parameters.

Preparation of samples. Twenty ml was withdrawn from the shake flask-culture to determine the glucose concentration, schizophyllan content and mycelial biomass. The withdrawn sample was equally divided into two lots and dispensed in centrifuge tubes. Centrifugation was undertaken at 13000 rpm for 30 minutes in Heraeus SEPATECH Biofuge 17RS at 20^oC (room temperature). The supernatant was collected for glucose determination and schizophyllan analysis.

Determination of mycelial biomass. The collected pellet was washed with deionized water followed by centrifugation at 13000 rpm for 30 minutes in Heraeus SEPATECH Biofuge 17RS at 20^oC (room temperature). The mycelial pellet was oven dried at 100^oC for 48 hr for the determination of the mycelial dry weight.

Analysis of schizophyllan. Five grams of the supernatant was mixed with 15 ml of isopropanol. This was subsequently placed at 4^oC for 48 hr to allow the precipitation of the shizophyllan. To separate the precipitate from isopropanol, the sample was centrifuged at 4^oc, 13,000 rpm for 10 minutes. The isopropanol was discarded and the suspended

schizophyllan was dried at 50 mbar for 48 hr using an oven set to 40°C and attached to a vacuum drier (KVW-Technik GmbH).

Determination of glucose concentration. Glucose content of the media was monitored using Accutrend® glucose strips (Roche, Inc., Germany) and read in Accutrend® GC (Roche, Inc., Germany).

RESULTS AND DISCUSSION

In this investigation, the possibility of utilizing coconut water as suitable medium for the biotechnological production of schizophyllan was tried. Coconut water, which is always available as waste product of coconut industry in developing countries of Asia and the Carribean, was evaluated in comparison with the existing semi-synthetic medium for the production of schizophyllan and a triple sugar enriched - basal semi synthetic medium containing the 3 sugars which are normally present in coconut water namely glucose, fructose and sucrose. Other researchers have used semi-synthetic medium for the growth of *S. commune* and its subsequent production of schizophyllan. For instance, Steiner et al (1987) utilized a medium consisting of 4% Avicel, 3.5% peptone and 0.5% $Ca(NO_3)_2 \cdot 4\ H_2O$ dissolved in 1000 ml distilled water. They were able to produce cellulase and xylanase in addition to schizophyllan. Rau (1999) on the other hand was able to establish the appropriate medium for the biotechnological production of schizophyllan which is composed of 30g glucose, 3g yeast extract, 1g KH_2PO_4 and 0.5g $MgSO_4 \cdot 7H_20$ dissolved in 1000 ml of deionized water under submerged condition. Using this formulation, he was able to produce schizophyllan as the major product. This formulated semi-synthetic medium is suitable in developed countries where synthetic glucose is readily available and cheaper than media derived from natural sources. Moreover, another medium formulation (10.3g glucose, 7g fructose, 9g sucrose, 3 g yeast extract, 1g KH_2PO_4 and 0.5g $MgSO_4 \cdot 7H_20$ dissolved in 1000 ml of deionized water) containing the three major sugars present in coconut water namely glucose, fructose and sucrose was also evaluated. In our desire to develop a natural medium from coconut water, we selected the formulation of Rau (1999) as reference due to its practicality and superiority in terms of stimulating the growth of *S. commune* and its efficient production of schizophyllan. As in any biotechnological production, we determined the appropriateness of coconut water as a suitable medium on the basis of schizophyllan production by *S. commune*. Coconut water is superior among the evaluated media. The peak of production of schizophyllan was recorded in coconut water a day earlier than the two semi-synthetic media. Coconut water produced 7.71g/ 1000 ml 4 days after incubation. Highest production of schizophyllan was recorded in basal semi-synthetic medium (6.69g/1000 ml) and the triple sugar – enriched medium (3.99g/1000 ml) 5 days after incubation. The peak of mycelial biomass production was recorded in all the media 5 days after incubation in the dark at 27°C and started to decelerate thereafter. It is surprising to note that despite the presence of the three important sugars in the triple sugar enriched medium, *S. commune* did not yield an appreciable amount of biomass and schizophyllan compared to the basal semi – synthetic medium and coconut water. Mycelial pellets produced in the triple sugar – enriched medium are irregular in size compared to the two culture media that produced uniform size of mycelial pellets (Fig 1). Moreover, it was observed that schizophyllan production is coupled with mycelial growth. This important finding implies that *S. commune* is already producing schizophyllan in the process of its mycelial proliferation. This notable observation further reaffirms the previous observation of Rau (1999) on the behavior of *S. commune* and the positive correlation between mycelial growth and schizophyllan production. It is interesting to note that coconut water could stimulate the mycelial colonization of *S. commune* with a concomittant production of schizophyllan despite of its limited glucose content (3.04g/1000 ml). As reported previously (http://www.geocities.com/vidhuofcms/nutrival.htm), coconut water derived from matured coconut contains primarily sugar and minerals with fats and proteins as minor constituents. The sugar may be in the form of reducing (glucose and fructose) and non-reducing (sucrose) where the level of the former decreases (from 83.3% to 10) while the latter (from 16.7 to 90%) increases as the coconut reaches its maturity stage. The better performance of *S. commune* to produce schizophyllan in coconut water derived from matured coconut that we used in this investigation may be partly attributed to its ability to secrete invertase (ß-fructofuranoside fructohydrolase, EC 3.2.1.26) that converts sucrose into glucose and fructose (Rojo et al 1994). *S. commune* is an interesting organism in fermentation. Being aggressive and non - fastidious, it has the ability to adapt to a changing nutritional environment. For instance, its ability to grow on cellulose – rich substrates resulted to the production of cellulase, xylanase and mannanase (Haltrich and Steiner, 1994). Even in a situation where carbon becomes limiting, it could switch to the utilization of glucan as carbon source due to its ability to produce ß- glucanases (Rau, 2005). Among the minerals (K, Na, Ca, P, Cu, S, Fe, Mn, Zn, Cu and Mg), potassium was the most abundant in young coconut and being replaced by sodium when the coconut matures. With regards to the protein content of the coconut water, the level increases from 0.13% to 0.29%. This level is enough to fulfill the nitrogen requirement of the growing mycelia in submerged condition (Rau 1999).

As shown in Table 1, coconut water is an appropriate medium in the production of schizophyllan in developing countries like the Philippines due to its technical feasibility and financial viability. Schizophyllan can be produced using coconut water as the medium with no direct cost in its production since coconut water is always free.

Table 1. Economics of schizophyllan production

PARTICULARS	QUANTITY	UNIT PRICE (US$)	ACTUAL PRICE (US$)	SCHIZOPHYLLAN PRODUCTION (G/1000 ML)	COST OF SCHIZOPHYLLAN (US$)*	DIRECT COST OF PRODUCTION (US$)	PROJECTED PROFIT (US$)
Components of T_1							
Glucose	30 g/1000ml	22.92/1000g	0.69				
Yeast extract	3 g/1000ml	18.24/100g	0.54	6.69	30.11	1.31	28.80
KH_2PO_4	1 g/1000ml	14.88/250g	0.06				
$MgSO_4 \cdot 7H_2O$	0.5 g/1000ml	21.00/500g	0.02				
Components of T_2							
Coconut	1000 ml of coconut water from 10 pieces of coconut	0.25	0.25	7.71	34.70	0.25	34.45
Components of T_3				3.99	17.96	1.99	15.97
Glucose	10.3 g/1000ml	22.92/1000g	0.24				
Fructose	7 g/1000ml	30.90/250g	0.86				
Sucrose	9 g/1000ml	30.60/1000g	0.27				
Yeast extract	3 g/1000ml	18.24/100g	0.54				
KH_2PO_4	1 g/1000ml	14.88/250g	0.06				
$MgSO_4 \cdot 7H_2O$	0.5 g/1000ml	21.00/500g	0.02				

T_1= Basal semi synthetic medium; T_2= Coconut water; T_3= Triple sugar enriched - semi synthetic medium
**Based from the price of commercial schizophyllan, 500g, US$2,250.00 (European Technologies, Inc., USA)*

However, when semi – synthetic medium is used, the cost ranges from US$1.31 to 1.99 in order to produce to 7 grams of schizophyllan.

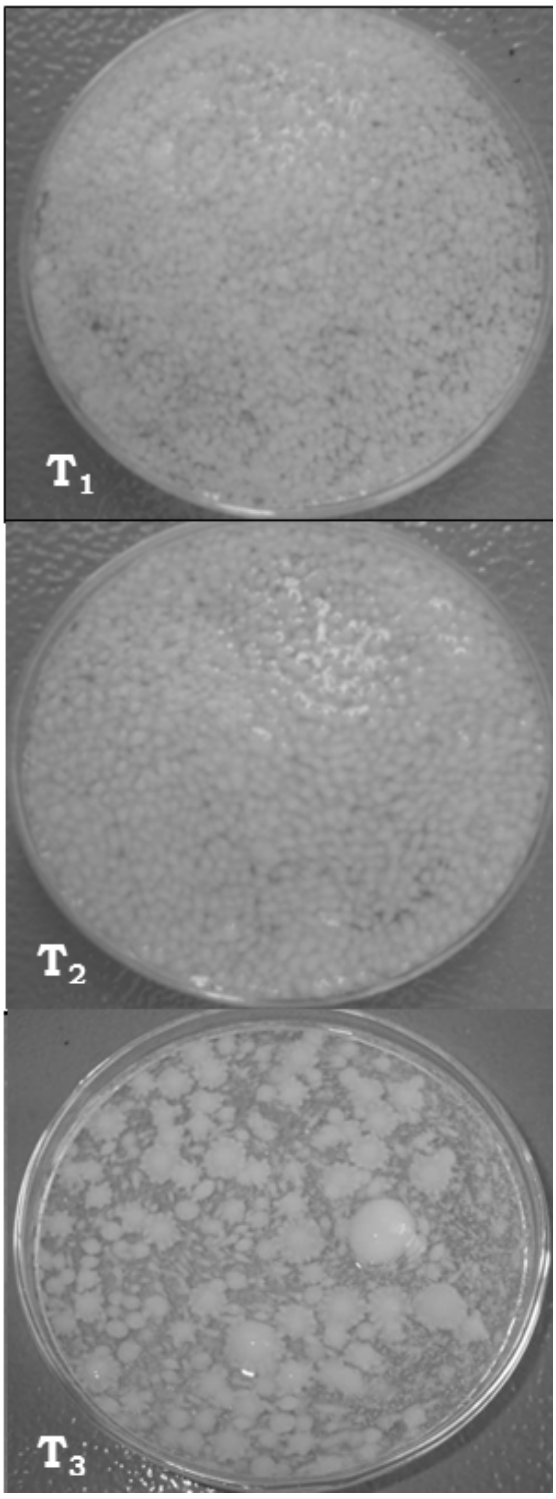

Figure 1. Mycelial pellets of *S. commune* embedded in schizophyllan – riched media 5 days after incubation in the dark at 27°C in shaking condition. (*Notes: T_1 – glucose – based basal semi synthetic broth (pH 5.1, unadjusted); T_2 - coconut water (pH 5.7, unadjusted) and T_3 – triple sugar enriched basal semi synthetic broth (pH 4.9, unadjusted)*

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