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Development of a Microbial Consortium for Hastening the Silkworm Pupal Residue (SWPR) Degradation

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ABSTARCT

Desilked silkworm pupae are rich in protein, nitrogen and other biological constituents, which can be utilized as nutrient source in crop production after composting. Bacterial population was dominant over fungi and actinobacteria throughout the process of degradation of silkworm pupa residue. Bacterial population (52×10^6 CFU/g) was found to be highest compared to fungi (21×10^3 CFU/g) and actinobacterial (19×10^3 CFU/g) population. All the bacterial isolates were rod shaped and non-endospore formers except SCVB-36. The bacterial isolates SCVB-31, SCVB-38 and SCVB-41 were gram positive. All of the isolates showed positive for the cellulase, pectinase, protease, amylase, lignolytic enzyme and chitinase production. Fungal isolate SCVF-09 (24 mm) showed highest zone followed by the bacterial isolate SCVB-38 (23 mm) for cellulase production. Bacterial isolate SCVB-36 (24 mm) showed highest pectinase activity followed by the fungal isolate SCVF-04 (22 mm) whereas, protease and amylase production was found in the bacterial isolates SCVB-31(18 mm) and SCVB-38 (17 mm), respectively. Lignolytic enzyme production ability was highest in fungal isolates. Fungal isolate SCVF-06 and SCVB-09 (99.46 and 103.8) showed significant reduction in the C:N ratio followed by actinobacterial isolate SCVA-08 (104.41) and bacterial isolate SCVB-36 (109.96) during decomposition of saw dust with silkworm pupae. Among twelve microbial isolates screened, four isolates were selected to form the consortium based on their ability to produce enzymes, carbon and nitrogen mineralization efficiency which includes two fungi (SCVF-06 and SCVF-09), one bacteria (SCVB-36) and one actinobacteria (SCVA-08).

Keywords: Silkworm pupal residue degradation, enzyme production, C:N ratio and microbial consortium

INDIA is the world's second largest producer of raw silk after China. The rearing of silkworm is done throughout the year. As a result, huge amount of pupal waste and silkworm litter are generated every year as a by-product of sericulture industry. The silkworm pupa is one of the major by-products of silk industry, which has been considered as waste in silk reeling unit (Patil *et al.*, 2013).

The major difficulty in the utilization of desilked silkworm pupae is that it cannot be stored for longer periods as it emits bad odour due to putrefaction. Disposal of silkworm pupae meal was a big problem in the silk factories. The silk producers after reeling out silk used to throw the dead pupae at the outskirts of the city, creating nuisance and health hazards. A great deal of work has been done on silk waste but no appreciable value of work has been done on pupal waste until 1959, even though the mulberry silkworm pupae consist of numerous biological constituents which are of great value as feed for animals including human beings, medicine and manure for crops. Such by-products which are presently discarded as waste can be utilized for financial gains and generation of value-based products. The silkworm litter is presently used as fodder and compost and the pupal waste is also utilized in oil extraction, as a substrate in biogas production and mushroom cultivation. The pupae of mulberry and non-mulberry silkworms have been extensively used as fertilizer, animal feed and edible insects in many countries, such as Japan, Korea, India and Thailand (Ramappa *et al*., 2015).

The pupae contain about 79.8 per cent of protein, 6.6 per cent fat and 5.1 per cent ash. Dried silkworm pupae contain about 8 per cent nitrogen. The crude protein extracted with 0.5 per cent sodium hydroxide contains 12.22 per cent nitrogen (Nagaraj and Basavanna, 1996). Since the pupae contains high amount of nitrogen and protein along with

micronutrients like zinc, copper, magnesium and manganese, there is a prospective potential for the bio-conversion of pupal waste to enriched compost and utilization as a nutrient source. Most of microorganisms which are involved in the degradation of the silkworm pupae are the chitinolytic microorganisms (Jie *et al*., 2009). They help to degrade the pupae and reduce the foul odour. Hence, there is a need to screen the microorganisms associated with the silkworm pupal degradation and use them for the efficient degradation of the same. Keeping the above points in view, the present study on "Development of a microbial consortium for hastening the silkworm pupal degradation" was conducted in the Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bangalore to develop an efficient decomposing consortium for composting of silkworm pupal residue.

Material and Methods

Collection of samples

Silkworm pupal residue (SWPR) samples were collected from silk reeling unit of Vijayapura, Chickballapur district of Karnataka.

Enumeration and isolation of SWPR degrading microorganisms

Five kilograms of silkworm pupal residue sample was kept for degradation for 60 days in triplicate. Optimum moisture approximately at 60 per cent was maintained by adding water and turning was done to ensure aeration during the process of degradation. Samples were drawn at 15 days of intervals (0, 7, 15, 30, 45 and 60 days) and subjected for studying general groups of microorganisms *i.e.*, Bacteria, Fungi and Actinobacteria and functional diversity of microorganisms associated with silkworm pupal degradation. Standard serial dilution plate count technique was adopted for the enumeration and isolation of bacteria, fungi and actinobacteria using spread plate method. Nutrient agar was used for the enumeration of bacteria, Kuster's agar / starch case in agar for actinobacteria and Rose Bengal agar for fungi. Bacterial, fungal and actinobacterial colony count was done after 2, 7 and 10 days incubation, respectively. The representative colonies were selected and were purified by sub culturing them in to new plates containing their respective media.

Morphological and biochemical characterization of microorganisms

The bacterial colonies were screened for the colony morphology, microscopic analysis (Gram reaction, cell shape and endospore formation) and biochemically characterized by evaluating IMViC, catalase, urease and H_2S production ability of the isolates were essentially carried out as per the standard procedures outlined by Cappuccino and Sherman (1992).

Screening of isolates for enzyme production

All the isolates (bacteria, fungi and actinobacteria) were screened for the ability to produce different enzymes such as cellulase, pectinase, protease, starch hydrolysis, lignolytic enzyme and chitinase as per the standard protocol outlined by Pointing (1999).

Carbon and nitrogen mineralization studies of selected isolates

The efficient isolates were selected based on enzyme production and were further screened for carbon and nitrogen mineralization efficiency in enhancing the speed of composting. Efficient isolates were individually inoculated to the beaker containing silkworm pupal residue and sawdust in 2:1 ratio for 30 days duration to test for carbon and nitrogen mineralization efficiency in three replicates. The initial total organic carbon (g/kg) and total nitrogen (%) was 447.3 and 1.23, respectively of the substrate used for the experiment. The optimum moisture approximately 60 per cent was maintained by adding water and regular turning was done to ensure aeration. The samples were drawn at 10, 20 and 30 days after degradation.The total organic carbon (TOC) content estimated using the standard dichromate oxidation method of Nelson and Sommers (1982). Total nitrogen was determined after digesting the sample with concentrated H_2SO_4 (1:20, w/v) followed by distillation (Bremner and Mulvaney, 1982).

Selection of microorganisms for consortium preparation

From the total of twelve isolates, four isolates including one bacterium, two fungal isolates and one actinobacterial isolate were selected. The selection was based on the efficiency of the isolate to produce specific enzyme, carbon and nitrogen mineralization efficiency which are having definite role in biodegradation for increasing the composting rate of silkworm pupal residue. The selected isolates were subjected to compatibility test by dual and triple inoculations *in vitro* on potato dextrose agar plates for testing their suitability for preparing the microbial consortium to be used for enhancing the speed of composting.

Molecular characterization of efficient silkworm pupal degrading isolates

The four efficient silkworm pupal degrading isolates (SCVA-31, SCVF-06, SCVF-09 and SCVA-08) which were selected for consortium preparation based on their enzyme production ability, carbon and nitrogen mineralization efficiency were molecularly identified based on 16S rRNA gene sequencing for bacterial and actinobacterial isolates and 18S rRNA gene sequencing for fungal isolates.

Results and Discussion

Succession of microorganisms during silkworm pupal degradation

The results obtained on the mean number of colony forming units of bacteria, fungi and actinobacteria isolated at different days of SWPR degradation is presented in the Table I. Among the microbial groups isolated, the bacterial population was dominant compared to fungi and actinobacteria throughout the period of silkworm pupal degradation. During initial days of SWPR degradation fungi and actinobacterial colonies were not found, but the activity of fungi and actinobacteria were found during 7^{th} and 15^{th} days, respectively, after degradation of SWPR. The bacterial population (98×10^6 CFU/g) was highest during 15^{th} day, whereas the population of fungi (21.0×10^3 CFU/g) was found to be highest during 30^{th} day of SWPR degradation. On 60^{th} day of silkworm pupal degradation, population of actinobacteria (19×10^3 CFU/g) found highest compared to population of bacteria and fungi.

Table I

Microbial Succession during silkworm pupal residue degradation

Days of degradation	Population of microorganisms		
	Bacteria (CFU x 10^6/g)	Fungi (CFU x 10^3/g)	Actinobacteria (CFU x 10^3/g)
0	5.0	0.0	0.0
7	23.0	1.0	0.0
15	98.0	7.0	3.0
30	52.0	21.0	10.0
45	12.0	16.5	12.0
60	5.0	11.0	19.0

Silkworm pupae are rich in nutrients that might have favored the growth of different groups of microorganisms. The bacterial and fungal population was found to increase at initial stage later there population decreased and actinobacterial population was favored in the later stage of degradation. This might be due to the temperature variation during SWPR degradation process. Usually aerobic composting process proceeds through a succession of microbial population beginning with mesophilic microorganisms. The release of temperature through microbial respiration favours the suppression of mesophilic microorganisms as well as the growth and multiplication of thermophilic microorganisms. Similar results were also observed by Shamala and Belagali (2012), while working on the composting processes of municipal solid wastes.

Morphological and biochemical characterization of bacterial isolates

The identical cultures from the same source of silkworm pupal compost during degradation were eliminated by selecting only representative isolates from identical colonies having the same morphological characters. All the four bacterial isolates were rod shaped and three were non-endospore formers (Table II). Three bacterial isolates showed positive for Gram reaction except the isolate SCVB-36. Most of the isolates were circular, mucoid and medium colonies on the NA plates with yellow, greenish and white to dense white pigmentation. All the four bacterial isolates showed negative for indole

TABLE II

Morphological and biochemical characterization of bacteria involved in silkworm pupal residue degradation

Isolates	Colony morphology	Gram reaction	Cell shape	Endospore formation	Indole test	Methyl red test	Voges proskauer test	Citrate utilization Test	Catalase test	Urease test	H_2S Production test
SCVB-31	Yellowish circular medium size	+	Rod	-	-	+	-	-	+	-	-
SCVB-36	Whitish, mucoid, medium size	-	Rod	+	-	-	+	+	-	-	-
SCVB-38	Greyish, circular, medium size	+	Rod	-	-	-	+	+	+	+	-
SCVB-41	Dense white, circular, big colony	+	Rod	-	-	-	+	-	-	-	-

and H_2S production tests. SCVB-38 showed positive for Vogue's Proskeur test, citrate utilization, catalase and urease tests whereas, other bacterial isolates were negative for almost all biochemical tests conducted. These results are in conformity with the study of Feng *et al.* (2011) where they have isolated 12 bacteria from the intestine of silkworm fed with different forage. Out of twelve isolates, eleven isolates were having rod shaped cells, nine isolates were gram negative and one was endospore producer and were positive for oxidase, catalase and methyl red test. Jie *et al.* (2009) also isolated G-254 *(Stenotrophomonas maltophilia*) a gram negative bacteria and having rod shaped from soil and water samples collected from the silkworm pupa storage house.

Screening of efficient silk worm pupal degradation microorganisms by plate assay for enzyme production

Activity of all the bacterial, fungal and actinobacterial isolates are presented in the Table III. Majority of the isolated organisms were positive for the production of all the six enzymes which were statistically significant among them. Among the three groups of organisms, fungal isolates SCVF-09(24 mm) and SCVF-10 (23 mm) showed highest zone followed by bacterial isolates SCVB-38 (23 mm) and SCVB-31 (23 mm) for cellulase production. Bacterial isolate, SCVB-36 (24 mm) showed highest pectinase activity followed by fungal isolate SCVF-04 (22 mm) which was significantly different compared to all the other isolates. Protease production and starch hydrolysis was found highest in bacterial isolate SCVB-31 (18 and 17 mm, respectively) and SCVB-38 (17 and 21 mm, respectively) compared to all the other isolates among the organisms. Lignolytic enzyme production ability was found highest in the fungal isolates SCVF-04, 06 and 09 (20 mm) compared to all the bacterial and actinobacterial isolates which was statistically significant among the different isolates. Chitinase producing efficiency was found highest in the bacterial isolate SCVB-36 (17 mm) and an actinobacterial isolates SCVA-08 (17 mm) followed by SCVA-12 (16 mm) compared to all the other isolates. Astrain G-254 (*Stenotrophomonas maltophilia*) with high chitinase activity and better silkworm puparium chitin degradation effect was screened near the storage plant of Guangdong Wengyuan Xinda Silk Joint Stock Company. After orient domestication, this strain could produce obvious transparent cycle on colloidal chitin plate (Jie *et al.,* 2009).

Carbon and Nitrogen mineralization by silkworm pupal degrading microorganisms

The results on the changes in total organic carbon, total nitrogen and C:N ratio during decomposition of saw dust with silkworm pupal residue are presented in Table IV. Among the organisms the fungal isolate SCVF-06 showed highest carbon and nitrogen mineralization ability followed by bacterial isolate SCVB-36 and actinobacterial isolate SCVA-08 throughout the period of silkworm pupal degradation. Significant decrease in C:N ratio was obtained in the treatment inoculated with the fungal isolate SCVF-06 (99.46) compared to all the other organisms. In general, there was a significant

TABLE III

Screening of efficient silkworm pupal degrading microorganisms for enzyme production

Isolates	Zone diameter (mm)					
	Cellulase	Pectinase	Protease	Starch hydrolysis	Lignolytic enzyme	Chitinase
SCVB-31	23 a	17 de	18 a	17 bc	06 de	15 bc
SCVB-36	20 b	24 a	16 d	18 b	15 b	17 a
SCVB-38	23 a	18 d	17 ab	21 a	15 b	14 cd
SCVB-41	20 b	17 be	13 c	15 de	05 e	11 fg
SCVF-04	19 bc	22 b	13 c	12 gh	20 a	08 h
SCVF-06	19 bc	20 c	12 cd	11 h	20 a	12 f
SCVF-09	24 a	17 de	05 g	13 fg	20 a	10 g
SCVF-10	23 a	14 f	08 f	12 gh	14 b	11 fg
SCVA-04	15 d	12 g	11 de	12 gh	10 c	14 cd
SCVA-08	16 d	14 f	10 e	14 ef	09 c	17 a
SCVA-10	18 c	16 e	16 b	13 fg	10 c	13 de
SCVA-12	16 d	14 f	11 de	16 cd	07 d	16 ab

Note : Mean values followed by the superscript in each column do not differ significantly at P=0.05 level by DMRT
SCVB: Bacterial isolates, SCVF: fungal isolates, SCVA: actinobacterial isolates

TABLE IV

Carbon and nitrogen mineralization by efficient silkworm pupal residue degrading microorganisms

Isolates	Days of degradation								
	10			20			30		
	TOC (g/kg)	TN (%)	C:N ratio	TOC (g/kg)	TN (%)	C:N ratio	TOC (g/kg)	TN (%)	C:N ratio
SCVB-31	364.50 e	1.29	282.55 c	244.00 c	1.37 h	178.4 a	183.75 b	1.52 f	120.80 a
SCVB-36	357.60 f	1.32	270.90 e	239.01 ef	1.39 g	171.94 b	173.75 f	1.58 e	109.96 c
SCVB-38	366.40 de	1.26	290.71 b	241.50 d	1.44 de	167.70 c	175.82 de	1.52 f	155.67 b
SCVB-41	372.80 b	1.29	288.99 b	248.25 a	1.42 f	174.82 ab	187.50 a	1.60 d	117.18 b
SCVF-04	342.00 i	1.33	257.00 f	227.01 h	1.44d e	157.64 d	169.80 g	1.62 c	104.80 d
SCVF-06	330.84 j	1.36	243.20 g	214.50 j	1.47 c	152.12 e	164.12 h	1.65 c	99.46 e
SCVF-09	350.00 g	1.25	280.00 c	227.10 h	1.52 a	149.40 e	170.25 g	1.64 b	103.80 d
SCVF-10	347.60 h	1.26	275.80 d	224.76 i	1.50 b	149.84 e	168.75 g	1.60 d	105.40 d
SCVA-04	368.40 c	1.32	279.09 c	240.30 de	1.44 d	165.72 c	179.75 c	1.60 b	109.60 c
SCVA-08	366.00 e	1.35	271.11 e	235.50 g	1.47 c	160.20 d	174.37 f	1.67 a	104.41 d
SCVA-10	376.80 a	1.28	294.30 a	246.00 b	1.45 d	167.32 c	176.87 d	1.58 e	119.40 a
SCVA-12	368.00 cd	1.24	296.77 a	238.50 f	1.43 ef	166.78 c	174.75 ef	1.61 cd	108.50 c

Note : Mean values followed by the superscript in each column do not differ significantly at P=0.05 level by DMRT
TOC: Total organic carbon, TN: Total nitrogen
SCVB: Bacterial isolates, SCVF: fungal isolates, SCVA: actinobacterial isolates

increase in N and decrease in organic carbon and C:N ratio was observed throughout the decomposition. At 30th day of decomposition, bacterial isolate SCVB-36 recorded highest mineralization ability of carbon and nitrogen, which was found to reduce highest C:N ratio of 109.96 followed by SCVB-41 (117.18), whereas, among the actinobacterial isolate, SCVA-08 (104.41) recorded highest reduction of C:N ratio followed by SCVA-04 (109.6). But the highest efficiency of mineralization of carbon and nitrogen was found in fungal isolates SCVF-06, SCVF-09 and SCVF-04 (99.46, 103.8 and 104.8 of C:N ratio, respectively) compared to all the other organisms during silkworm pupal degradation.

The changes in the C:N ratio reflects organic matter decomposition and stabilization during composting process because microorganisms used carbon as source of energy and nitrogen for building cell structure. In the initial stage of composting, intense mineralization processes take place, which were manifested by considerable decrease and increase in total organic carbon and nitrogen, respectively in all the treatments as a result the C:N ratio decrease consistently as composting progress. These results are in agreement with the findings of Pramanik *et al*. (2007) the organic carbon content decreased during composting of different organic wastes (cow dung, grasses, aquatic weeds and municipal solid wastes) and increased the total nitrogen content thus decreased the C:N ratio of compost as compared to the initial organic substrates.

Selection of isolates for consortium preparation

Among twelve microbial cultures screened, four cultures were selected to form the consortium based on their ability to produce enzymes,carbon and nitrogen mineralization efficiency (Table V).

TABLE V

Selection of isolates for consortium preparation

Isolates	Molecular identification
SCVB-36	*Bacillus licheniformis*
SCVF-06	*Aspergillus aculeatus*
SCVF-09	*Penicillium simplicissimum*
SCVA-08	*Streptomyces noursei*

Microbial consortium which includes two fungi (SCVF-06 and SCVF-09), one bacteria (SCVB-36) and one actinobacteria (SCVA-08). They were molecularly identified as *Bacillus licheniformis* (SCVB-36), *Aspergillus aculeatus* (SCVF-06), *Penicillium simplicissimum* (SCVF-09) and *Streptomyces noursei* (SCVA-08). Kumar *et al.* (2008) developed a fungal consortium consisting of *Aspergillus nidulans, Scytalidium thermophilus* and *Humicola* species to compost paddy straw and found it effective in converting paddy straw into nutritionally rich compost, thereby leading to economical and environmental friendly disposal of cropresidue.

The present study reports development of a consortium of microorganisms comprising bacteria, fungi and actinobacteria for quick aerobic composting. Since no single organism is able to produces all the enzymes necessary for the degradation of all types of organic waste material, there is a need to use a consortium of microorganisms which can act synergistically for the rapid conversion of different organic waste materials. Selection of the microorganisms is based on production of six important enzymes capable of degrading major components of organic waste materials, the consortium can be applied to wide range of organic waste materials. Application of the consortium helps not only to reduce time of composting, cost of production and chances of pathogenic contamination, but also to develop quickly the quality compost for sustainable production of agriculture.

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