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BIOMASS PRODUCTION AND FORMULATION OF *Bacillus subtilis* FOR BIOLOGICAL CONTROL

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ABSTRACT

Bacillus subtilis is a widespread bacterium found in soil, water, and air. It controls the growth of certain harmful bacteria and fungi, presumably by competing for nutrients, growth sites on plants, and by directly colonizing and attaching to fungal pathogens. When applied to seeds, it colonizes the developing root system of the plants and continues to live on the root system and provides protection throughout the growing season. The study on biomass production and formulation of *B. subtilis* for biological control was conducted in the laboratory of Department of Plant Pathology, College of Agriculture, University of the Philippines Los Baños (UPLB-CA), College, Laguna from May to July 2005. The objective of the study was to determine the optimum pH and a good carbon source for biomass production of *B. subtilis* and to develop a seed treatment formulation of *B. subtilis* as biological control agent. Results showed that the optimum pH for growth of *B. subtilis* was pH 6 (1.85 x 10^9 cfu/ml). In laboratory tests for biomass production using cassava flour, corn flour, rice flour, and brown sugar as carbon sources, it grew best in brown sugar plus yeast extract medium (6.8 x 10^8 cfu ml^{-1} in sterile distilled water and 7.8 x 10^8 cfu ml^{-1} in coconut water). In test for bacterial biomass carriers, talc proved to be the best in terms of number of bacteria recovered from the seeds (3.98 x 10^5 cfu $seed^{-1}$).

[***Keywords***: *Bacillus subtilis*, biomass, formulations, biological control]

INTRODUCTION

Bacillus subtilis is a ubiquitous bacterium commonly recovered from water, soil, air, and decomposing plant residue. It is a spore-forming bacterium which, when applied to seeds, colonizes the developing root system of the plants. The bacterium competes with and thereby suppresses plant disease fungal organisms such as *Rhizoctonia, Fusarium, Aspergillus,* and others. The bacterium continues to live on the root system and provides protection throughout the growing season (EPA 2003).

Leibinger *et al.* (1997) observed that selected antagonistic isolates of *B. subtilis, Aureobasidium pullulans,* and *Rhodotorula glutinis* reduced the size and number of lesions on wounded apples caused by the postharvest pathogens *Penicillium expansum, Botrytis cinerea*, and *Pezicula malicorticis*. They applied combinations of the antagonistic bacteria to apple trees in the field late in the growing season of two consecutive years. They found that population sizes of the introduced antagonists on apple surfaces increased in the field following application of treatments until harvest. After transfer of the fruit from the field into cold storage, the populations of the introduced antagonists remained higher than in the control treatments.

In South Africa, Korsten *et al.* (1997) found that preharvest applications of *B. subtilis* field sprays integrated with copper oxychloride or benomyl consistently reduced the severity of avocado black spot caused by *Pseudocercospora purpurea.* In the greenhouse trial, Schisler *et al.* (2002) observed that *B. subtilis* strain AS 43.3 decreased the severity of *Fusarium* head blight caused by *Giberella zeae* in as much as 90%.

Another report mentioned that *B. subtilis* produces an antibiotic, called iturin, which is effective against various pathogenic fungi, such as *Sclerotina fruticola* causing brown rot on picked stone fruit. *B. subtilis* has also been tested for control *Verticillium* and *Streptomyces gramicifaciens* causing root rot on cucumber, corky rot on tomato, and carnation wilt. Following seed treatment, *B. subtilis* colonized the developing root system, competed with disease organisms that attacked root system, and hence suppressed disease caused by *Fusarium* spp. and *Rhizoctonia* spp. (Anonymous 2002).

Canaday and Ownley (1999) observed that populations of *B. subtilis* on snap bean seedling roots were proportional to the rate of *B. subtilis* applied. Moreover, neither variety nor chemical seed treatment significantly affected root colonization by *B. subtilis*. Combining of chemical seed treatment with commercial formulation of *B. subtilis* at 1 oz/100 lb seed

significantly increased mean yields. The objectives of the study were to determine the optimum pH and a good carbon source for biomass production of *B. subtilis*, and to develop a seed treatment formulation of *B. subtilis* as biological control agent.

MATERIALS AND METHODS

The study was conducted in the laboratory of Department of Plant Pathology, College of Agriculture, University of the Philippines Los Baños (UPLB-CA), College, Laguna from May to July 2005.

Determination of Optimum pH

The medium used in this experiment was brown sugar (10 g) and yeast extract (2.5 g) in 1000 ml of sterile distilled water (SDW). Forty-five ml of the medium was dispensed into 500 ml E-flasks and pH of the medium was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 before sterilization at 15 psi for 15 minutes.

B. subtilis from 36-hour culture slants on potato dextrose peptone agar (PDPA) medium (250 g potato, 20 g dextrose, 15 g peptone, 20 g agar in 1000 ml SDW) was suspended in SDW (10 ml SDW per slant) and the bacterial suspension was stirred with a Vortex mixer. Ten percent (5 ml) of bacterial suspension was inoculated into each flask medium and shaken at 50 rpm for 10 hours using Lab-Line@ Orbit shaker. Each treatment was replicated three times. The optical density (OD_{600nm}) was recorded using a spectrophotometer (Spectronic 20). The OD readings were transformed into standard curve of correlation between OD and bacterial population to determine the bacterial population.

Determination of Carbon Sources

The experiment was arranged in factorial completely random design consisting of three factors. The first factor was two solvents (coconut water and SDW), the second factor was four carbon sources (1% cassava flour, 1% corn flour, 1% rice flour and 1% brown sugar) and substrate with no carbon content, and the third factor was yeast extract (0% and 0.25%). Detail of chemical compositions of the carbon sources is presented on Table 1.

The treatments were arranged as follows:

Medium A_1 = SDW
Medium A_2 = Coconut water (CW)
Medium B_1 = SDW + 0.25% yeast extract (YE)
Medium B_2 = CW + 0.25% YE

Table 1. Constituents of the different carbon sources used in the media preparation (mg 100 g^{-1}).

Contituents	Cassava[1]	Corn[1]	Rice[2]	Brown sugar[3]
Water	0.062	0.012	-	-
Carbohydrate	0.0347	0.071	0.078	97,300
Protein	0.002	0.010	0.008	-
Fat	0.003	0.0045	0	-
Calcium	33	12	20	85
Iron	0.70	2.50	0.80	1.30
Phosporous	0.09	0.27	-	3.90
Potassium	-	-	-	100
Magnesium		-	30	23
Vitamin A	tr	tr	-	-
Thiamine, B1	0.06	0.35	0.03	0.008
Riboflavin, B2	0.03	0.13	0.036	0.007
Niacin	0.06	2	3	0.082
Vitamin C	36	0	-	0.50
Zinc	-	-	1	0.18
Amylose	-	-	0.076	-

Sources: [1]Okigbo (2005); [2]Juliano and Bechtel (1985); [3]Dhampure Specialty Sugars Ltd (2003).

Medium C_1 = SDW + cassava flour
Medium C_2 = CW + cassava flour
Medium D_1 = SDW + cassava flour + 0.25% YE
Medium D_2 = CW + cassava flour + 0.25% YE
Medium E_1 = SDW + corn flour
Medium E_2 = CW + corn flour
Medium F_1 = SDW + corn flour + 0.25% YE
Medium F_2 = CW + corn flour + 0.25% YE
Medium G_1 = SDW + broken rice flour
Medium G_2 = CW + broken rice flour
Medium H_1 = SDW + broken rice flour + 0.25% YE
Medium H_2 = CW + broken rice flour + 0.25% YE
Medium I_1 = SDW + brown sugar
Medium I_2 = CW + brown sugar
Medium J_1 = SDW + brown sugar + 0.25% YE
Medium J_2 = CW + brown sugar + 0.25% YE

All media were adjusted to pH 6 with 1 N HCl. Each medium was dispensed into 18 test tubes (7.2 ml per test tube) and autoclaved at 15 psi for 15 minutes.

B. subtilis from 36-hour culture was suspended in SDW (10 ml SDW per slant) and the bacterial suspension was stirred in a Vortex mixer. Ten percent (0.8 ml) of bacterial suspension was inoculated into each test tube and shaken at 50 rpm. Uninoculated test tubes were prepared as control. Each treatment was replicated three times. The growth of bacteria in the different test media was recorded at 2, 4, 6, 8, 10, and 12 hours using Spectronic 20 at OD_{600nm}. Data of the OD readings were transformed into standard curve.

Biomass Production of *B. subtilis*

B. subtilis was cultured in potato dextrose broth (PDB - 250 g of peeled and sliced potato and 20 g of dextrose in 1000 ml of distilled water). The PDB was dispensed in five 1-L flasks at 180 ml per flask, and then autoclaved at 15 psi for 15 minutes. After sterilization, each flask was inoculated with 20 ml (10%) of 36-hour old SDW suspense of B. *subtilis*. The culture was shaken for 36 hours. The biomass was harvested by centrifugation at 5000 rpm for 6 minutes. The centrifuged cells were used in the formulation.

Formulation of *B. subtilis*

The inert carriers and additives used in the formulations were:

A. Talc + 0.25% YE + 1% carboximethyl cellulose (CMC).
B. Rice flour + 0.25% YE + 1% CMC.
C. Cassava starch + 0.25% YE + 1% CMC.
D. Corn starch + 0.25% YE + 1% CMC.
E. Powdered rice bran + 0.25 YE + 1% CMC.
F. Mt. Pinatubo ash + 0.25% YE + 1% CMC.
G Charcoal powder + 0.25% YE + 1% CMC.

Twenty-five grams of inert carrier, 0.25% YE, and 1% CMC were mixed on a sterile aluminum tray. The mixture was placed in 500-ml flasks and autoclaved at 15 psi for 30 minutes. Twenty ml of bacterial cells were added into the inert carrier under aseptic conditions, then the mixture was air dried in a laminar flow chamber for 48 hours. After drying, the formulated product was packed in sterile polypropylene bags, sealed and stored at room temperature until further use.

Analyses of Bacterial Population from Corn Seeds Mixed with Formulated *B. subtilis*

The bacterial population in the formulated biomass was determined as follows: One gram of the bacterial formulation was added to 100 g corn seeds wetted with 1 ml SDW in a sterile plastic bag. The mixture was shaken until the seeds were thoroughly coated with the formulation. Three corn seeds coated with the formulation were taken randomly and placed separately into test tubes containing 10 ml SDW. With a sterile pipette, 0.1 ml from a 10^{-2} dilution was placed onto PDPA plates and spread using a sterile glass rod hockey. The plates were sealed and incubated in inverted position. After 24 hours, bacterial colonies were counted.

RESULTS AND DISCUSSION

Determination of Optimum pH

The optimum pH obtained for *B. subtilis* in brown sugar yeast extract medium after shaking at 50 rpm for 10 hours was pH 6 (Fig. 1). For most bacteria, the optimum pH is near the neutral point (pH 7). Certain bacteria are acid tolerant and will survive at reduced pH levels. Ozcan and Altinalan (2001) reported that to obtain optimal α-amylase activity, the pH should be 5-6. Montealegre *et al*. (2003) found that *B. subtilis* 639 and *B. lentimorbus* 640 grew best at pH 5.

Determination of Carbon Sources

Results showed that the growth rate of *B. subtilis* in all test media was slow during the first 4 hours, then became faster until it reached its peak at 10 hours. The growth of *B. subtilis* in media containing cassava flour, corn flour, broken rice flour, and brown sugar using SDW as solvent with and without yeast extract are presented in Figure 2. Bacterial growth in media with 0.25% yeast extract was significantly higher than those grown in media without yeast extract. This might be due to the nutrient content of yeast extract. Neogen (2005) reported that yeast extract is the water-soluble portion of autolyzed yeast containing vitamin B complex. It is an excellent stimulator of bacterial growth. It provides vitamins, nitrogen, amino acids, and carbon in microbiological culture media.

The growth of *B. subtilis* in media containing cassava flour, corn flour, broken rice flour, and brown

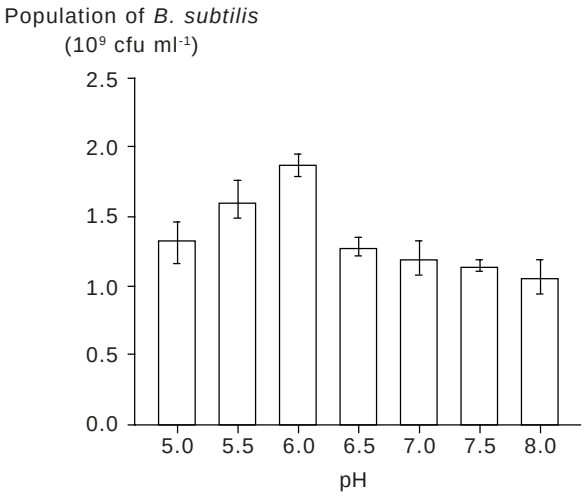

Fig. 1. Effect of pH on growth of *Bacillus subtilis* in brown sugar yeast extract medium after shaking at 50 rpm for 10 hours.

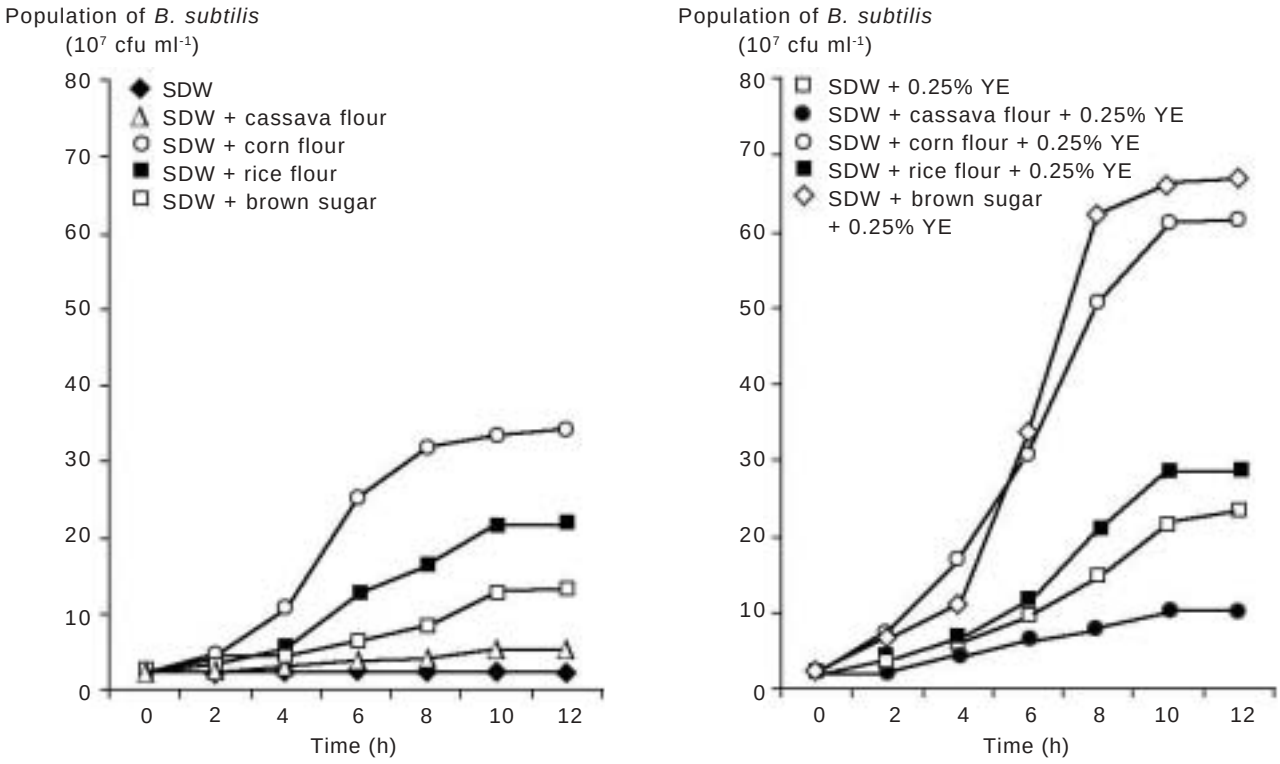

Fig. 2. Growth of *Bacillus subtilis* in different carbon sources (cassava flour, corn flour, broken rice flour, brown sugar) diluted in sterile distilled water (SDW) without yeast extract (left) and with 0.25% yeast extract (right).

sugar using coconut water as solvent with and without yeast are presented in Figure 3. The same growth trend in media using SDW as solvent was observed. Again, the best growth was obtained in brown sugar plus 0.25% YE. This might be due to the high mineral contents in brown sugar (calcium, iron and phosphorous) as compared to other media tested (Table 1). The data showed that *B. subtilis* required growth factors (from yeast extract) for optimal biomass production. Todar (2005) reported that most *Bacillus* species are versatile chemoheterotrophs capable of respiration using a variety of simple organic compounds (sugars, amino acids, organic acids). In some cases, they also ferment carbohydrates in a mixed reaction that typically produces glycerol and butanediol. A few species, such as *Bacillus megaterium*, require no organic growth factors; others may require amino acids, B-vitamins, or both.

B. subtilis grew well in media using coconut water (Fig. 3). This might be due to the high carbohydrates and mineral content of coconut water (Ohler 1999).

Evaluation of Inert Carrier

The best carrier was determined based on the number of bacterial colonies found on the formulation-coated seeds. The inert carriers evaluated were talc, rice flour, cassava starch, corn starch, powdered rice bran, Mt. Pinatubo ash, and charcoal powder.

Results showed that the highest bacterial population (3.9×10^5 cfu seed^{-1}) was on seed coated with the talc base formulation, and the lowest (5.0×10^4 cfu/seed) was on seed with the starch base formulation (Table 2). The ability of talc to stick better on the surface of the seeds compared to the other carriers might explain this observation.

Talc has been used as inert carrier in seed treatment formulation for *Bacillus* plant growth promoting rhizobacteria (PGPR) strain to suppress rice sheath blight disease caused by *R. solani* (Nandakumar *et al.* 2001) and to control downy mildew on pearl millet (Raj *et al.* 2003). All PGPR *Bacillus* spp. tested also promoted the vegetative and reproductive growth of both rice and pearl millet.

CONCLUSION

The optimum pH obtained for *B. subtilis* in brown sugar yeast extract medium after shaking at 50 rpm for 10 hours was pH 6. *B. subtilis* grew best in brown sugar plus yeast extract medium and grew well in media wherein coconut water was used.

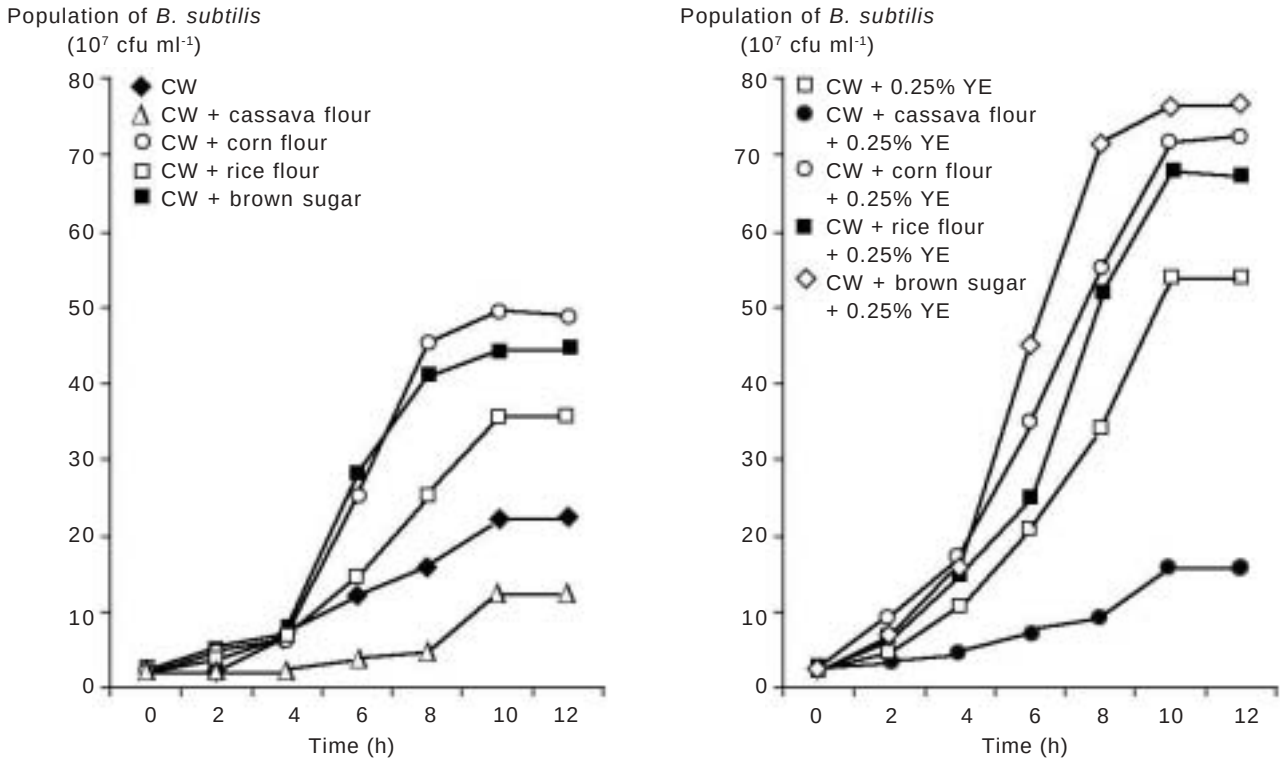

Fig. 3. Growth of *Bacillus subtilis* in different carbon sources diluted in coconut water (CW) without yeast extract (left) and with 0.25% yeast extract (right).

Table 2. Population of *Bacillus subtilis* in formulated product and in corn seed coated with the formulated product amended with 0.25% yeast extract and 1% carboxymethyl cellulose[1)].

Treatments	Bacterial population (10^3 cfu seed^{-1})
Talc	398.00a
Rice flour	
Cassava starch	70.00d
Corn starch	55.00d
Powdered rice bran	97.33c
Mt. Pinatubo ash	100.00c
Charcoal powder	63.00d
Control (plain corn seed)	0.00e
5% LSD	23.38
CV (%)	10.00

[1)]Data are averages of three plates.
In a column, means followed by a common letter are not significantly different at the 5% level by LSD.

Several bacterial biomass carriers namely, talc, rice flour, cassava starch, cornstarch, powdered rice bran, Mt. Pinatubo ash, and powdered charcoal were evaluated. Of these, talc proved to be the best in terms of bacterial colonies recovered from coated seeds.

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