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Research Article

Biodegradation of Organic Waste Using *Bacillus* Species Isolated from Soil

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Abstract

Organic waste can be enzymatically degraded by microbes. In this study, the *Bacillus* species were isolated from soil and identified as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus macquariensis*, *Bacillus brevis*, and *Bacillus circulans* which were optimized considering pH (5, 7, 9) and temperature (37°C, 45°C, 55°C) for the maximum production of amylase, gelatinase, lipase, and cellulase, principally for the degradation of organic waste. The maximum production of amylase was found at 37°C with pH 7 and 9, gelatinase and lipase at 37°C with pH 5,7,9 by almost all identified species. Similarly, the production of cellulase was shown by *Bacillus licheniformis* only at 45°C, pH 5. The degradation was confirmed by the analysis of the solid content of degraded waste. The maximum degradation of starch and lipid-containing waste was shown by *Bacillus macquariensis* whereas *Bacillus circulans* were able to degrade gelatin-containing waste effectively. *Bacillus* species showed a synergistic effect in biodegradation. *Bacillus subtilis* and *Bacillus licheniformis* used in ratios 1:1 and 1:2 were found to be effective degraders of lipid and starch-containing waste respectively. *Bacillus macquariensis*, *Bacillus brevis*, and *Bacillus circulans* used in ratio 1:1:1 showed effective degradation of gelatin-containing waste. The degradation of the organic waste by multi-enzyme producer *Bacillus* species can be the most effective and eco-friendly method and their optimization for enzyme production can be beneficial for commercial enzyme production as well as for biotechnological applications.

Keywords: organic waste; *Bacillus* species; amylase; gelatinase; lipase; biodegradation

Introduction

Organic waste includes food waste, green waste, wood waste which is a biodegradable material that can be broken down into simple organic molecules and can be converted into renewable biogas and compost by microorganisms under controlled conditions (Leow *et al.*, 2018). The metabolites produced by the microorganism break down the complex waste into simpler forms and then utilize it as a source of nutrients converting them into safe by-products (Saha and Santra, 2014).

Bacillus is Gram positive rod that is endospore former, chemoheterotrophs, aerobic or facultative anaerobes, catalase producer, and motile by the means of peritrichous flagella. The wide range of physiological abilities of genus *Bacillus* allows them to grow in every environmental condition as they are capable of forming extremely resistant spores and are predominantly found in soil (Amim *et al.*, 2015). Bacteria capable of producing amylase enzymes are *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus licheniformis*, *Bacillus brevis*, *Bacillus macquariensis*, *Lactobacillus sps*, *Proteus sps*, *Pseudomonas sps* etc. (Pokhrel *et al*., 2013). Amylase mainly α-amylase, β-amylase, and glucoamylase are of great importance in many biotechnological processes including starch degradation, pharmaceuticals, etc. (Singh *et al*., 2015). Different bacteria express different forms of gelatinase and the bacteria include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Clostridium perfringens*, *Serratia marcescens*, *Bacillus subtilis*, *Bacillus licheniformis*, etc. (Balan *et al*., 2012). Cellulase is a complex enzyme that comprises endoglucanase, exoglucanase, and β-glucosidase which synergistically hydrolyze cellulose to cello-biose, glucose, and oligosaccharides and also owing to the crystalline and amorphous complex structure of cellulose (Nigam, 2013). *Bacillus subtilis* LFS3 is able to secrete both acidophilic and thermophilic cellulase (Basyal and Yildiz, 2017). *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus alcalophilus*, *Pseudomonas*, *Staphylococcus* spp, *Alcaligeannes* spp, *Chromobacterium* spp etc. are potent Lipase producers. Lipase produced by *Bacillus* species shows interesting properties which make them a potential for biotechnological applications (Mazhar *et al*., 2017). Though enzymes are produced by the plant, animal, and microbes but the economically important enzymes are mostly recovered from microorganisms because the number of enzymes from plants and animal is limited (Volesky *et al*., 2008).

With the increment in population growth rate, the globe is facing the issue of rapid waste generation causing disposal and environmental problems because it cannot be used directly, and there require some improvement in its physical and chemical properties (Wierzba and Nabrdalik, 2005). Though the dumping method is one of the most common disposal techniques, there also arises a problem due to the presence of some toxic chemicals in waste which ultimately decreases the quality of soil and causes diseases in plants, animals, and humans (Forastiere *et al*., 2011). Different methods were widely used for the remediation but they were not found to be ecofriendly. Accepting the method of biodegradation by using microbes not only solve the problem of waste management but also balance the ecosystem (Rastogi *et al*., 2009). The degradation of organic waste using microbes plays a significant role in creating an ecofriendly environment. *Bacillus* species are the major group of bacteria that are widely used for biodegradation because of their capacity to produce various enzymes. Pollutants such as Polyethylene was found to be effectively degraded by *Bacillus subtilis* (Vimala and Mathew, 2016). *Bacillus polymyxa* and *Bacillus cereus* were able to degrade hard keratins (Laba and Rodziewicz, 2014). In natural condition, the low availability of substrate, competition with microbes, unfavorable environmental condition (pH, moisture, temperature, aeration), etc. causes slow biodegradation. Factors that directly or indirectly affect the process should be checked and controlled to rapid the process, therefore the optimization of such parameters is important for the effective degradation of waste (Joutey *et al*., 2013). The degradation of organic waste was found to be highly efficient using microbial consortium and degradation capacity depends on its functional and structural stability (Mirdamadian *et al*., 2011).

This study is undertaken to isolate *Bacillus* from the topsoil of different areas which includes compost soil, rhizospheric soil, night soil, and soil from the kitchen garden, characterize them and assess their ability to produce amylase, gelatinase, cellulase, and Lipase enzymes. This study gives an idea about the pattern of distribution of *Bacillus* species in the soil along with the different parameters that play important role in the optimization of the enzymes from the intended species. Furthermore, it aids in generating ideas about the isolation of strains of potential amylase, gelatinase, cellulase, and lipase producers which is useful for the biodegradation of organic waste. This study also gives the idea about the effect of pH and temperature in enzyme production along with the synergistic effect of *Bacillus* species in the biodegradation of organic waste. Finding the optimized condition of enzyme production by *Bacillus* species not only helps in biodegradation but also provides the idea for commercial enzyme production and many other biotechnological applications.

Materials and Methods

The research was done from 5th November 2019 to 15th February 2020 at Laboratory of Microbiology, Department of Microbiology, St. Xavier's College, Maitighar, Kathmandu, Nepal.

Sample Collection, Isolation, and Identification of Bacillus Species

Soil samples were collected in a sterile zip-seal plastic bag from the depth of 10 cm. 1g of soil sample was taken in the test tube containing 9 ml sterile normal saline and was heated at 80°C for 10 minutes in the water bath. 1ml suspension was taken from the tube and serially diluted up to 10^{-7} followed by pour plating on Nutrient Agar. The plates were kept for incubation at 37°C for 24 hours (Manzum and Mamun, 2019). The isolated colonies were subjected to Gram staining. The colonies showing Gram-positive rod were further selected for identification according to the Bergey's Manual of Determinative Bacteriology (Table 1).

Primary Screening for Enzymatic Activity

Amylolytic Activity

The primary screening for the amylolytic activity of the identified *Bacillus* species was performed by streaking the organisms on 1% starch agar plates and incubating them at 37°C for 48 hours. After incubation, the plates were flooded with iodine solution. Iodine solution was then dispensed with caution and a zone of hydrolysis was observed (Sonune and Garode, 2018).

Table 1: Identification tests of isolates according to Bergey's Manual of Determinative Bacteriology

Tests	Colony code				
	A4	**A6**	**A9**	**A11**	**A12**
Voges Proskauer	-ve	+ve	-ve	-ve	+ve
Citrate Utilization	-ve	+ve	+ve	+ve	+ve
Acid from arabinose	-ve		-ve	+ve	
Starch hydrolysis	+ve	+ve	+ve	+ve	+ve
6.5% NaCl growth	+ve	+ve	+ve	-ve	+ve
Swollen cell (containing spore)	+ve	+ve	+ve	+ve	+ve
Cell diameter ≥ 1µm (width)		-ve		-ve	
Growth at 55°c		+ve		-ve	
Identified *Bacillus* species	*Bacillus macquariensis*	*Bacillus licheniformis*	*Bacillus brevis*	*Bacillus circulans*	*Bacillus subtilis*

Result shown is the identified *Bacillus* species according to Bergey's Manual of Determinative Bacteriology

Gelatinolytic Activity

Identified *Bacillus* species were streaked on 1% gelatin agar plates and incubated at 37°C for 48 hours. After incubation, the plates were flooded with 15% $HgCl_2$ solution and then dispensed with caution and a zone of hydrolysis was observed (Manandhar and Sharma, 2013).

Cellulolytic Activity

Identified *Bacillus* species were streaked on CMC agar plates and incubated at 37°C for 72 hours. After incubation, the plates were flooded with 0.1% congo red solution. Then plates were left undisturbed for 20 minutes. Finally, destaining was done by using a 1M NaCl solution to observe the clear zone around the growth (Roopa *et al*., 2017).

Lipolytic Activity

Identified *Bacillus* species were streaked on 1% tween 20 agar media without calcium chloride and incubated at 37°C for 72 hours. After incubation at 37°C for 72 hours, the individual plates were flooded with copper sulphate solution. The copper sulphate solution was then dispensed with caution and a zone of hydrolysis was observed (Manandhar and Sharma, 2013).

Optimization of Bacillus species for enzyme production

The isolates showing a clear zone of hydrolysis in primary screening were taken for the optimization for enzyme production. The media used for the optimization of amylase, lipase, cellulase, and gelatinase were 1% starch agar, tween 20 agar without calcium chloride, CMC agar, and 1% gelatin agar respectively. The spot inoculation was performed. The experiments were done by adopting search technique i.e., varying parameters (Lugani *et al*., 2015). After the incubation time, the respective media were flooded by the respective reagents as done in primary screening and then the clear zone was observed. The effect of different pH 5, 7, and 9 on selected isolates i.e., *Bacillus* species was monitored on amylase and gelatinase activity after 48 hours of incubation whereas, cellulase and lipase activity after 72 hours of incubation. Selection of optimum temperature for amylase, gelatinase, cellulase, and lipase production for screened *Bacillus* species was done by incubating respective inoculated media at different temperatures (37°C, 45°C, and 55°C) for 48 hours for amylolytic and gelatinolytic activity and 72 hours for the cellulolytic and lipolytic activity.

Biodegradation of organic waste using Bacillus species

The organic waste (starch, gelatin, and lipid-containing waste) treatment was conducted using identified *Bacillus* species individually. The isolated organisms were inoculated in nutrient broth and incubated at 37°C for 48 hours and then the culture was compared to 0.5 McFarland standard. 1ml aliquot of fully grown single bacterial culture was inoculated into a sterilized jar containing 25g of each sterilized standard organic waste (starch, lipid, gelatin containing waste) and incubated at respective optimized temperature for 25 days and analyzed for solids content, pH, and bacterial growth. The mixture of organisms was also used for waste treatment. *Bacillus subtilis* and *Bacillus licheniformis* were used in ratio 1:1, 1:2 and 2:1 whereas *Bacillus macquariensis*, *Bacillus brevis* and *Bacillus circulans* were used in ratio 1:1:1. Controls were set in a sterilized jar containing 25g of each sterilized organic waste without inoculating the organisms. The bacterial numbers were enumerated by pour plating serially diluted organic waste samples into nutrient agar. According to Korea Testing and Research Institute, the solid content of the organic waste was determined using the standard drying method (An *et al*., 2018). Degraded organic wastes were placed into a pre-weighed petri dish and dried in an oven at 105°C until they exhibited constant weight. After drying, the petri dish was reweighed to calculate the solid content using the following equation.

Solid content (%) = (W2-W0)/ (W1-W0)

Where,

W0 represents the weight of the petri dish (g)

W1 represents the weight of the degraded waste and petri dish before drying (g) and

W2 represents the weight of the degraded waste and petri dish after drying (g).

Result and Discussions

Optimization of Bacillus species for enzyme (amylase, gelatinase, cellulase, and lipase) production

Among 40 isolates, 5 were identified as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus macquariensis*, *Bacillus brevis* and *Bacillus circulans*. The identified *Bacillus* species were optimized for enzyme production which was qualitatively evaluated by measuring the zone of hydrolysis around the bacterial growth after the required incubation period. The production of extracellular amylase can be affected by the pH of the media and incubation temperature. In the case of *Bacillus subtilis*, the greatest amylolytic activity was observed at pH 7 and pH 9 incubated at 45°C and 37°C respectively. This finding correlates with the study carried out by (Singh *et al*., 2015). *Bacillus licheniformis* showed the greatest amylolytic activity at pH 5, incubated at 55°C. This finding was reported to be similar to the research done (MuraliKrishnan *et al*., 2017; Allah *et al*., 2018). *Bacillus macquariensis* showed the greatest amylolytic activity at pH 7, 9 incubated at 45°C. The greatest amylolytic activity was shown by *Bacillus brevis* at pH 7, incubated at 37°C. This finding correlates with the study carried out by (Vishnu *et al*., 2014). In the case of *Bacillus circulans*, the greatest amylolytic activity was observed at pH 9 incubated at 55°C (Table 2).

The incubation time for the optimization of gelatinase was performed for 48 hours (Balan *et al*., 2012). All the isolated species of *Bacillus* were able to produce gelatinase enzyme. According to our study, the *Bacillus subtilis* showed the highest gelatinase production at pH 5 and incubation temperature 45°C. *Bacillus licheniformis* showed the highest gelatinase production at pH 9 and incubation temperature 45°C. The maximum gelatinase production was observed at pH 7 and 9 incubated at 37°C by *Bacillus brevis*. In the case of *Bacillus circulans* and *Bacillus macquariensis*, they showed greatest gelatinolytic activity at pH 5, 7, and 9 incubated at 37°C. Most of the gelatinase producers were found to be a mesophilic type with an optimal temperature of 37°C which correlates with the study done (Sai-ut *et al*., 2014; Banerjee *et al*., 1999). Another study (Balan *et al*., 2012; Mazotto *et al*., 2011) reported that the optimum temperature for gelatinase production was 35°C and 50°C-70°C and the optimum pH was 7-14 and 7.5 respectively. The result of optimization of gelatinase was found to be different than our findings which indicate that the optimum pH and temperature for gelatin production is variable (Table 3). Cellulolytic activity of *Bacillus* species was evaluated after 72 hrs of incubation. Among them, the *Bacillus licheniformis* was only the species found to be capable of producing cellulase enzyme. The greatest cellulolytic activity was observed at pH 5 incubated at 45°C. A similar result was reported in another study also (Acharya and Chaudhary, 2012; Behera *et al*., 2016).

Table 2: Optimization for amylase production by *Bacillus* species

***Bacillus* spp.**	**Optimized condition**		**Zone of hydrolysis (mm)**
	pH	**Temp.**	
Bacillus subtilis	9	37°C	2
	7	45°C	2
Bacillus licheniformis	5	55°C	5
Bacillus macquariensis	7, 9	45°C	5
Bacillus brevis	7	37°C	5
Bacillus circulans	9	55°C	8

Data shown is the optimized parameters (pH and temperature) for maximum amylase production by *Bacillus* species

Table 3: Optimization for gelatinase production by *Bacillus* species

***Bacillus* spp.**	**Optimized condition**		**Zone of hydrolysis (mm)**
	pH	**Temp.**	
Bacillus subtilis	5	45°C	5
Bacillus licheniformis	9	45°C	9
Bacillus macquariensis	5,7,9	37°C	5
Bacillus brevis	7,9	37°C	15
Bacillus circulans	5,7,9	37°C	20

Data shown is the optimized parameters (pH and temperature) for maximum gelatinase production by *Bacillus* species

For lipolytic activity, the chosen incubation time was 72 hrs. (Mazhar *et al*., 2017; Sarkar *et al*., 1998). In the case of *Bacillus subtilis*, the greatest lipolytic activity was observed at pH 7 incubated at 45°C and 37°C. This finding correlates with the study carried out by (Mazhar *et al*., 2017). *Bacillus licheniformis* showed the greatest lipolytic activity at pH 9, incubated at 37°C which was found to be similar to the study (Sangeetha *et al*., 2010; Bhosale *et al*., 2015). pH 8 and 30°C were found to be optimized conditions for lipase production by *Bacillus licheniformis* reported by (Anbu and Hur, 2014) and a similar study carried out by (Sethi and Prasad, 2013) has also reported pH 8 and 50°C as an optimized condition. Another study showed different optimized incubation temperatures of lipase production by *Bacillus licheniformis* which indicates that the temperature of lipase production is variable. *Bacillus macquariensis* showed the greatest lipolytic activity at pH 7, 9 incubated at 45°C. *Bacillus brevis* showed the greatest lipolytic activity at pH 5, incubated at 37°C. In the case of *Bacillus circulans*, the maximum lipase production was observed at pH 5, 37°C, pH 5,7, 45°C, and pH 5, 7, 9, 55°C (Table 4).

Degradation of organic waste (starch, gelatin, and lipid-containing) using Bacillus species

After the optimization of enzyme production, the organic wastes were subjected to biodegradation for 25 days. After the specified time, the solid content (Table 5) and pH of degraded organic waste were analyzed along with the microbial load. The maximum degradation of starch-containing waste was shown by *Bacillus macquariensis* as indicated by the percentage of solid content (21%) of the waste whereas the minimum degradation was shown by *Bacillus circulans* (41%) which is in accord with the findings by (An *et al*., 2018). The degradation rate was found to be similar of *Bacillus subtilis* and *Bacillus brevis* as indicated by the percentage of solid content i.e. (38%) in both cases. 34% solid content was found on the starch-containing waste degraded by *Bacillus licheniformis.* The solid content of control was 85% which proved that the biodegradation was effective.

The degradation of gelatin-containing waste was also evaluated. The maximum degradation of gelatin-containing waste was shown by *Bacillus circulans* as indicated by the percentage of solid content (41%) of the waste whereas the minimum degradation was shown by *Bacillus licheniformis* (91%). The solid content was found to be 48%, 47%, and 34% of the gelatin waste degraded by *Bacillus macquariensis, Bacillus brevis,* and *Bacillus subtilis* respectively. The solid content of control was 91% which proved that the *Bacillus licheniformis* was unable to produce gelatinase enzymes.

Lipid-containing waste was also subjected to biodegradation. The maximum degradation of lipid-containing waste was shown by *Bacillus macquariensis* as indicated by the percentage of solid content (62%) of the waste whereas the minimum degradation was shown by *Bacillus brevis* (97%). The solid content was found to be 83%, 87%, 96%, and 98% of the gelatin waste degraded by *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus circulans,* and control respectively. Effective degradation was not shown by *Bacillus circulans* and *Bacillus brevis.*

Table 4: Optimization for lipase production by *Bacillus* species

***Bacillus* spp.**	**Optimized condition**		**Zone of hydrolysis (mm)**
	pH	**Temp**	
Bacillus subtilis	7	37°C, 45°C	2
Bacillus licheniformis	9	37°C	4
Bacillus macquariensis	5	37°C	1
Bacillus brevis	5	37°C	1
	5	37°C	1
Bacillus circulans	5,7	45°C	1
	5,7,9	55°C	1

Data shown is the optimized parameters (pH and temperature) for maximum lipase production by *Bacillus* species

Table 5: Solid content of degraded organic waste

***Bacillus* species**	**Solid content (%)**		
	Starch-containing waste	**Gelatin-containing waste**	**Lipid-containing waste**
Bacillus subtilis	38	85	83
Bacillus licheniformis	34	91	87
Bacillus macquariensis	21	48	62
Bacillus brevis	38	47	97
Bacillus circulans	41	41	96
Control	85	91	98

Data shown is the degradation of organic waste by *Bacillus* species. The solid content of degraded organic waste in percentage.

Synergistic effect of Bacillus species in the degradation of organic waste (starch, gelatin, and lipid-containing).

The result of the synergistic effect of *Bacillus* species on the degradation of organic waste (starch, gelatin, and lipid-containing) is shown in (Table 6). Starch-containing waste was found to be effectively degraded by *Bacillus subtilis* and *Bacillus licheniformis* (1:2) as analyzed by the percentage (32%) of the solid content of degraded waste. The solid content was found to be 33% and 85% of the starch-containing waste degraded by *Bacillus subtilis* and *Bacillus licheniformis* (2:1) and control respectively. *Bacillus subtilis* and *Bacillus licheniformis* (1:1) and *Bacillus macquariensis*, *Bacillus brevis* and *Bacillus circulans* (1:1:1) showed similar degradation rate indicated by the percentage of solid content (40%).

The effective degradation of gelatin-containing waste was shown by *Bacillus macquariensis*, *Bacillus brevis,* and *Bacillus* (1:1:1) as analyzed by the percentage (34%) of the solid content of degraded waste. The solid content was found to be 80%, 84%, and 85% of the gelatin-containing waste degraded by *Bacillus subtilis* and *Bacillus licheniformis* used in the ratio 1:1, 1:2, and 2:1 respectively. The control showed 91% solid content.

Bacillus subtilis and *Bacillus licheniformis* (1:1) showed effective degradation of lipid-containing waste as indicated by the reduced percentage (53%) of the solid content of degraded waste (Fig. 1). The solid content was found to be 87% and 98% of the lipid-containing waste degraded by *Bacillus subtilis* and *Bacillus licheniformis* (1:2) and control respectively. Similar degradation of the lipid-containing waste was shown by *Bacillus subtilis* and *Bacillus licheniformis* (2:1) and *Bacillus macquariensis*, *Bacillus brevis*, and *Bacillus* (1:1:1) as indicated by the percentage of the solid content (82%).

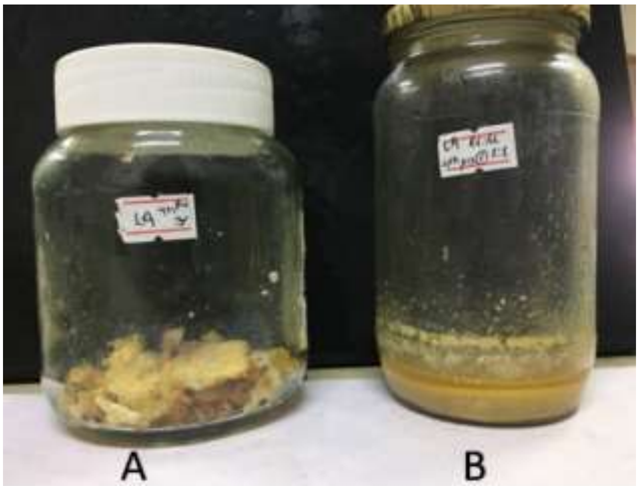

Fig. 1: Biodegradation of lipid-containing waste using *Bacillus subtilis* and *Bacillus licheniformis* (1:1) synergistically. A. Control; B. *Bacillus subtilis* and *Bacillus licheniformis* (1:1)

The mixture of *Bacillus* subtilis and *Bacillus licheniformis* was found to be effective in organic waste treatment. This outcome might be due to the secretion of multifunctional enzymes by the organism. The determination of pH and enumeration of microorganisms was also performed. The number of organisms in degraded organic waste (starch, gelatin, and lipid-containing waste) was found to be TMTC which indicates that the waste might be the appropriate medium for its growth which was also mentioned in the study by (An *et al*., 2018). The pH of degraded starch-containing waste and lipid-containing was found to be acidic and neutral whereas degraded gelatin-containing waste was found to be neutral and alkaline. Finally, the biochemical test of the isolated colony was also performed. The isolated Gram-positive rod showed all tests positive for *Bacillus* when subjected to different biochemical tests which confirmed that the inoculated organism was only the *Bacillus* species responsible for biodegradation.

Table 6: Solid content of degraded organic waste using *Bacillus* species synergistically

***Bacillus* species**	**Ratio**	**Solid content (%)**		
		Starch-containing waste	**Gelatin-containing waste**	**Lipid-containing waste**
Bacillus subtilis and *Bacillus licheniformis*	1:1	40	80	53
Bacillus subtilis and *Bacillus licheniformis*	1:2	32	84	87
Bacillus subtilis and *Bacillus licheniformis*	2:1	33	85	82
Bacillus macquariensis, Bacillus brevis and *Bacillus circulans*	1:1:1	40	34	82
Control		85	91	98

Data shown is the degradation of organic waste by *Bacillus* species synergistically and the solid content of degraded organic waste is expressed in percentage.

Author's Contribution

All authors jointly_designed the research plan; B Rana Chhetri, P Silwal, P Jyapu, Y Maharjan, T Lamsal performed experimental works, collected the required data & prepared the manuscript. A Basnet_analysed the data & critically revised the manuscript; Final form of manuscript was approved by all authors.

Conflict of Interest

The authors declare that there is no conflict of interest with present publication.

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