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Screening and Molecular Characterization of Extracellular Lipase Producing Bacillus Species from Coconut Oil Mill Soil

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ABSTRACT

Lipases (triacylglycerol acylhydrolases E.C 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial application. In view of this significance of lipase we performed screening and molecular characterization of extracellular lipase producing *Bacillus* species in soil samples collected from coconut oil mill. The dominant Lipase producing organisms were isolated from oil-spilled areas of the groundnut oil extracting industry at Salem. The isolates were identified as *Bacillus* spp, the influence of growth period on lipase production of *Bacillus spp*. (B1 – B5) was assessed by culturing it on media for 48hrs. Further, the influence of substrate concentration (2 to 4 %) on lipase production was also assessed on the optimized substrate, which maximized the lipase production. Isolated *Bacillus spp.*(B1 – B5) were assayed for extra cellular lipase production. One unit of lipase activity was defined as the amount of enzyme releasing one mole of free fatty acid in one minute under standard assay condition. The purification method consists of Ammonium Sulphate precipitation, Dialysis and Column chromatography and Molecular determination by SDS PAGE. In genotypic characterization, RAPD analysis was done to characterize the organism.

Keywords: *Lipase, Bacillus spp., SDS PAGE and RAPD.*

1. INTRODUCTION

Lipases are important enzyme with significant commercial applications in industries. Lipases catalyze the hydrolysis of triacyl glycerols to glycerol and free fatty acids, in contrast to esterases. Lipases are activated only when adsorbed to an oil-water interface and do not hydrolyze dissolved substrates in the bulk fluid. A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Among the various factors that influence lipase production during culture, the type of carbon substrates and inducers, have a profound effect on the production of microbial lipases because microbial lipases function is to break down insoluble lipidic substrates so that they can be more easily absorbed (**Saxena** *et al*., 1999). Lipases find promising applications in organic chemical processing, detergent formulations, synthesis of biosurfactants, the chemical and paper industries, nutrition, cosmetics, and pharmaceutical processing. Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding the uses of these enzymes. Increased productivity of lipase during the fermentation process is of great importance, and lower cost of production could promote new industrial applications. However, interest in bacterial lipases has increased because they are more stable than those from other organisms, especially when exposed to high temperatures and other severe conditions (**Sugihara** *et al*., 1991). Recently, there has been considerable interest in the basic properties and industrial applications of thermostable lipases from mesophiles and thermophiles. The present study is to investigate the extracellular lipase producing *Bacillus* Species isolated from coconut oil mill soil.

2. MATERIALS AND METHODS

Soil sample were aseptically collected from oil-spilled areas of the oil extracting industry. Both dilution plate and enrichment method were used for the initial screening and isolation of bacterial species. For the enrichment method, 1g of samples were subjected to heat treatment for 10 min at 80ºC in a water bath in order to kill most of the vegetative cells and thus to eliminate non-spore forming bacteria (**Mora***et al*., 1998). After heat treatment, the samples were transferred into 100 ml of Tributyrin medium. Incubation was performed in a rotary shaker at 50^0C until turbidity obtained. Then 500 µl of the broth was plated on Tributyrin medium. For the dilution plate method 1g of samples were transferred in 9 ml of 0.85% saline water. After pasteurization at 80^0C for 10 min, 1 ml aliquot from each of the samples was transferred in 9 ml of 0.85% saline water and 6 fold dilutions were prepared. One ml of dilution was plated on Tributyrin medium plates and incubated for 48-72 h at 37^0C. Single colonies with different morphologies were picked and purified using streak plate method.

Screening for Lipase Activity

The media described were used in lipase screening. After inoculation of the isolates, the plates were incubated for

3-4 days at 50^{O}C. Opaque halos around the colonies were taken as the indication of lipase activity (**Haba***et al.,* 2000).

pH Optimization

The Effect of pH values was carried out to determine the optimum pH value for lipase productivities by all five *Bacilli spp* (B1 – B5). The pH was adjusted at pH 6 values for the production media using 6 N NaOH or 6 N HCl. Effect of different inoculum size of the *Bacillus spp.* was used. Lipase production using SSF in different production vessels: Enzyme production was studied in 250 ml.

Temperature Optimization

The effect of temperature values was carried out to determine the optimum temperature value for lipase productivities by all five *Bacilli spp.* (B1 – B5). Lipase production was optimum of temperature in 37°C lipase production was studied by incubating the production medium at 37^{0}C temperature (B1 – B5).

Lipid Optimization by Substrates

By using different substrate sources such as olive oil, coconut oil, Groundnut oil and Palm oil, their effects on lipase production by the selected *Bacillus spp*. was assessed at optimum pH (pH 6) and temperature (37°C). Further, the influence of substrate concentration (2% & 4%) on lipase production was also assessed on the optimized substrate, which maximized the lipase production.

Enzyme Assay

Isolated *Bacillus spp.* (B1 – B5) was assayed for extra cellular lipase enzyme production using titrimetric method.

Lipase Activity

One unit of lipase activity was defined as the amount of enzyme releasing one mole of free fatty acid in one minute under standard assay condition.

$$\text{Lipase activity (µg/ml/min)} = \frac{\text{Volume of alkali consumed X Normality of NaOH}}{\text{Time of incubation X Volume of enzyme solution}}$$

Lipase Purification

The lipase was purified by Ammonium Sulphate precipitation, Dialysis, Column chromatography followed by SDS Gel Electrophoresis

Immobilization of Enzyme

The crude enzyme was diluted to 1:10 dilution. The enzyme preparation thus prepared was preserved in a refrigerator and used. A mixture of 10ml of enzyme and 25ml of 3.6% sodium alginate solutions was reacted with 4% CaCl2 solutions to get enzyme entrapped beads, which were repeatedly washed with distilled water and then suspended in it for determination of enzyme activity by DNSA method.

Isolation of Genomic DNA

About 2 ml of broth culture was taken and centrifuged at 8000 rpm for 10 minutes, 1 ml of lysis buffer was added to the pellets and incubated in water bath at 40°C for 5 minutes. After a brief spin at 10,000 rpm for 10 min, about 750µl of supernatant was collected and equal volume of phenol: Chloroform: isoamyl alcohol was added in the ratio of 25:24:1. The tubes were gently mixed by inverting and incubated in ice for 5 minutes. Followed by a spin at 10,000 rpm for 10 min the aqueous layer was collected in a separate 1.5 tubes and equal volume of ice cold ethanol was added and then incubated in ice for 5 min. Then the tubes were centrifuged at 10000 rpm for 10 min. The supernatant was discarded and the pellet (DNA) was dissolved in 50µl of TE buffer. The quality of DNA was tested using spectrophotometer using 260/280 ratio.

Polymerase Chain Reaction

RAPD experiments were performed using the primer OPI-07 (5'-CAGCGACAAG-3') reported by Mora et al. 1998. PCR reaction was carried out in a volume of 25ul containing 1 ul of bacterial genomic DNA solution, 12.5 ul of 2X PCR master mix. The cycle conditions were followed as per Mora et al. 1998. After the reactions, 10 ul of product was electrophoresed in 1.5 % (v/v) agarose gel in TBE buffer and analyzed.

Results

Enrichment culture technique enabled the isolation of strains with lipolytic activity in tributyrin media plates In total, 25 isolates were collected from the soil sample and among them; five isolates (B1 to B5) showed high lipolytic activity (Table. 1).The lipolytic microbes were further screened and characterized by their features and reactions and then identified as Gram positive, rod shaped motile organism (Table.2). Finally the morphological and biochemical test indicated that the suspected organisms were *Bacillus spp*. The efficiency of lipolytic *Bacillus spp.* (B1 - B5) was assayed with different substrates like coconut oil, Palm oil, olive oil and also at varied medium substrate concentration at 48 hrs. Among the tested substrates, ***Bacillus stearothermophilus****B2* showed maximum activity (48 µg/ml/min) in coconut oil at pH 6 (Table.3) Among the *Bacillus spp*. tested; maximum lipase activity was achieved by the following *Bacillus*

spp. B1, B4 and B5 at 37°C for 24 to 48 hours (Table 4). In a trial to precipitate lipase by ammonium sulphate, results revealed that 30 % saturation was proved to be the best concentration for maximal specific activity. The most active protein fractions raise the purification fold many times from the origin (Table.7)

Estimation

A continuous increase of enzyme activity was due to the increase of enzyme concentration (53.3 µg/ml/min), where it reached up to 53.3 µg/ml/min for the purified lipase. One tenth (4 %) tributyrin was the best substrate concentration for the purified lipase activity It was found that the enzyme activity decreased above this particular concentration (2) % for lipase (Table.5&6).

Molecular Weight Determination

The overall purification steps protocol resulted in raising the purification fold for lipase after applying DEAE cellulose. This would possibly be due to removal of inhibitory and /or high molecular weight contaminants during these purification stages.(Table.6). The molecular weight of single band 36 KDa Produced enzymes were immobilized calcium alginate beads.

Isolation of DNA

The DNA was isolated using phenol:chloroform extraction method. The pelleted DNA was dissolved in TE buffer. The isolated DNA was confirmed by Agarose Gel Electrophoresis method.

Amplification of DNA

The isolated DNA was Amplified using polymerase chain reaction to molecular characterized the isolated Bacillus species. Because the isolated Bacillus species have shown similar results in its phenotypic characters such as morphology and Biochemical characteristics so reveal the variation among the isolates the genotypic characterization is very essential for this purpose. The isolated DNA along with random primers have used for the Amplification purpose. Amplification of STR region mostly yields one distinct DNA Fragment of minimum 1940.10 bp and the maximum of 11,000 bp in length **(Fig 1)**

For the molecular identification of five isolate strains RAPD profiles were obtained with short random yielding similar Amplified profiles. The image of gel was further analyzed by using non linear dynamics software program. The dendrogram of the representative isolates and the reference strains were shown in (Fig 1).

Table: 1 Sample

Sample	Isolates	Dominative Isolate Name
Oil Mill waste soil	25	B_1, B_2, B_3, B_4, B_5

The results revealed that 25 isolates of lipase activity the dominative bacteria Bacillus sp. Were listed in the above table.

Table: 2Biochemical and morphological characterization

S .No	Tests	B_1	B_2	B_3	B_4	B_5
1	Gram's Staining	+	+	+	+	+
2	Motility test	+	+	+	+	+
3.	Endospore Staining	+	+	+	+	+
4.	Indole Test	-	-	-	-	-
5.	Methyl red Test	-	-	-	-	+
6	VP Test	-	-	-	-	-
7	Citrate Utilization Test	-	-	+	+	-
8.	Urease Test	-	-	-	-	-
9.	Starch hydrolases	+	+	+	+	+
10.	Casein Hydrolases	+	+	+	+	+
11.	Gelatin Hydrolases	+	+	+	+	+
12.	Nitrate reduction Test	+	+	-	+	-
13.	Oxidase Test	+	+	+	+	+
14.	Catalase Test	-	-	-	-	-
15.	Glucose Test	+	+	+	+	+
16.	Lactose Test	+	+	+	+	+
17.	Sucrose Test	+	+	+	+	+
18.	Manitol Test	-	+	+	+	+

(+ Positive - Negative)The identified biochemical and morphological of organism B_1 - *Bacillus Puplliae,* B_2 - *Bacillus stearothermophilus,* B_3 - *Bacillus amyloliquifaciems*B_4 *Bacillus magaterium,* B_5*Bacillus subtilis.*

Table: 3 Effect of pH-6 for the Production of Lipase

S. No	Samples	pH	Vol.of Alkali Consumed (ml)	Time (min)	µg/ ml/ min= Vol. alkali consumed× Strength of alkali / Vol. of enzyme Sample× Time (min)
1	*Bacillus puplliae*	6.0	0.3	1.29	18.16
2	*Bacillus stearothermophilus*		0.2	1.10	14.5
3	*Bacillus amylolicefacieus*		0.3	1.20	20.0
4	*Bacillus magaterium*		0.5	1.29	31.0
5	*Bacillus subtilis*		0.2	1.0	16.0

Table: 4 Effect of temperature 37^{O}C for the production of Lipase

S. No	Samples	Temperature	Vol.of Alkali Consumed (ml)	Time (min)	µg/ ml/ min= Vol. alkali consumed× Strength of alkali / Vol. of enzyme Sample× Time (min)
1	*Bacillus puplliae*	37^{o}C	0.1	0.30	26.6
2	*Bacillus stearothermophilus*		0.1	0.27	29.6
3	*Bacillus amylolicefacieus*		0.1	0.26	30.76
4	*Bacillus magaterium*		0.2	0.30	45.7
5	*Bacillus subtilis*		0.1	0.20	40.0

Table: 5 Effect of Substrate concentration (2%) on Production of Lipase

S. No	Samples	Substrates	Vol.of Alkali Consumed (ml)	Time (min)	µg/ ml/ min= Vol. alkali consumed× Strength of alkali / Vol. of enzyme Sample× Time (min)
1	*Bacillus puplliae*	Groundnut oil	0.6	3.47	13.83
		Coconut oil	0.7	3.20	17.5
		Pam oil	0.1	0.22	36.36
		Olive oil	0.3	2.48	9.67
2	*Bacillus stearothermophilus*	Groundnut oil	0.3	1.10	23.76
		Coconut oil	0.4	1.10	29.09
		Pam oil	0.1	4.10	19.6
		Olive oil	0.9	3.20	22.5
3	*Bacillus amylolicefacieus*	Groundnut oil	0.5	3.0	13.33
		Coconut oil	0.6	1.0	48.0
		Pam oil	0.6	1.10	47.52
		Olive oil	0.2	0.40	40.0
4	*Bacillus magaterium*	Groundnut oil	0.5	1.20	33.3
		Coconut oil	0.7	1.25	44.8
		Pam oil	0.7	3.20	17.5
		Olive oil	0.8	2.20	29.09
5	*Bacillus subtilis*	Groundnut oil	0.1	4.11	19.6
		Coconut oil	0.1	0.30	16.0
		Pam oil	0.2	1.20	13.3
		Olive oil	0.2	0.34	28.57

Table: 6 Effect of substrate concentration (4%) on production of Lipase

S. No	Samples	Substrates	Vol. of Alkali Consumed (ml)	Time (min)	µg/ ml/ min= Vol. alkali consumed× Strength of alkali / Vol. of enzyme Sample× Time (min)
1	*Bacillus puplliae*	Groundnut oil	0.3	0.44	32.87
		Coconut oil	0.5	3.00	13.33
		Pam oil	0.7	3.20	17.50
		Olive oil	0.6	1.32	36.36
2	*Bacillus stearothermophilus*	Groundnut oil	0.2	1.20	13.30
		Coconut oil	0.1	0.40	12.12
		Pam oil	0.3	1.28	18.75
		Olive oil	0.1	4.10	19.60
3	*Bacillus amylolicefacieus*	Groundnut oil	0.4	1.10	29.09
		Coconut oil	0.6	3.00	48.00
		Pam oil	0.2	0.50	19.27
		Olive oil	0.4	1.10	29.09
4	*Bacillus magaterium*	Groundnut oil	0.7	3.20	17.50
		Coconut oil	0.6	3.47	13.83
		Pam oil	0.3	2.48	09.67
		Olive oil	0.5	1.20	33.33
5	*Bacillus subtilis*	Groundnut oil	0.5	3.00	13.33
		Coconut oil	0.1	4.10	19.60
		Pam oil	0.2	1.10	14.55
		Olive oil	0.2	0.58	16.60

Table: 7 Purified Enzyme activity (APS)

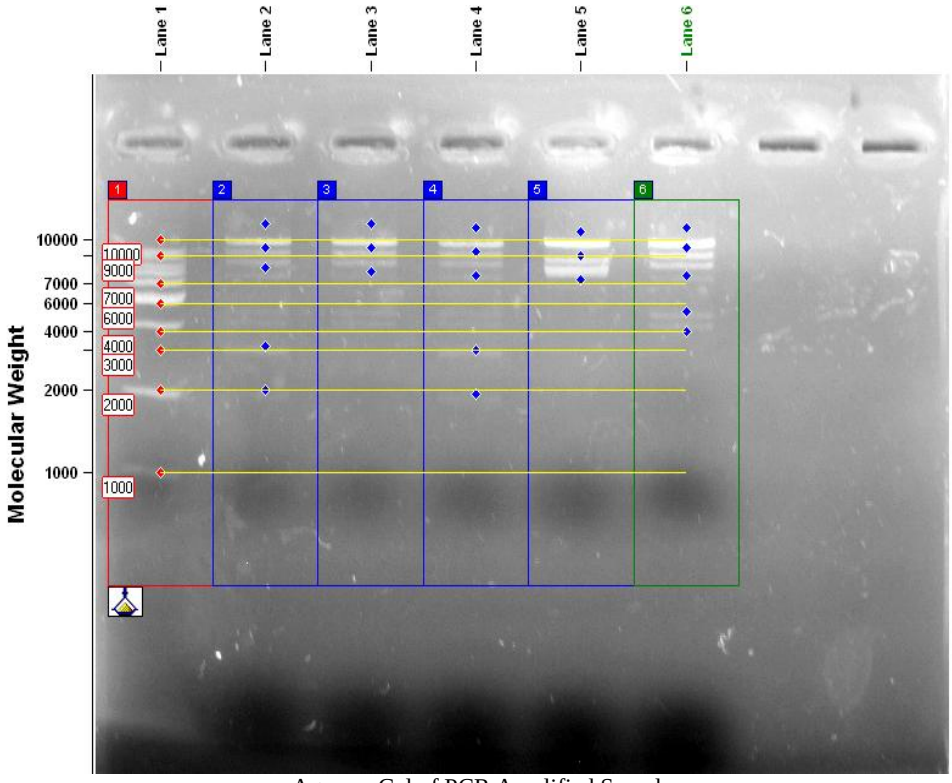
S.No	Samples	Vol of alkali consumed (ml)	Time (min)	µg/ ml/ min= Vol. alkali consumed× Strength of alkali / Vol. of enzyme Sample× Time (min)
1	*Bacillus puplliae*	0.3	0.44	32.87
2	*Bacillus stearothermophilus*	0.6	3.00	48.00
3	*Bacillus amylolicefacieus*	0.1	0.22	36.36
4	*Bacillus magaterium*	0.4	1.10	29.09
5	*Bacillus subtilis*	0.6	1.10	47.52

Table: 8 Purified Enzyme activity (Dialysis)

S.No	Samples	Vol of alkali consumed (ml)	Time (min)	µg/ ml/ min= Vol. alkali consumed × Strength of alkali / Vol. of enzyme Sample × Time (min)
1	*Bacillus puplliae*	0.1	0.21	38.09
2	*Bacillus stearothermophilus*	0.2	0.31	51.6
3	*Bacillus amylolicefacieus*	0.2	0.42	38.05
4	*Bacillus magaterium*	0.1	0.25	32.0
5	*Bacillus subtilis*	0.2	0.50	48.3

Table: 9 Purified Enzyme activity (Column Chromatography)

S.No	Samples	Vol of alkali consumed (ml)	Time (min)	µg/ ml/ min= Vol. alkali consumed × Strength of alkali / Vol. of enzyme Sample × Time (min)
1	*Bacillus puplliae*	0.1	0.20	40.0
2	*Bacillus stearothermophilus*	0.1	0.15	53.3
3	*Bacillus amylolicefacieus*	0.1	0.20	40.0
4	*Bacillus magaterium*	0.1	0.23	34.9
5	*Bacillus subtilis*	0.1	0.16	50.0



Agarose Gel of PCR Amplified Samples

Fig 1 Molecular Characterization Results

3. DISCUSSION

In the present study, the lipase producing bacterial strains were isolated from coconut oil mill soil and identified as *Bacillus spp.* (B1 to B5). Among the different substrates tested, olive oil was found to be suitable for enhancing the lipase production by the isolated and screened *Bacillus* strains and the maximum lipase activity (53.3 µg/ml/min) was recorded by *Bacillus spp*. 5 (B2). Furthermore, irrespective of the substrates tested, the lipase activity was maximum at pH 6.0. In pH5 and pH7 high medium pH tested, the lipase activity was less.

In previous, One hundred and fifty-three bacterial isolates were grown at 55°C and at pH 9 in order to produce a thermoalkalostable lipase to be used as additive to detergent formulations. A screening test of lipolytic productivity of all bacterial isolates resulted in the fact that only two bacterial isolates were found to be the best lipolytic enzymes producer (Table 1). From the industrial point of view, in order to produce low cost thermo alkalo stable lipase, the two most potent bacterial isolates. This result is in consistence with the earlier report of **Achamma** *et al.,* (2003); they inferred that the lipase activity of *Bacillus spp*. was maximum at pH 7 during the 24 h of culture period. In the present study, in all the

tested substrates and also in all the media pH, the tested *Bacillus* strains showed maximum activity during 24 h of the culture period. On further increase in culture period to 72 h, the lipase activity decreased. High levels of lipase activity were also obtained in *Bacillus* strains when olive oil was used as the substrate. **Rohit** *et al.,* (2001) reported that the lipase production was more when vegetable oil, olive oil, soya bean oil, sunflower oil and gingili oil were used as the carbon source. In the present study, the influence of medium temperature indicated that the lipase production of the isolated strains was higher (0.001 to 0.0021 µg/ml/min) at 37°C when compared to those at 27 and 47°C. Here also maximum activity was displayed by *Bacillus spp.* 5 (B5). **Walavalkar and Bapat** (2001) have reported that, the lipase activity of *staphylococcus spp*. was maximum at 37°C. **Lakshmi***et al.,* (1999) reported that the production of lipase was high in medium added with vegetable oil than the medium added with glucose. In contradiction, **Banerjiee** *et al.,* (1993) reported that some microorganisms showed higher activities when grown in medium containing glucose. **Novotny** *et al.,* (1988) reported that olive oil in combination with glucose increases lipase activity and in most cases and also the presence of olive oil, together with glucose or glycerol in the medium significantly decreased both lipase and esterase levels. They also further inferred that, if olive oil was used as the only carbon source for growth, the enzyme activities of *Candida guilliermondii* and *yeast spp*. showed a four to five fold increases. As reported by **Nakashima** *et al.,* (1988), the presence of olive oil as growth medium greatly enhanced the lipase activity of *Bacillus* strain 5(B1) in the present study. **Fadiloglu band Erkmen** (1999) also reported that olive oil in combination with other nitrogen sources enhanced the lipase production, but the presence of carbon source in the olive oil significantly ($P < 0.01$) decreased the lipase activity and biomass content. They also reported that organic nitrogen sources were found to increase lipase synthesis by *Candida rugosa* grown in the presence of olive oil. In earlier, the lipase active fractions were concentrated again under vacuum and were further purified by gel permeation. The purified enzyme had lipase activity 168 U/mL and a specific lipase activity in these fractions was 1479 U/mg protein. A representative profile as summarized in the Table 9. The purified lipase was incubated with various concentrations of triolein in the emulsion and the final concentrations of the triolein in the reaction mixture ranged from 0.5 to 4.0mM. Several molecular biological typing methods have been developed to improve the discriminative power of typing methods for the genus Bacillus These include amplification of a parasite DNA sequence by either a specific PCR or a random amplified polymorphic DNA (RAPD) PCR or detection of restriction fragment length polymorphism (RFLP) by southern hybridization with DNA specific probes. The last two methods have drawbacks RFLP analysis is a time consuming technique and large amounts of purified DNA are needed where as RAPD analysis requires strict conditions to obtained reproducibility between different Laboratories and generates complex patterns. In contrast specific PCR – based methods are attractive because of their rapidly and also culturing parasites can be avoided. However most causes level of Polymorphism found with coding or repeated non coding PCR – amplified sequences is not refined enough to distinguish between closely related strains (**Harris** *et al.,* 1990). Micro satellite DNA sequences tandem of a sample nucleotide motif are distributed abundantly in the eukaryotic genomes and may reveal important strain polymorphisms. However until now only two micro satellites showing size polymorphisms have been identified and characterized for Bacillus usually to study micro satellites investigators screen a genomic DNA library and then evaluate the micro satellite size polymorphism by PCR amplification of electrophoresis on acrylamide gels RAPD analysis are useful for differentiating bacteria at the strain level. Diversity studies have recently been undertaken to assess host or ecological specialization in this *Bacillus spp*. Previous methods such as isoenzymes and rDNA analysis detected little or no genetic variation among isolates of the *Bacillus spp.* Therefore random amplified polymorphic DNA (RAPD) was used to screen multiple regions of anonymous DNA detected extensive polymorphisms although there was no evidence of host or ecological specialization used restriction fragment length polymorphism analysis of the large non transcribed spacer of rDNA repeats of a worldwide collection of Bacillus the distribution of identified nuclear type patterns suggested that gene flow was occurring across North America and the another tentatively concluded that the relative risk of releasing a single native isolate of this *Bacillus spp.* across Canada was likely to below. A more precise evaluation of the genetic structure of Canadian populations of *Bacillus spp*. was described using RAPD analysis which provides multilocus finger prints and is more sensitive to low variation the analysis revealed that *Bacillus spp.*

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