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Factorial Design for Optimization of Rice Straw Incorporation into Soil Using *Micromonospora chalcea*

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Abstract: A Box-Wilson central composite design was applied to study the effect of *Micromonospora chalcea* inoculum-size, municipal sludge and rice straw ratios on decomposition of the straw in sandy soil. The experiment included 20 runs with five levels for each of the three factors. Results indicated highly significant effect of sludge ($p = 0.005$) on the straw decomposition and organic carbon release. Although inoculum size of *M. chalcea* was not significantly effective, it showed a significant interaction with sludge ($p = 0.021$). Probably, indicating a critical importance of sludge as source of essential nutrients for supporting *M. chalcea* growth. Extracellular enzyme profiling of *M. chalcea* revealed a general wide activity including: polysaccharide hydrolases, proteases, lipases, phosphatases and amino-peptidases. Response surface methodology was employed for the optimization of rice straw decomposition by *M. chalcea*. Conditions that supported highest carbon release were incorporating 100 g straw with 40 g sludge into 100 g sandy soil, leading to 10 folds reduction in time of straw decomposition compared to that before optimization. On the other hand, sludge ratios above 81% have retarded the decomposition process even under straw ratio as low as 10%.

Key words: Response surface analysis, actinomycetes, lignocellulose decomposition, extracellular enzymes

INTRODUCTION

The microbial decomposition of lignocellulosic agricultural wastes is recently recognized as a major renewable resource, with potential for conversion to a variety of products such as soil biofertilizers, industrial enzymes, animal feeds and biofuels (Howard *et al*., 2003). However, cellulose crystallinity and lignin - which offers cellulose physical protection against cellulolytic enzymes-limit the decomposition of polysaccharides present in lignocellulose residues for biotechnological application (Lynd *et al*., 2002). Rice straw is one example of the lignocellulosic wastes that is often disposed by biomass burning; a practice which is not restricted to developing countries alone, but is considered a global phenomenon (Levine, 1996). Rice straw composed mainly of cellulose and hemicellulose encrusted by lignin, in addition to a small amount of protein which makes it high in C:N ratio and persistent to microbial decomposition compared to straw from other protein-rich grains such as wheat and barely (Parr *et al*., 1992).

Many actinomycetes can degrade cellulose and solubilize the lignin structure as their primary metabolic activity at high nitrogen levels compared to white rot fungi (Eriksson *et al*., 1990). Previous studies shown that actinomycete strains, particularly *Streptomyces*, *Micromonospora* and *Nocardioides* are capable of attacking the lignocellulosic components of rice straw, causing significant release of carbon and to degrade synthetic organic dyes that are structurally related to lignin (El-Shatoury *et al*., 2005a, b; Abdulla and El-Shatoury, 2006). The addition of sludge, as a rich source of nitrogen and labile carbon, enhanced the decomposition of straw by the above mentioned actinomycete strains and was suggested to accelerate its incorporation into soil.

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Traditional methods of optimizing straw decomposition have involved changing one variable, while fixing the others at a certain level. This single dimensional approach is time consuming and fails to locate optimal conditions because it doesn't consider the effect of possible interactions between variables. Factorial design and response surface analysis, first described by Box and Wilson (1951), is an experimental strategy for seeking the optimum conditions for a multivariable system. This technique has been widely used in applied sciences such as optimizing enzyme production and assessment of drug interactions (Kalil *et al*., 2000); however, it has not been well exploited to optimize decomposition of agriculture wastes by microorganisms. Although few previous studies have focused on response surface optimization of lignocellulosic enzymes in fermentation media (Cacchio *et al*., 2001; Trupkin *et al*., 2003), this is the first report-to the best of our knowledge-regarding optimization of agriculture wastes incorporation into soil.

The goal of this study is to study the effect of various ratios of sludge, rice straw and *M. chalcea* inoculum on accelerating the straw decomposition in soil and to investigate the extracellular enzyme profile of *M. chalcea* that may contribute to accelerating the decomposition process. Response surface optimization technique was applied to determine the optimal conditions for accelerating straw incorporation into soil.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

Micromonospora chalcea strain isolated by Abdulla and El-Shatoury (2006) was used in the experiment. The inoculum was produced from spore suspension preserved at -20°C, inoculated into yeast extract dextrose broth and incubated at 28°C, 100 rpm for 5 days. Mycelia were harvested by centrifugation, homogenized, washed twice in phosphate buffer and used for straw incorporation and enzyme profiling experiments.

Straw Incorporation Factorial Design

A Box-Wilson Central Composite Design (CCD) with five levels for each of three factors (straw, sludge and inoculum size) was studied which required 20 experimental runs, including 6 central points (Table 1). Central points provide additional degree of freedom for error estimation, which increase power when testing the significance of effects (Carvalho *et al*., 1997). All runs were performed in

Table 1: Values for the 20 runs in the CCD

Run	Straw	Sludge	Inoculum
1	100.00	55.00	1.65
2	81.75	81.75	0.84
3	55.00	55.00	0.30
4	55.00	55.00	1.65
5	55.00	55.00	3.00
6	55.00	55.00	1.65
7	55.00	100.00	1.65
8	81.75	81.75	2.45
9	55.00	10.00	1.65
10	81.75	28.24	0.84
11	28.24	28.24	0.84
12	55.00	55.00	1.65
13	10.00	55.00	1.65
14	28.24	28.24	2.45
15	81.75	28.24	2.45
16	28.24	81.75	2.45
17	55.00	55.00	1.65
18	55.00	55.00	1.65
19	55.00	55.00	1.65
20	28.24	81.75	0.84

Table 2: Activity of extracellular enzymes from *Micromonpspora chalcea* measured on solid media

Recorded activity	Enzyme
+	Amylase
-	Lactase
-	Urease
+	Catalase
+	Gelatinase
-	Casein hydrolysate
+	Chitinase
+	Pectinase
+	Cellulase
-	Hemicellulase
+	Caboxymethylcellulase

Table 3: Activity of extracellular enzymes from *Micromonpspora chalcea* measured using API-ZYM® system

Recorded activity[a]	Enzyme
Phosphatases	
3	Alkaline phosphatases
3	Acid phosphatases
3	Phosphohydrolase
Esterases	
4	Lipase
4	Esterase lipase
3	Esterase
Amino-peptidases	
4	Leucine arylamidase
2	Valine arylamidase
2	Cystine arylamidase
Proteases	
5	Chemotrypsin
3	Trypsin
Glycosyl-Hydrolases	
2	α-galactosidase
3	β- glucosidase
4	N-acetyl- β-glucosaminidase
5	α-glucosidase
5	β-galactosidase
0	β-glucuronidase
0	α-mannosidase
0	α-fucosidase

[a] Zero corresponds to a negative reaction, 5 to a reaction of maximum color intensity. For the purpose of this study, values were reported as low activity (1), moderate activity (2-3) and high activity (4-5)

duplicates using 17×12×7 cm plastic pots containing 100 g sandy soil. Thermally treated municipal sludge (to eliminate pathogens), coarsely chopped rice straw and *M. chalcea* harvested mycelia were mixed thoroughly with soil in the pots according to the corresponding levels for each run and incubated under laboratory conditions. Moisture content was adjusted gravimetrically at 40-50% by water spraying and mixing. Organic carbon was detected by wet oxidation using modified Walkely-Black method (Alf and Nannipieri, 1995). Samples were analyzed at intervals and statistical analysis was performed using multiple regressions and ANOVA using Minitab v12 statistical computing package.

Enzyme Profiling

Extracellular enzyme profiling was determined on solid media and using the API-ZYM® system (bioMérieux). Extracellular enzymes detection on solid media (Table 2) was performed according to the methods of (Wollum II, 1982; Williams *et al*., 1983). Enzyme screening plates were inoculated with 5 ML of *M. chalcea* mycelia suspension and scored for enzymatic activities after incubation at 28°C for 7-14 days.

The API-ZYM® system (bioMérieux) is a semi-quantitative method allowing examination of 19 hydrolysates (Table 2) in a strip with a series of microcupules containing dehydrated chromogenic substrates of 19 different enzymes against a control (substrate free) microcupule. A 65 µL of

M. chalcea mycelia suspension were dispensed into each of the 20 microcupules of the API-ZYM®, incubated and color reactions were performed according to the producer instructions. Readings were scored using the API-ZYM® color chart ranging from 0 (negative) to 5 (maximum) (Table 3).

RESULTS AND DISCUSSION

Results for change in carbon as a function of time in the CCD are shown in Fig. 1. The statistical analysis were performed with data obtained at 10 days of incorporation as there was no significant increase in the available organic carbon for most of the runs ($p>0.05$) after this time. Also, organic carbon has significantly decreased after 25 days ($p = 0.009$), indicating the start of mineralization of carbon, particularly in runs number 2, 8 and 20.

The effect estimate for each variable (straw, sludge and inoculum) and their interactions at 10 days of experiment were determined and reported in Table 4. The decomposition process performance was measured by the release of organic carbon response. Both t-test and p-value confirmed the highly significant effect of sludge on the straw decomposition and organic carbon release. Similar conclusion was reported by Strauss *et al*. (2003); since sludge is relatively rich in nitrogen and phosphorus, its co-incorporation with straw is advantageous and complements the high carbon content in rice straw. Although inoculum size was not effective in this factorial design ($p = 0.066$), it exhibited a significant interaction with sludge ($p = 0.021$). This result indicates a critical importance of sludge as source of essential nutrients for supporting *M. chalcea* growth. In addition, *M. chalcea* is probably well adapted to metabolize organic matter in sludge and maintain good mycelia growth; therefore variation in inoculum size was not significantly effective. A previous pilot study (Hayashida *et al*., 1988) descried complete composting of one ton of poultry feces by *Streptomyces*, *Thermoactinomyces* and *Thermomonospora* strains after 10 days incubation over a wide temperature range (15-60°C) in 30 cm depth containers. That pilot study showed satisfactory growth and efficient penetration of mycelia into feces in 30 h under optimal conditions. In order to assess the metabolic ability of *M. chalcea*, its extracellular enzymatic profiling was investigated (Table 2 and 3). The strain showed wide extracellular enzymatic abilities, including polysaccharide hydrolases, esterases, amino-peptidases, proteases, lipases, phosphatases, pectinase and chitinase. Tiquia (2002) have reported a particular abundance of

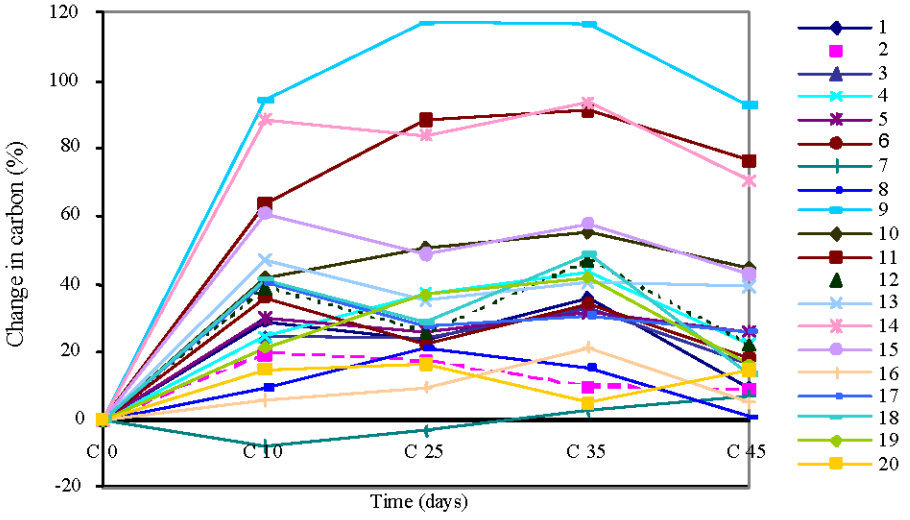

Fig. 1: Percentage of change in organic carbon as a function of time for the 20 runs in the central composite experimental design

Table 4: Main effects and interactions analysis from the CCD for carbon after 10 days of incorporation

Factor	Coef.	SD	t-value	p-value
Straw	0.018	0.5611	0.033	0.974
Sludge	1.985	0.5611	3.537	0.005
Inoculum	38.515	18.7022	2.059	0.066
Straw*Sludge	0.000	0.0049	0.096	0.925
Straw*Inoculum	-0.010	0.1629	-0.061	0.953
Sludge*Inoculum	-0.445	0.1629	-2.734	0.021

Values in bold indicates significance at $p<0.05$

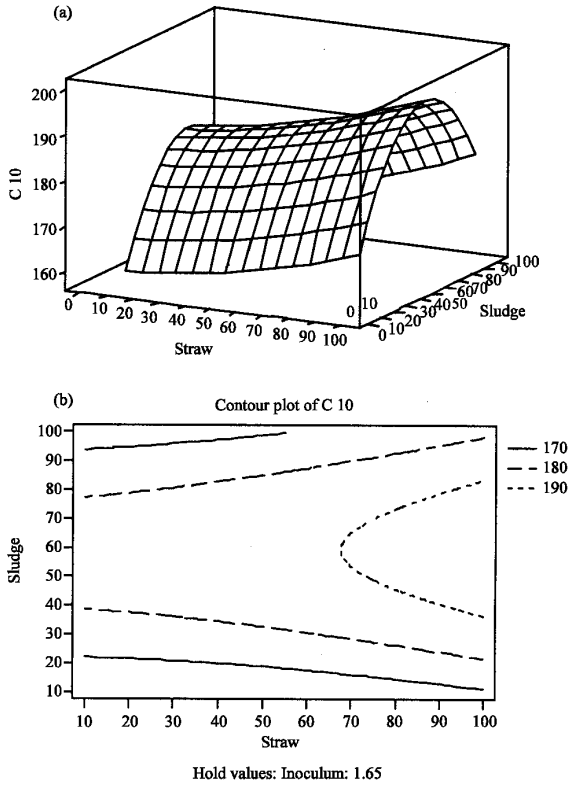

Fig. 2: Response surface (a) and contour plot (b) for the release of carbon as a function of straw and sludge percentage, with 1.65% *Micromonospora* inoculum, after 10 days of incorporation into sand soil

polysaccharide hydrolases, esterases and amino-peptidases during manure composting. The enzymatic ability of *M. chalcea* is, thus, well suited for successful growth in straw-sludge environment, attacking the lignocellulosic material of straw and may explain the significant organic carbon release after 10 days of inoculation. The resulted partially decomposed lignocellulose represents a good source of labile carbon for other indigenous microflora in the experiment to sustain the decomposition process.

Estimation of the effect for the variables (straw, sludge and inoculum) and their interactions at 10 days of experiment using nitrogen content response was also determined. Regression analysis indicated no significant effect of any of the three variables on nitrogen content ($p>0.05$). Moreover, no significant interactions between variable was obtained (data not shown). Therefore, nitrogen content was not considered an adequate response for measuring the straw decomposition process in this study.

Table 5: Analysis of variance for the CCD at 10 days of incorporation

Source of variance	df	Sum of square	Adjusted sum of square	Adjusted mean square	F
Regression	9	1934.0	1934.0	214.9	2.2[a]
Residual Error	10	978.9	978.9	97.9	
Lack of Fit	5	248.3	248.3	49.7	
Pure Error	5	730.6	730.6	146.1	0.34[b]
Total	19	2912.9			

Regression coefficient: R = 0.96. [a] calculated F-value (regression/residual), [b] critical F-value (lack of fit/pure error)

A second order model equation was established, based on ANOVA (Table 5), to describe the increase of carbon as function of straw and sludge ratio in soil, for 10 days of incorporation experiment. The p-value for lack-of-fit was 0.911 indicating the adequacy of this full quadratic model to fit the data. Based on the F-test, the model is predictive since its calculated F-value is greater than critical F-value and the regression coefficient (0.96) is close to unity, indicating that 96% of the variations in the response are explained by this model, as it is well established that values above 0.9 are considered very good (Haaland, 1989).

The full quadratic model was used to generate response surfaces for the analysis of the variable effects on organic carbon release. As seen in Fig. 2, an increase in both straw and sludge ratio can lead to accelerated straw decomposition with subsequent increase in organic carbon release. However, sludge ratios above 81 g/100 g soil have retarded the release of organic carbon, even under straw ratio as low as 10% in soil. This result may indicate an inhibitory effect of high nitrogen concentrations in sludge on the decomposition process. Previous studies have shown that nitrogen may actually inhibit the decomposition of the lignin fraction of litter either by inhibiting synthesis of lignocellulolytic enzymes or by reacting with breakdown products to form other compounds that resist decay (Hobbie and Vitousek, 2000; Fog, 1988).

In conclusion, the extracellular enzyme profiling of *M. chalcea* revealed presence of various enzymatic groups that are involved in lignocellulose decomposition. On the basis of response surface analysis, the optimum conditions to accelerate rice straw decomposition in soil were incorporating 100% straw with 40% sludge and 1.65% inoculum of *M. chalcea*. At these conditions, a value of 190 C mg g^{-1} was obtained after 10 days. This represents a reduction of 10 folds in the time required for straw decomposition, as obtained by Shiga *et al*. (1985). Therefore, with the effective decomposition of rice straw and simultaneous reduction in time, its incorporation into soil using *M. chalcea* can be regarded as possible and economically attractive technique for safe disposal of rice straw.

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