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Serratia liquefaciens from IMO: Antibacterial and Chitinolytic Potentials

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Abstract

Indigenous microorganisms (IMOs) and effective microorganisms (EMs) have long been used in natural farming as substitutes for conventional fertilizers and pesticides. While the microbial composition of EMs is well-documented, the identity of microbes in IMOs are obscure and may vary depending on the source. In this study, bacteria in IMOs collected from a local community in Bukidnon were identified and screened for antimicrobial and chitinolytic potential. *Serratia liquefaciens* exhibited antimicrobial potential against the tomato bacterial wilt pathogen *Ralstonia solanacearum* (Smith). *Serratia liquefaciens*-impregnated discs inhibited the growth of *R. solanacearum* with an average annular zone of inhibition radii 5.3 mm, indicating considerable antimicrobial activity. *S. liquefaciens* was also evaluated for chitinolytic potential using shrimp exoskeletons. Results showed that the chitin mass exposed to *S. liquefaciens* was significantly reduced compared to the control (n=30, T= -3.04, p-value=0.0043). The results of this study infer that the presence of *S. liquefaciens* in IMOs may contribute to the inherent potentials of indigenous microorganisms to inhibit microbial pathogens and possible biodegradation activity.

Keywords: *Serratia liquefaciens*; indigenous microorganisms (IMOs); antimicrobial property; *Ralstonia solanaceaurm*; biodegradation; chitin; Bukidnon; Philippines.

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1. Introduction

An understanding of the composition of microorganisms found in the inoculants used in natural farming is a must for every farmer practicing this popular and established alternative to conventional farming. The mixture of microorganisms found in the commercialized natural farming microbial inoculant called Effective Microorganisms (EM), has been known to include phototrophic and lactic acid bacteria, fermenting yeasts, and actinomycetes which are compatible with one another and can coexist in liquid culture [1,2]. These microbes are listed as ingredients in every bottle of EM sold from agricultural supply shops. On the other hand, the composition of the non-commercialized counterpart of EM, called indigenous microorganisms (IMOs), is less established, more so that the IMOs are collected locally and would surely differ in their microbial contents.

IMOs, along with EMs, have long been used in natural farming as substitutes for conventional fertilizers and pesticides. The microbes in IMOs are collected on cooked rice placed inside paper-sealed wooden boxes that are in turn placed under leaf litters, preferably of bamboo plants [3] in undisturbed areas [4]. These microbes are valuable in natural farming because of their abilities to fix atmospheric nitrogen, decompose organic wastes and residues, enhance nutrient cycling, detoxify pesticides, produce chemicals that stimulate plant growth, and suppress plant diseases [1] through antagonistic activities that hinder the growth of several fungal and bacterial plant pathogens [2]. The identities of the microbes present in IMOs are however unknown.

Isolation and characterization of IMOs from Ifugao, Philippines have yielded eight bacterial and three fungal species [3], the characterizations of which were only up to the colony morphology level. One of the bacterial colonies had a translucent-red color, similar to the one observed by the current researchers on their isolation of the microbes found in the IMOs collected from Bukidnon, Philippines. Upon identification of the said microbe, it was found out to be *Serratia liquefaciens*. *Serratia* have been known to be part of the plant growth-promoting rhizospheres (PGPRs) associated with the roots of legumes at numbers estimated to be at four million cells per gram of the rhizospheric soil [5] and strains of *S. liquefaciens* and *S. plymuthica* have been reported to inhabit the rhizospheres of sugar beet and grapes, respectively [6,7].

Serratia is a genus of gram-negative bacteria belonging to the family Enterobacteriaceae. The type species of the genus is *S. marcescens*, an opportunistic pathogen [8]. A hallmark of the many biotypes of *Serratia* is the production of a nondiffusible red pigment called prodigiosin, or 2-methyl-3-amyl-6-methoxyprodigiosene [9]. Prodigiosin has been found to have bacteriostatic activity [10] and has been tested against gram-positive and gram-negative bacteria like *Staphylococcus aureus* and *Escherichia coli*, respectively, showing more significant activity against the gram-positives [11,12].

Prodigiosin's anti-fungal properties were also explored using molds like *Aspergillus niger*, *Mucor* sp and *Rhizopus* sp [11], although *Serratia*'s anti-fungal activities are most likely from their ability to produce three types of chitinases: ChiA, ChiB, ChiC and a chitobiosidase [13]. These enzymes must act together like how the different components of a machinery work [14] so that they can degrade the chitin found in the cell walls of fungi and exoskeletons of insects, thus making *Serratia* a potential biocontrol against fungal crop pathogens and insect pests [15]. *Serratia* was also successfully grown with supernatants of shrimp shells as the sole carbon or

nitrogen source, using the enzymes chitosanase and protease to metabolize the polysaccharide [16]. Such bioconversion and degradation of chitinous wastes have been considered as alternative disposal methods to chemical treatments which have drawbacks because of the usage of harsh chemicals [17]. In fact, other bacteria like *Bacillus cereus* and *Exiguobacterium acetylicum* have already been tested for biodegradation of shrimp shell wastes [17].

There are enough compelling reasons to consider investigating *Serratia*'s contributions to the valuable ability of the IMOs to speed up the decomposition of organic wastes and suppress plant diseases – the presence of *Serratia* in the rhizospheres of plants and in the IMOs collected from various parts of the Philippines and the bacteria's widely popular ability to produce prodigiosin and chitinases. This study focuses on *S. liquefaciens*, the species identified from the IMOs collected in Bukidnon. The antibacterial activity of *S. liquefaciens* against the economically-important tomato bacterial wilt pathogen, *Ralstonia solanacearum* (Smith), was evaluated, and its chitinolytic ability was assessed using shrimp exoskeletons.

2. Materials and methods

2.1 Sampling Site: The IMOs were collected from an undisturbed vegetated area in Pualas, Baungon, Bukidnon (Figure 1). A microwavable plastic container was used as an IMO-collecting box. It was filled with rice, a traditional substrate for collecting microbes in natural farming, covered then sterilized. When the container has cooled sufficiently, it was set up at the sampling site and covered with leaf litter. The box was brought back to the laboratory after seven days.

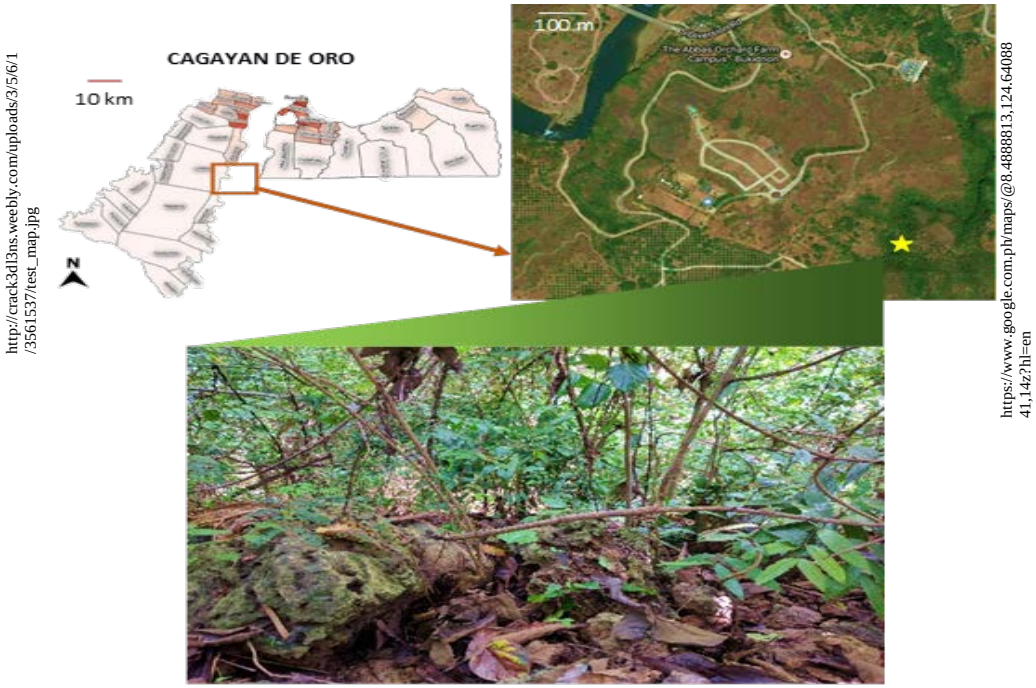

Figure 1: The sampling site at Pualas, Baungon, Bukidnon (top). The ground was covered with leaf litter and is moderately shaded by the leaves of the plants throughout the day (bottom).

2.2 Isolation and Characterization

Methods for serial dilution, plating and other subsequent laboratory procedures done were taken from Anyanwu and his colleagues [3] with some modifications. Different points in the IMO box, based on the visual characteristics of the molds, were swabbed and inoculated into a vial with 10 mL of peptone water. The sample was then serially-diluted up to 10^{-4} dilution and were spread-plated onto nutrient agar media (0.05 mL inoculum per plate). The plates were incubated at 28℃ [11, 18, 19] for 24 h and red-colored colonies were isolated.

Gram-stained cells of the suspected *Serratia* were examined at 1000x magnification. The cells' motility and their ability to ferment lactose were observed through stab inoculation in EMB agar.

For the determination of physiological characters, IMViC test was done with *Escherichia coli* as positive control. The API 20E System (BioMerieux) was used to confirm the identity of the microorganism of interest. A 0.5 McFarland Standard was followed to prepare the inoculant for the API strip. After 24 hours, the results were recorded and reported online via the API Web.

2.3 Antimicrobial Assay Against **Ralstonia solanacearum**

For the test of the antimicrobial potential of *S. liquefaciens* against *R. solanacearum* (Smith), plate antagonism assay [11] was done. The media used was nutrient agar. A pure culture of *R. solanacearum* (Smith) was obtained from the Philippine National Culture of Microorganisms (PNCM).

Prior to the preparation of the antimicrobial assays, separate suspensions of *S. liquefaciens* and *R. solanacearum* (Smith) were prepared in sterile distilled water following a 0.5 McFarland standard [20]. Using sterile micropipettes, 50 µL of the *R. solanacearum* (Smith) suspension was dispensed at the center of the nutrient agar medium and was spread-plated. Two sterile cardboard discs were placed approximately 3 cm apart. On the experimental disc (labelled "S"), 10 µL of the suspension with *S. liquefaciens* was dispensed. The other disc served as a negative control (labelled "-"). A third disc with cefoxitin served as a positive control (labelled "+"). Thirty replicates were made and the plates were incubated at 28℃ [11, 18, 19]. The annular radii of the resulting zones of inhibition per treatment were measured in millimeters using a ruler and their means computed.

2.4 Chitin Biodegradation Assay

In preparation for the chitin-biodegradation assay, a suspension of *S. liquefaciens* was made using the McFarland 3 standard [20]. The inoculum for the suspension was obtained from a 24-h nutrient agar culture of the microbes. These were then suspended in sterile distilled water and vortexed. The turbidity of the suspension was adjusted by visual comparison to the McFarland standard and 5 mL sterile nutrient broth (NB) was mixed with the suspension.

Uncooked shrimp exoskeletons were washed, dried, cut into smaller pieces so that they would fit through the mouth of a 25-mL test tube, weighed and sterilized. Aliquots of sterile NB were added into each of the tubes containing the exoskeletons. Thirty of these tubes were inoculated with a loopful of *S. liquefaciens*; these were

the experimental set-ups. The other thirty were left uninoculated; these were the control set-ups. The tubes were incubated at 33℃ as the enzyme chitinase, ChiA, was found to be active and most stable at temperatures between 30℃ and 45℃ [21]. The shrimp exoskeletons were weighed again after 30 days of exposure to *S. liquefaciens* and changes in their masses were noted. A two-sample t-test was performed on the data using Microsoft Excel 2016.

3. Results and discussion

3.1 Isolation and Characterization

The isolated red colonies from the IMOs in Bukidnon started off as translucent-white in color. It was observed that disturbance and exposure to room temperature (much cooler than 37℃) triggered the change in color of the colonies. This was in line with the reported optimal conditions for prodigiosin-production in *Serratia*: temperatures of around 28℃ [18,22], pH of 7, and aeration disturbance of 200 rev/min [18]. The disturbed colonies started changing in color from translucent pink and ultimately ended up as dull red (Figure 2).

Ten species of *Serratia* have been described so far, of which only *S. plymuthica*, *S. rubidaea* and the popularly opportunistic pathogen, *S. marcescens* [23] are known to be capable of producing the red pigment, prodigiosin [7,12,18], which is also known to be bacteriostatic and antibacterial [10,12,18,19,22,24] among many of its other properties and potential uses [24,25]. However, many other types of bacteria, other than *Serratia*, can manifest the production of red pigments [10], and there are species of *Serratia* that do not produce any pigments at all [18].

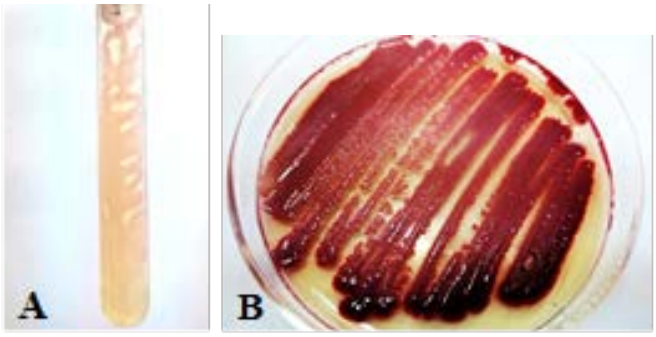

Figure 2: Isolated red colonies were originally white, turned translucent pink when disturbed (A) and became dull red as they aged (B). Media used were nutrient agar.

Gram-staining revealed gram negative, rod-shaped cells and stab inoculation results in EMB suggested motile and non-lactose-fermenting microbes (Figure 3). Oxidase tests also had negative results.

The microbe showed negative results for indole production but had variable results with the methyl red test; initially it turned positive, indicating the presence of acidic byproducts, but gradually changed towards a negative result with further incubation. The microorganism of interest showed positive results for the Voges-Proskauer and citrate utilization tests. It was noted that *E. coli* which was supposed to be negative for the citrate

test appeared to be positive (Figure 4).

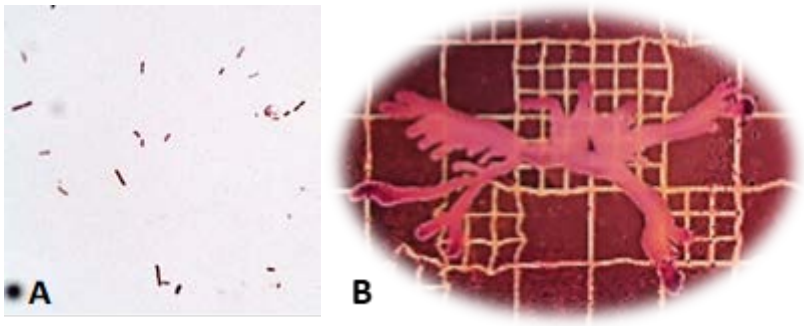

Figure 3: Gram negative rods observed at 1000x magnification (A). The spreading pattern of the colony from the original stab inoculation in EMB agar indicated motility (B). The pale interior color of the colony suggested negative lactose fermentation.

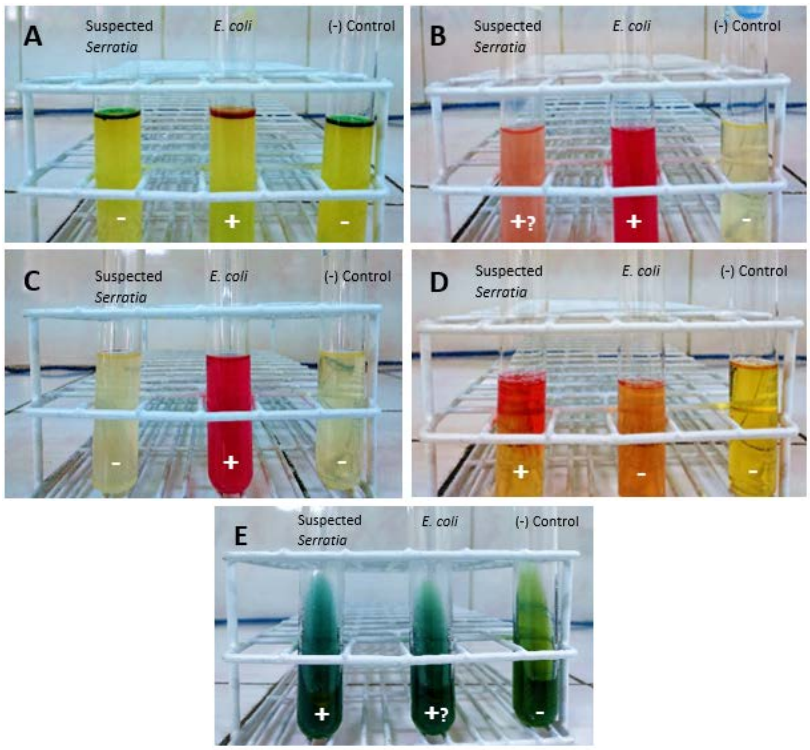

Figure 4: The IMViC tests for the suspected *Serratia* culture; indole test after 48h (A), methyl red test after 48h (B), methyl red test after 72h (C), Voges-Proskauer test after 48h (D) and citrate test after 48h (E).

The microbe was finally identified to be *Serratia liquefaciens* with 96% probability of species identification (Figure 5). This seemed to be unlikely at first since *S. liquefaciens* has not been reported to produce prodigiosin at all. However, notes on the characteristics of *S. liquefaciens* from the American Type Culture Collection (ATCC) described its colonies to be translucent with pink pigmentation which develops as the culture ages [26]. In fact, pigmented *Serratia* have been shown to cause infections in much less frequency than the non-pigmented *Serratia* [11]. *Serratia* have also been found to live in the rhizospheres of leguminous plants at a density of four

million cells per gram of soil [5] and strains of *S. liquefaciens* and *S. plymuthica* have been reported to inhabit the rhizospheres of sugar beet and grapes, respectively [6,7]. Strains of these bacteria were said to produce plant-growth-promoting chemicals, have anti-fungal properties, encourage the establishment of nitrogen-fixing symbionts and act as insect pest pathogens [6,7]. Although *S. liquefaciens* has been reported to be present in plant rhizospheres [6], this study was the first documentation on the presence of this bacterium in IMOs.

GOOD IDENTIFICATION

Strip	API 20 E V4.1						
Profile	7307763						
Note							
Significant taxa		% ID	T	Tests against			
Serratia liquefaciens		96.0	0.67	ADH	1%		
Next taxon		% ID	T	Tests against			
Serratia marcescens		3.8	0.42	ADH	0%	ARA	25%

Figure 5: The API 20E results after 24 hours. The microbe of interest was identified to be *Serratia liquefaciens.*

3.2 Antimicrobial Assay Against Ralstonia solanacearum

The zones of inhibition caused by the antibiotic cefoxitin (+) towards *R. solanacearum* averaged at 13 mm in annular radii width (Table 1). As expected, no zones of inhibition were observed around the negative control discs (-).

The experimental discs (S) dispensed with 10 µL of the 0.5 McFarland suspensions of *S. liquefaciens* showed evidences of the growth of the said microorganism through the presence of red pigments around the discs. Furthermore, zones of inhibition were observed between the experimental discs and the *R. solanacearum*, which averaged at 5.3 mm in annular radii width, despite being uneven around the discs.

This unevenness is attributed to two things: the presence of actively-dividing cells, which produced the antimicrobial agents, most likely the red pigments around the experimental discs, and the nondiffusible nature of this red pigment in agar [9]. The shapes of the zones of inhibition often followed the pattern of the spread of the red pigment (Figure 5).

3.3 Chitin Biodegradation Assay

The difference between the initial and final masses of the exoskeletons were computed and expressed as percent loss of masses. Results showed that the differences between the initial and final dry masses of the exoskeletons exposed to *S. liquefaciens* were significantly higher than those not exposed to the bacteria (T = -3.04, p-value = 0.0043) (Table 2).

Table 1: Observed widths of the annular radii (in mm) of the zones of inhibition in the antimicrobial assay against R. solanacearum.

n	(−)	(+)	S
1	1	11	4
2	0	13	2
3	1	13	5
4	1	13	5
5	0	14	6
6	0	14	4
7	1	13	6
8	0	13	6
9	0	13	4
10	1	13	5
11	1	13	5
12	0	14	5
13	0	13	6
14	0	13	6
15	0	13	10
16	0	12	4
17	0	13	6
18	0	12	6
19	0	13	6
20	0	12	5
21	0	13	6
22	0	14	4
23	0	13	6
24	0	13	6
25	0	13	4
26	0	13	5
27	0	13	5
28	0	14	5
29	0	13	6
30	0	13	6
Mean	0.2	13	5.3
sd^2	0.17	0.41	1.73

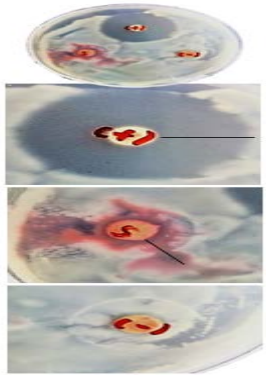

Figure 5: The result of the antimicrobial assay of S. liquefaciens (S) against R. solanacearum. The antibiotic cefoxitin served as the positive control (+). The negative control was a sterile disc (-). The lines represent the annular radii.

Table 2: Mean percent loss of masses of the shrimp exoskeletons in the control and experimental set-ups of the chitin biodegradation assay.

n	CONTROL (without *S. liquefaciens*)				EXPERIMENTAL (with *S. liquefaciens*)			
	Initial (g)	Final (g)	Loss (g)	% Loss	Initial (g)	Final (g)	Loss (g)	% Loss
1	0.0070	0.0039	0.0031	0.44	0.0100	0.0052	0.0048	0.48
2	0.0033	0.0012	0.0021	0.64	0.0068	0.0039	0.0029	0.43
3	0.0085	0.0062	0.0023	0.27	0.0123	0.0066	0.0057	0.46
4	0.0141	0.0115	0.0026	0.18	0.0202	0.0115	0.0087	0.43
5	0.0085	0.0039	0.0046	0.54	0.0186	0.0104	0.0082	0.44
6	0.0082	0.0049	0.0033	0.40	0.0105	0.0050	0.0055	0.52
7	0.0049	0.0030	0.0019	0.39	0.0067	0.0036	0.0031	0.46
8	0.0101	0.0021	0.0080	0.79	0.0110	0.0061	0.0049	0.44
9	0.0072	0.0046	0.0026	0.36	0.0065	0.0038	0.0027	0.41
10	0.0056	0.0029	0.0027	0.48	0.0138	0.0056	0.0082	0.59
11	0.0064	0.0052	0.0012	0.19	0.0124	0.0064	0.0060	0.48
12	0.0094	0.0080	0.0014	0.15	0.0087	0.0051	0.0036	0.41
13	0.0042	0.0014	0.0028	0.67	0.0142	0.0072	0.0070	0.49
14	0.0161	0.0098	0.0063	0.39	0.0107	0.0054	0.0053	0.50
15	0.0055	0.0035	0.0020	0.36	0.0088	0.0046	0.0042	0.48
16	0.0063	0.0050	0.0013	0.21	0.0147	0.0083	0.0064	0.43
17	0.0092	0.0073	0.0019	0.21	0.0154	0.0087	0.0067	0.43
18	0.0068	0.0037	0.0031	0.46	0.0116	0.0046	0.0070	0.60
19	0.0053	0.0040	0.0013	0.24	0.0129	0.0067	0.0062	0.48
20	0.0060	0.0049	0.0011	0.18	0.0093	0.0054	0.0039	0.42
21	0.0072	0.0043	0.0029	0.40	0.0124	0.0059	0.0065	0.52
22	0.0045	0.0031	0.0014	0.31	0.0102	0.0051	0.0051	0.50
23	0.0114	0.0098	0.0016	0.14	0.0110	0.0073	0.0037	0.34
24	0.0049	0.0021	0.0028	0.57	0.0074	0.0036	0.0038	0.51
25	0.0057	0.0036	0.0021	0.37	0.0128	0.0068	0.0060	0.47
26	0.0071	0.0040	0.0031	0.44	0.0163	0.0081	0.0082	0.50
27	0.0093	0.0048	0.0045	0.48	0.0161	0.0093	0.0068	0.42
28	0.0043	0.0028	0.0015	0.35	0.0098	0.0060	0.0038	0.39
29	0.0106	0.0069	0.0037	0.35	0.0141	0.0055	0.0086	0.61
30	0.0075	0.0053	0.0022	0.29	0.0148	0.0084	0.0064	0.43
Mean				0.38				0.47

	CONTROL	EXPERIMENTAL	T-value
Mean % loss	0.38	0.47	-3.04**
sd	0.16	0.06	

** = highly significant ($\alpha \leq 0.01$)

4. Conclusion

Serratia liquefaciens has been found to be among the microorganisms present in the IMOs collected from Bukidnon, Philippines. *S. liquefaciens* showed antibacterial potential against the tomato bacterial wilt pathogen *Ralstonia solanacearum*, and has demonstrated significant biodegradation activity for chitinous shrimp exoskeletons. These antimicrobial and chitinolytic properties will not only be useful in accelerating the biodegradation rate of many kitchen wastes, but also give *S. liquefaciens* and its source IMOs the prospect of

being used as biocontrol agents against the bacterial and fungal diseases and insect pests of many economically-important crops.

5. Recommendations

S. liquefaciens was just one of the many microbes isolated from the IMOs collected from Pualas, Baungon, Bukidnon, Philippines. Despite this, *S. liquefaciens* has shown potentials in terms of antimicrobial and chitinolytic activities. It is however imperative to characterize the other microbes present in IMOs and their potentials for use in natural farming investigated. Additionally, the *S. liquefaciens* used in this study were isolated from IMO 1. Further processing of IMO 1 through the addition of raw sugar to promote the process of fermentation potentially changes the composition of microbes in the subsequent IMO 2. Detection of the presence of *S. liquefaciens* in IMO 2 is therefore recommended. Finally, it is inherent for the sources of IMOs to vary because these microbes are supposed to be collected from the localities where they will be used. As such, the composition of IMOs may vary according to the place and time of their collection. The identification of the microbes present in these different IMOs is therefore recommended to establish the common and unique species of microbes present among them and to infer on how these microbes interact with each other and collectively give the IMOs the characteristics that make them useful in natural farming.

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