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Biochemical and genetically identification of solvent tolerant novel *Bacillus subtilis* Pa2

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Abstract

A total of 25 isolates originating from hydrocarbon contaminated soil samples were successfully isolated via direct plating method using different growth medium. Eight isolates demonstrated high enzymatic potential (lipase, protease and amylase). Out of the total isolates, 86% isolates showed hydrocarbon degradation efficiency. These isolates were screened for lipolytic activity using tributyrine as substrate. Isolate Pa2 was found to produce the highest lipase yield on solid medium and later identified as *Bacillus subtilis* by biochemical test and 16s ribosomal RNA sequence. Stability studies in various organic solvents at 20% (v/v) showed that lipase from *B. subtilis Pa2* was stable under different solvents like acetone, hexane and benzene. However, the activity was greatly reduced in ethyl alcohol.

Keywords: Lipase; Organic solvent tolerant; Enzyme production; *Bacillus subtilis*

Introduction

Enzymes are the best catalyst known till date. They are the most remarkable and highly specialized proteins with an extraordinary catalytic power, even higher than synthetic catalysts. Hence, they occupy a unique position in synthetic chemistry due to their high selectivity's and rapid catalysis under ambient conditions.

The world enzyme demands are satisfied by 12 major producers and 400 minor suppliers. Around 60% of the total world supply of industrial enzymes is produced in Europe. At least 75% of all industrial enzymes (including lipases) are hydrolytic in action.

Among all the enzymes, hydrolases are the most employed class of enzymes for industrial biotransformations. It is estimated that approximately 80% of all industrially used enzymes are hydrolases (Hari Krishna et al. 2002). These enzymes catalyse the hydrolylic cleavage of C-O, C-N, C-C and other bond like P- O bonds in phosphates. Their applications are very diverse including hydrolysis of polysaccharides, nitriles, proteins, lipids and esterification of fatty acids. Most of these enzymes are used in processing type reactions to degrade proteins, carbohydrates and lipids in detergent formulations and in food industry (*l.c.*).

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface (Martinelle et al. 1995) 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. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Many lipases are active in organic solvents where they catalyze a number of useful reactions including esterification (Chowdary et al. 2001; Hamsaveni et al. 2001; Kiran et al. 2001a; Kiyota et al. 2001; Rao and Divakar, 2001), transesterification, regioselective acylation of glycols and menthols, and synthesis of peptides (Ducret et al. 1998; Zhang et al. 2001) and other chemicals (Therisod et al.1987; Weber et al. 1999; Bornscheuer, 2000; Liese et al. 2000; Azim et al. 2001). Lipases are now becoming as important industrially as the proteases and carbohydrases.

Substrates for the lipase are mostly insoluble or partially soluble in water and organic solvents or organic–aqueous solutions do favour some reactions. Organic solvents may also provide many advantages viz., (1) relatively high solubility of substrates, (2) relative ease of recovery of products in organic phase, (3) possibility of reducing the degree of undesirable substrate and/or product inhibition in organic solvent-water biphasic systems and (4) ability to shift the reaction equilibrium in the synthetic direction by continuously removing the products with organic solvents in organic solvent-water two phase systems. Nevertheless, organic solvents are generally known to have detrimental effect on microorganisms and the enzyme it produces (Ogino et al. 1994). In the presence of organic solvents, most organisms lose their functions and cease growing. Enzymes are often denatured and inactivated by organic solvents. Besides that a number of lipases have been isolated from unicellular bacteria,

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mainly those produced by various species of genus *Pseudomonas and Bacillus (*Chin et al. 2003).

So far, the number of organic solvent tolerant bacteria is limited and most of the isolated organic solvent tolerant strains are *Pseudomonas (*Aono et al.1992; Cruden et al.1992; Inoue et al. 1991; Nakajima et al.1992; Ogino et al. 1994). This paper aim describes to an organic solvent tolerant bacterium, its identification and lipase production.

Environmental consequences are foreseen. Through the use of the proper lignin-degrading fungi, at least 30% electrical energy can be saved in mechanical pulping and paper strength properties are improved (Kirk et al. 1993; Kirk et al. 1994; Akhtar et al. 1996; Akhtar et al. 1997; Akhtar et al. 1998; Akhtar 2000). In addition, the fungal pretreatment for mechanical pulping has less environmental impact than chemical pretreatments (Sykes 1994; Singh et al. 2010).

The objective of this research work is to investigate the ligninolytic enzymes; peroxidase, manganese peroxidase, laccase, and cellulase production patterns during lignin degradation by *P. coccineus* and *C. versicolor* used and applied in the biopulping of *Acacia mangium* wood chips.

Material and methods

Enrichment and Isolation of microorganisms

Soil samples were collected from various oil contaminated sites such as petrol pumps at Patan, Himmatnagar, Sathamba, Bayad, Khedbrahmha and oil wells at Mehsana of North Gujarat region, India. Microorganisms were isolated by enrichment technique. Soil samples were added to Tributyrine broth containing (g/lit): peptone, 5; Yeast Extract, 3; tributyrine, 10; pH is adjust to 7 by adding separately sterilized sodium carbonate (20% w/v). Plates were incubated at 37^0C for 48 h and pure cultures were obtained of those isolates showing prominent growth on tributyrine agar. Among all the isolates, Pa2 secreted maximum lipase on solid media.

Preparation of inoculum

Maximum secretion of lipase was observed for the isolate Pa2 in the broad range of pH-6 to 10. One loopful of culture from the slant was inoculated in a 250 ml Erlenmeyer flask containing 100 ml of sterile Tributyrine broth, pH 7.5 and incubated at 37°C on rotary shaker at 180 rpm for 24 h to obtain actively growing culture.

Lipase production

Lipid carbon sources seem to be generally essential for obtaining a high lipase yield; however, scientists have produced good yields even in the absence of fats and oils. During the study, eight bacterial strains showed good enzymatic potential. Out of total eight isolates, Pa2 was good lipase producer and grown in production medium. Growth and lipase activity was measured calorimetrically. Production medium contain (g\l) peptone, 30; yeast extract, 10; NaCl, 5 and Olive Oil. 10. Young culture of Pa2 was also inoculated in the production medium containing different solvents (10% v/v) and incubated at 37^oC and at 180 rpm in an environmental shaker.

Lipase activity

Lipase activity was estimated by initial release of p-Nitrophenol from p-Nitrophenylacetate at 37^oC and measured at 410nm. One unit of enzyme activity is calculated as the amount of enzyme that released 1µg of pNP per minute under assay conditions. (Winkler et al. 1979).

Hydrocarbon degrading ability

The hydrocarbon degradation ability is checked in Bushnell - Hass broth (BH broth). Different hydrocarbon substrates viz., Kerosene, Servo 2T Oil, and Diesel were added at 5% v/v concentration in BH broth. Inoculum was prepared by growing Pa2 culture in nutrient broth and the cells are harvesting by centrifugation, washed twice with sterile distilled water, suspended in normal saline. From this, 0.1ml is inoculated in BH broth and incubated on rotary shaker at 37^0C at180 rpm. Growth is check after 48, 72, and 96h of incubation.

Solvent tolerant and degrading ability

The solvent degradation ability is check in Bushnell - Hass broth (BH broth). 10% and 20%v/v different Solvent substrate viz., Benzene, Toluene, Methanol, n-Hexane and Xylene were taken in BH broth. Inoculums are prepared by growing the culture in nutrient broth and the cells harvested by centrifuging the culture, washed twice with sterile distilled water and suspended in normal saline. From this, 0.1ml is inoculated in BH broth and incubated on rotary shaker at 37^0C and 180 rpm. Lipase activity and growth are checked after 48h and 72h of incubation.

Identification and taxonomical studies

The isolated strain is identified according to method described in "Bergey's Manual of Determinative Bacteriology" (Holt et al.1994) and also via 16S rRNA sequence. 16S rRNA sequence is amplified via PCR using specific primers. For further characterization of strain Pa2, a phylogenetic tree based on comparison of 16S rRNA sequence is constructed based on comparison of 16S rRNA sequence of this strain and those of type strains of *Bacillus* species. All sequences were aligned on CLUSTAL W1.75 (Thompson et al. 1994). Phylogenic tree constructed in Biology Workbench 2.0 (http://workbench.sdsc.edu/).

Result and Discussion

Screening

A total of 25 isolates were isolated by direct plating method using 1% (w/v) of Tributyrine. Out of these, only isolate Pa2 show remarkable activity after 48 h. Isolate Pa2 was able to grown at 10% and 20% (v/v) concentration of solvents in production medium where Acetone and benzene exhibited good growth of Pa2 in production medium (Figure 2). Therefore, this strain is selected for further study.

Identification and taxonomic study

Table 1 shows the results of the morphological and biochemical characteristics of strain Pa2. The colony appeared to be irregular, rough, flat, entire and opaque on nutrient agar. The bacterium is a Gram-positive rod, sporulating and occurring singly or in pairs. It is able to hydrolyze gelatin, starch, tributyrine, glycerol and produces acid in glucose, fructose and sucrose broth. It is also able to reduce nitrate to ammonia. This characteristic corresponds to genus *Bacillus*. Me5 cannot reduce nitrate to ammonia the above show a typical characteristic of the genus *Bacillus*. Me5 was utilized sucrose. While other two species of Gram negative Ba1 and Hi1 is utilize glucose but Hi1 is ferment glucose.

Table 1: Biochemical tests

No.	Test	Results of Biochemical Test			
		Pa2	Ba1	Me5	Hi1
	Carbohadrates utilization				-
1	Lactose	-	-	-	-
2	Maltose	-	-	-	-
3	Fructose	+	-	-	+
4	D-Glucose	+	+	D	+
5	Galactose	-	-	-	+
6	Sucrose	+	-	+	-
7	L-Arabinose	d	ND	ND	ND
8	Raffinose	-	ND	ND	ND
9	Melibiose	-	ND	ND	ND
10	Mannose	+	ND	ND	ND
11	Inulin	+	ND	ND	ND
12	Glycerol	+	+	+	D
13	Inositol	+	ND	ND	ND
14	Sorbitol	+	ND	ND	ND
15	Mannitol	+	d	-	-
16	Ribose	+	ND	ND	ND
17	Rhamnose	-	ND	ND	ND
18	Cellobiose	-	ND	ND	ND
19	Xylose	d	-	-	-
20	Esculin	-	ND	ND	ND
21	ONPG	-	ND	ND	ND
22	Adonitol	-	ND	ND	ND
23	Citrate	d	+	-	+
24	Sorbise	-	ND	ND	ND
25	sodium gluconate	-	ND	ND	ND
26	Urease production	-	ND	ND	ND
27	Catalase production	+	+	+	+
28	starch Utilization	+	+	+	+
29	Gelatin Utilization	+	+	+	+
30	Tributyrine Utilization	+	+	+	+
31	Voges- proskauer	+	-	-	+
32	Indol formation	-	-	-	-
33	Lysine decarboxylase	d	d	D	-
34	Ornithin decarboxylase	d	ND	ND	ND
35	Deamination of Phenylalanine	-	ND	ND	ND
36	Nitrate Reduction	+	-	-	D
	Gas from Glucose	-	-	-	+
	H2S production	-	-	-	-
	Groth in Nacl				
	2%	+	+	+	+
	5%	+	-	-	-
	10%	ND	ND	ND	ND

Results are average of triplicate observation. *Note*: (+) positive results; (-) negative results; (d) variable results. ND not done

Phylogenetic tree analysis

16S rDNA sequences of 5 different *Bacillus* species obtained from NCBI database are compared. Sequences are analyzed using neighbur-joining method according to the models of Thompson et al 1994. Comparison study showed highest homology (99%) with *B. subtilis*. Phylogenetic relationship of closely related microorganisms is showing in Figure 2. 16s rDNA sequence was submitted in European Molecular Biology Laboratory and it's Accession No. HE573240.

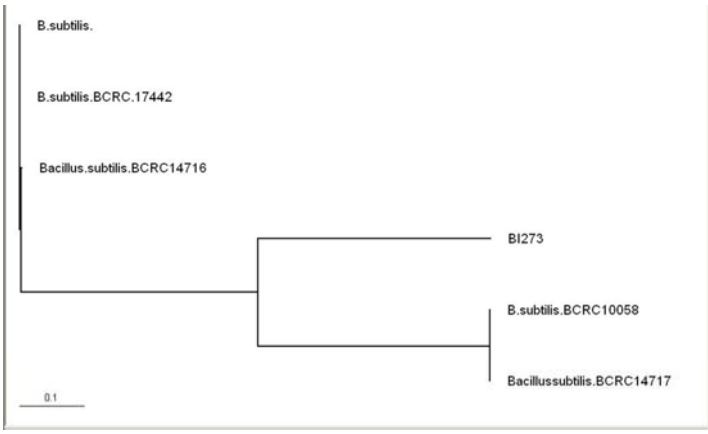

Figure 1: Phylogenic tree and 16s rDNA sequence

DISTANCE MATRIX : BI273(Pa2) . 0.000 0.730 0.721 0.721 0.731 0.731 .*Bacillus.subtilis*.BCRC14716 0.730 0.000 0.727 0.728 0.004 0.004 .*B.subtilis*.BCRC10058 0.721 0.727 0.000 0.000 0.731 0.731 .*Bacillussubtilis*.BCRC14717 0.721 0.728 0.000 0.000 0.732 0.732 .*B.subtilis*. 0.731 0.004 0.731 0.732 0.000 0.000 *B.subtilis*.BCRC.17442 0.731 0.004 0.731 0.732 0.000 0.000

Effect of organic solvents on lipase production

In recent years, few lipases that were organic solvent tolerant from *Bacillus* species has been isolated (Chin et al 2003). It is well known that effect of organic solvents on lipase activity differs from lipase to lipase. Activity of the lipase have been stimulated in presence of 10% (v/v) acetone and benzene in the production medium while ethyl alcohol shows negative effect on lipase production.

Enzymes are often much more stable in solutions containing hydrophilic or hydrophobic organic solutions than in organic solvent-free aqueous solution. (Ogino H et al. 1994). The replacement of some water molecules of an enzyme with solvent molecules sometimes stabilizes the structure of the enzyme isolation and production of lipase by *B. subtilis.*

Hydrocarbon degradation ability

Microorganisms from the different hydrocarbon contaminated soil are isolated and investigated for their capacities of enzyme production under different physiological conditions. (Margesin et al. 2000). Further, the correlation between enzyme activity and hydrocarbon degradation has been worked out particularly for lipase (*l.c.*). However, other authors found distinctly lower rates of oil hydrolysis in oil-contaminated soils than in normal soils. (*l.c.*) A soil lipase activity is a valuable tool to monitor oil biodegradation in freshly diesel oil-contaminated soils (Margesin et al. 1999). Probably due to a high content of available aliphatic compounds Pa2 showed hydrocarbon degradation abilities (Diesel, Kerosene and 2T oil). Hydrocarbon degradation and lipase activity are somewhat related, so lipase producing pa2 may be used for biodegradation of petroleum hydrocarbons

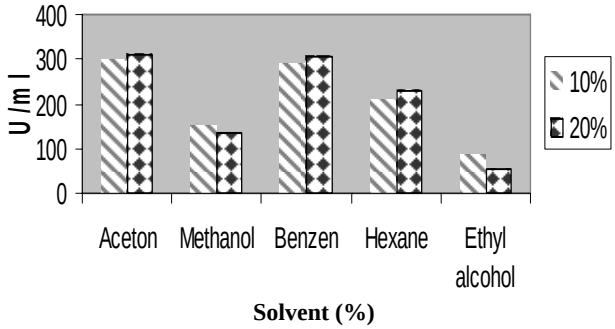

Figure 2: Percentage of solvent tolerance on lipase activity.

Conclusion

A new organic solvent tolerant *B. subtilis* strain Pa2 that is capable of growing in presence of solvent as well as show hydrocarbon degradation abilities. *B. subtilis strain Pa2* is a Gram-positive spore-forming bacterium, usually used for the subtilisine antibacterial substance production and Amylase production. We have successfully isolated a new strain of *B. subtilis* Pa2 that is stable in solvent and in addition produce an organic solvent-stable lipase therefore is very useful for bacterial reaction processes and also it can be used as bioremediation agent

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