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Preparation and Characterization of Cutinase Inducible Substrate, and Screening and Selection of *Pseudomonas cepacia* NRRL B 2320 for Enhanced Production of Cutinase

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In this study, fifteen different microorganisms grown on medium containing Tween 80 or olive oil or cutin, were screened for their possible cutinase producing potential. Among fifteen microorganisms, *Pseudomonas cepacia* NRRL B 2320, *Pseudomonas fluorescens* NRRL B 3178 and *Pseudomonas geniculata* NRRL B1606 were capable of growing on the medium containing cutin as sole source of carbon and their culture filtrates showed enzyme activity towards p-nitrophenyl butyrate (p-NPB). The essential substrate for production of cutinase, cutin, was prepared from tomatoes in our laboratory and characterized. Cutinase activity was further confirmed by cutinase specific substrate, p-nitrophenyl (16 methyl sulphone ester) hexadecanoate (p-NMSH), where, *Pseudomonas cepacia* NRRL 2320 have shown highest activity of 40 U/mg and 41.2 x10^{-2} U/mg towards both p-NPB and p-NMSH, respectively. However, further attempts have been made to replace cutin with different oils, cutin monomer and other carbon sources for the production of cutinase. This is the first report on the production of cutinase from different *Pseudomonas species* other than *P. putida* and *P. mendocina*.

Key words: Lipase; Esterase; Cutinase; Cutin; *Pseudomonas sp*.

Cutinases (3.1.1.74) are hydrolytic enzymes, the smallest member of α/β hydrolase family that degrades cutin. Cutin is an insoluble three dimensional polymeric network of esterified hydroxyl and epoxy fatty acids of C_{16} and C_{18} family[1]. This comprises the structural component of the plant cuticle. Cutin is embedded in and covered by coating cuticular waxes typically composed of chloroform/methanol-soluble lipids such as hydrocarbons and wax esters[1]. Cutinases have potential use in dairy industry for hydrolysis of milk fat, house hold detergents, oleochemical industry, synthesis of structured triglycerides polymers and surfactants[2], synthesis of ingredients for personal-care products, synthesis of pharmaceuticals and agrochemicals containing one or more chiral centers[3,4]. At low water activities, transesterification of fats and oils or stereo selective esterification of alcohols can be achieved using cutinase. Mostly, phytopathogenic fungi are reported to be capable of producing cutinases. Extensive studies have been done on the production, purification and characterization of fungal cutinases[5-10]. In contrast, there are only few studies on bacteria that appear capable of cutin degradation[11-15]. Bacterial cutinases are different from fungal cutinase in respect of their thermal and high pH stability. It has been observed by Sebastian *et al.*[16], that the bacterial cutinases are stable even at 70°C, whereas, fungal cutinases loses its activity at 45°C. Previously, few studies

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have been done on for the production of cutinase from *Pseudomonas sp* using apple cutin as substrate[15,16]. As far our knowledge goes, cutin, the primary carbon source/inducer for cutinase production is not available commercially. So, to start work with cutinase, cutin needs to be prepared in the laboratory. In our study, we have prepared cutin from tomatoes, which is a low-cost cutin source as compared to apple cutin in India. Previously, only two reports are available on production of cutinase from *Streptomyces spp*[11] and *Botrytis cinera*[17] using tomato cutin as a carbon source. To the best of our knowledge, only one report is available till date on the production of cutinase, where flaxseed oil had been successfully used in place of cutin[18]. In our present study, we have screened for efficient cutinase producer among natural isolates and procured strains from different microbial culture collection centers using different media. Primarily, the enzyme activity was measured using p-NPB and p-NPP (p-nitrophenyl palmitate). Cutinase specific substrate, p-NMSH was synthesized and characterized in our laboratory as described previously by Degani *et al*.[19]. The production of cutinase was confirmed using p-NMSH as substrate in the assay. We have also tried for alternative inducer/substrate for cutinase production by replacing cutin with different oils (mustard oil, soybean oil, and sunflower oil), oleic acid and cutin monomer (16-hydroxyhexadecanoic acid). The effect of different carbon sources with and without cutin on the production of cutinase was also studied.

MATERIALS AND METHODS

Preparation of cutin

Cutin was prepared from fresh tomato peels using method described by Walton and Kolattukudy[20]. Tomato peels collected by known quantity of fresh tomatoes were boiled, washed and dried properly. Then peels were boiled in oxalic acid (4g/L)/ ammonium oxalate (16g/L) buffer for 3-4 hours. After digesting with enzymes (cellulase (sigma, USA) and pectinase (Himedia, India)) for 18h at 30°C, peels were subjected to extensive solvent extraction with methanol-chloroform in soxhlet apparatus. These peels were ground to powder (< 20 mesh) to get cutin. This cutin was further characterized by FT-IR and AFM studies.

Preparation of p-nitrophenyl (16-methyl sulphone) ester (p-NMSH)

The cutinase specific substrate, (p-nitrophenyl (16-methyl sulphone ester) hexadecanoate) is prepared using the method described by Degani *et al*.[19]. One equivalent of 16-hydroxyhexadecanoic acid (3 g) mixed with 2 equivalent of methane sulfonyl chloride (1.7 ml) in dry dichloromethane. Thereafter, 5 ml of triethylamine was added and stirred for 1.5 h at the -20°C under N_2 atmosphere. Then 1.1 equivalents of p-nitrophenol (PNP, 1.6 g) was added and stirred for another 6 h at the same temperature. The mixture was then left to reach room temperature under stirring. The disappearance of the reactant PNP, was tested by thin layer chromatography (TLC). At the end of the reaction, 10% sodium bicarbonate (50 ml) was added to the mixture and the organic products were separated in a separating funnel with dichloromethane (3 x 30 ml). The organic phase was dried above sodium sulfate, filtered and concentrated to dryness in a rotary vacuum evaporator. The resulted powder was freed from byproducts using silica gel column with a mixture of dichloromethane (90%) and hexane (10%) as a mobile phase and stored in the darkness at -20°C until use. The substrate was characterized by H^1NMR spectroscopy.

Microorganisms and maintenance medium

Different *Pseudomonas* strains used for this study are *P. syringae pathovar lachrymans* van Hall 1902 NRRL B799, *P. cepacia* (also known as *Burkholderia cepacia*) NRRL B2320, *P. fluorescens* NRRL B14678, NRRL B10, NRRL B11, NRRL B253, NRRL B1603, NRRL B3178, NRRL B2641, *P. geniculata* NRRL B1606, NRRL B1612, NRRL B1888, and NRRL B1890 procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA and *P. cepacia* NCIM 5029 was procured from National Collection of Industrial Microorganisms, Pune, India. Four strains are isolated from rotten tomatoes in our laboratory. All microorganisms were grown on nutrient agar medium at 28°C and maintained at 4±1 °C.

Isolation of microorganisms from rotten tomatoes

The fresh tomatoes were kept at 37ºC for two days and the outer part of the tomatoes were washed with autoclaved distilled water. The tomato washed water then spread on the solid medium

containing 1% Tween 80. The microorganisms grown on medium containing Tween 80 again cultured on medium containing olive oil. The finally four microorganisms were isolated which are able to grow in the presence of Tween 80 as well as olive oil.

Primary screening of cutinase producing microorganisms

The microorganisms are primarily screened for cutinase activity by plate technique. The composition of medium used in g/L: meat extract 3.0, polyoxyethylene-sorbitan mono laurate (Tween 20) 5.0, $CaCl_2.2H_2O$ 0.4, agar 15.0. The plates were incubated at 28° C for 48 h. In order to enhance the contrast, 5 ml of Lugols iodine solution was added to each plate and removed after 1 min[21].

Production of enzyme seed culture medium

The medium used for the development of seed culture contained (g/L): glucose 6.0, beef extract 3.0, peptone 15.0, urea 6.0, KH_2PO_4 2.0, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 5.0 and pH 7. The seed culture medium was inoculated with a loop full of the microorganisms freshly grown on nutrient agar slant. The culture was then incubated for 10 h (to reach culture OD at 600nm: (0.6~0.8) at 28°C and 180 rpm[22].

Production medium

The following media were used for the production of cutinase (g/L): beef extract 3.0, peptone 15.0, urea 6.0, KH_2PO_4 2.0, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 5.0 with glucose (6g/L) or glucose (6g/L) with Tween 80 (10g/L) or glucose (6g/L) with olive oil (10g/L) or glucose (6g/L) with cutin (4g/L) or cutin (4g/L). The production of cutinase was carried out in 250 ml Erlenmeyer flask containing 50 ml medium. The medium was inoculated with 2% seed culture having OD of 0.8 at 600nm wavelength. The cultures were grown for 120 h at 28°C and 180 rpm[22]. The effect of mustards oil, oleic acids, soybean oil, sunflower oil and 16-hydroxyhexadecanoic acid, and different carbon sources (lactose, maltose, fructose, galactose, sucrose, xylose and starch) with or without cutin was observed on the production of cutinase.

Cutinase assay

Cutinase activity of culture filtrate was measured by modified method of Winkler and Stuckman[23] using p-nitrophenyl butyrate (p-NPB) and p-nitrophenyl palmitate (p-NPP) (Sigma) as substrate. One enzyme unit is defined as the amount of enzyme required to release one micro-molar of p-nitrophenol/min under assay condition. Cutinase activity was further confirmed with cutinase specific substrate, p-NMSH[19]. The cutinase production from *P. cepacia* NRRL B 2320 was confirmed using cutinase specific substrate, p-nitrophenyl (16 methyl sulphone ester) hexadecanoate (p-NMSH), which was prepared in our laboratory. Assay was performed using the method described in enzyme assay, with the change in incubation period for 1 hour instead of 5 min at 55°C. One unit of enzyme activity is defined as release of 1 µmol of *p*-nitrophenol per min. Specific activity was represented as U/mg of protein. Protein estimation was done by Lowry method[24] using BSA (sigma) as standard.

RESULTS AND DISCUSSION

Characterization of cutin and p-nitrophenyl (16 methyl sulphone ester) hexadecanoate (p-NMSH)

The cutin was prepared from tomatoes as described in Materials & Methods and characterized using AFM and FT-IR studies. AFM was used successfully to image the surface morphology and elucidate structure details of amorphous and crystalline polymers, and phase separated macromolecular systems[25]. The topography of prepared cutin at different stages of preparation was obtained using AFM. The gradual removal of big agglomerates as observed from Fig. 1a, 1b and 1c was evidence of the removal of other unwanted substances (pectin, cellulose and waxes) present in the tomato peels. Cutin isolated from tomato fruits mainly formed by esterification of secondary hydroxyl groups of the dihydroxy fatty acids monomers. Previously, FT-IR spectroscopy was used to characterize (*in situ*) the functional chemical groups of isolated cuticles and their interactions with exogenous chemicals at the cuticular level[25]. In the present investigation, experiments have been performed to obtain FT-IR of cutin (Fig. 2), and absorption around 1630 and 1500 cm^{-1} were due to the stretching of C=C bonds and stretching of benzenoid ring, respectively.

The observed H^1NMR spectra (Fig. 3) of the synthesized p-NMSH were comparable with the previous report[19]. There is a sharp peak between 1.1-1.3 ppm is due to the presence of a long chain fatty acid at the one end of which

nitrophenol group is attached giving peaks at 8.26 ppm and 7.26 ppm and the existence of sulphonyl group at other end confirmed by peaks at 4.22 ppm and 2.97 ppm.

Screening of microorganisms using different media

For screening and selection of potential cutinase producer, bacteria were grown on different plates containing primary screening media which composed of meat extract, Tween 20, $CaCl_2$ and agar. A clear zone was observed around the colonies of bacteria producing lipase/esterase/cutinase. The clear zone formed is due to the precipitation of calcium laurate around each colony of the strains producing lipase/esterase/cutinase. Among the tested bacteria, most of the procured strains (except *P. fluorescens* NRRL B14678) and two isolated bacteria showed clear zone around their colonies. All of these bacteria have ability to produce lipase/esterase/cutinase. To distinguish between lipase/esterase and cutinase producer, the selected bacteria were then grown on different fermentation media containing either glucose or glucose with Tween 80 or glucose with olive oil or glucose with cutin or cutin as carbon source. The effect of Tween 80 and olive oil on p-NPBase and p-NPPase activity of different microorganisms have been studied. As cutinase is an intermediate between lipase and esterase, initially experiments were performed to select the efficient lipase and esterase producer and then the cutinase producer. It was found that the affinity towards the substrates (p-NPB and p-NPP) varies with the inducers used in the media. Among the different fermentation media used, when all bacteria were grown on medium containing glucose showed relatively higher activity with p-NPB than p-NPP (Fig. 4a). The highest production of p-NPBase (esterase/cutinase) was observed to be 11.08 U/mg, 8.77 U/mg and 7.09 U/mg from *P. cepacia* NRRL B 2320, *P. geniculata* NRRL B 1606 and *P. fluorescens* NRRL B 3178, respectively. Similarly, when they were grown on medium containing glucose with Tween 80 (Polyoxyethylenesorbitan monooleate) most of them showed higher activity of p-NPBase (Fig. 4b). It was observed that the glucose alone and glucose with Tween 80 have induced the esterases from ten microorganisms, which were having higher

Table 1. p-NPBase, p-NPPase and p-NMSHase activity of *P. cepacia* B2320 in the presence of different inducers and carbon sources

Inducer/Carbon sources	PNPB (U/mg)	PNPP(U/mg)	P-NMSH $x10^{-2}$ (U/mg)
Mastard Oil	45.79±1.7	2.20±.043	13.6±0.49
Oleic acid	1.18±.0.35	0.41±0.022	3.1±.0.07
Sunflower oil	42.34±2.63	1.42±0.89	10.4±0.42
Soybean oil	46.87±1.10	1.45±0.66	11.0±0.84
16-HDA	9.74±2.59	0	5.8±0.21
Lactose	12.48±1.84	0.39±0.27	2.01±0.03
Lactose+cutin	36.09±0.85	0.21±0.13	28.51±0.12
Fructose	10.26±0.96	1.30±0.52	3.32±0.14
Fructose+cutin	43.82±4.71	0.62±0.16	12.09±0.00
Maltose	12.42±1.96	0.87±0.35	2.19±0.11
Maltose+cutin	31.29±2.37	1.36±0.21	8.05±0.49
Sucrose	5.38±0.80	2.64±0.89	3.30±0.13
Sucrose+cutin	53.75±0.78	1.76±0.12	16.61±1.23
Xylose	8.03±0.37	0.88±0.82	1.87±0.30
Xylose+cuitn	35.23±0.82	0.51±0.21	15.02±2.48
Galactose	17.58±1.25	0.48±0.12	6.37±0.93
Galactose+Cutin	42.40±2.35	1.27±0.05	12.81±0.40
Starch	15.70±3.22	0.95±0.00	2.95±0.07
Starch+cutin	47.94±4.21	1.74±0.75	40.83±1.66
Glucose	11.08±1.29	5.08±0.36	1.0±0.03
Cutin	40.15±2.17	0.41±0.01	41.2±0.26
Glucose+Cutin	32.54±2.93	2.76±0.19	13.90±0.29

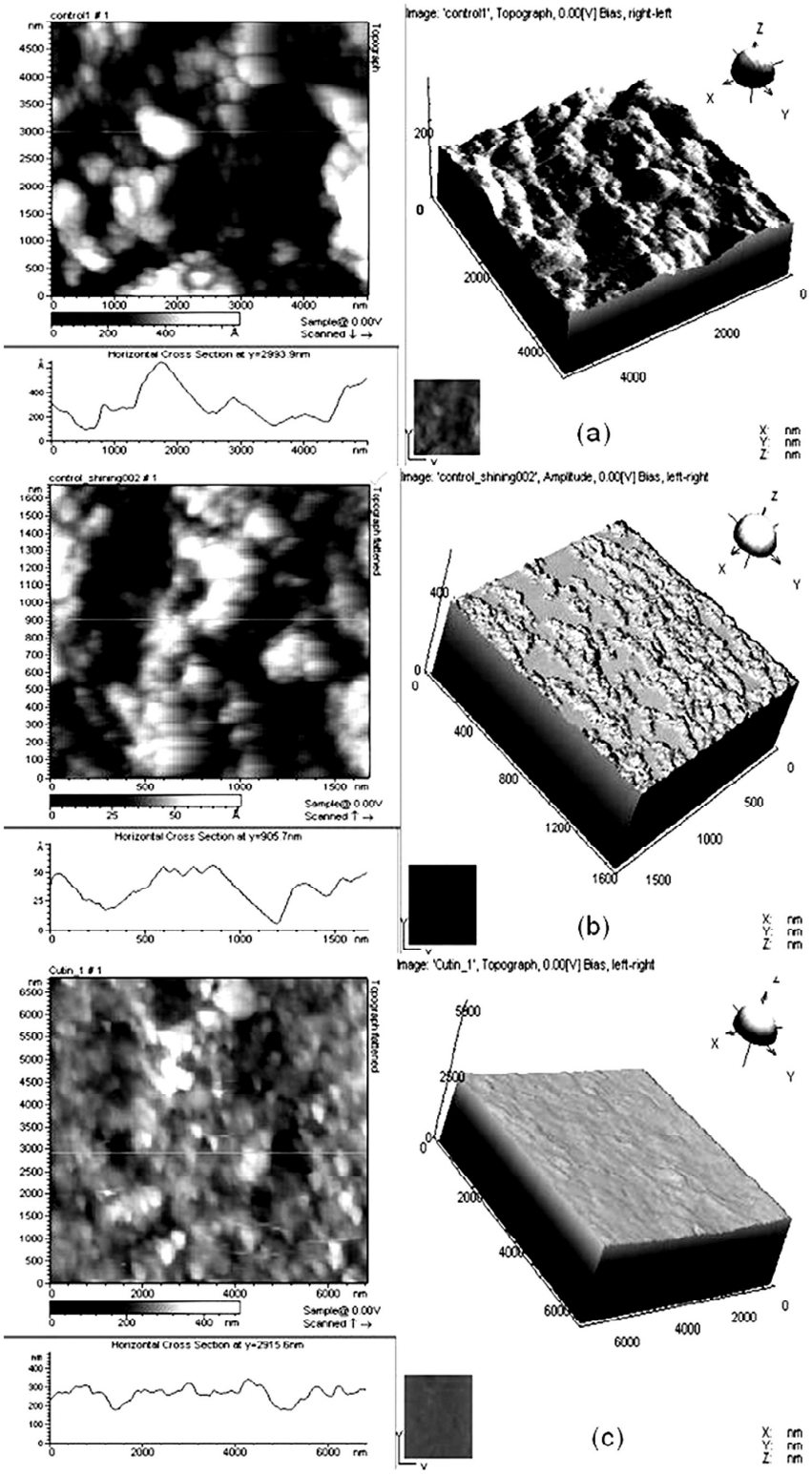

Fig. 1. AFM image of (a) untreated tomato peels (b) enzyme treated tomato peels and (c) Cutin.

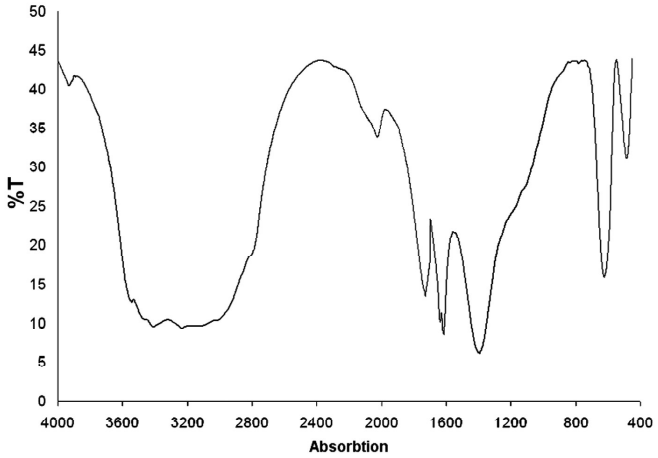

Fig. 2. FT-IR spectra of cutin prepared from tomato peels

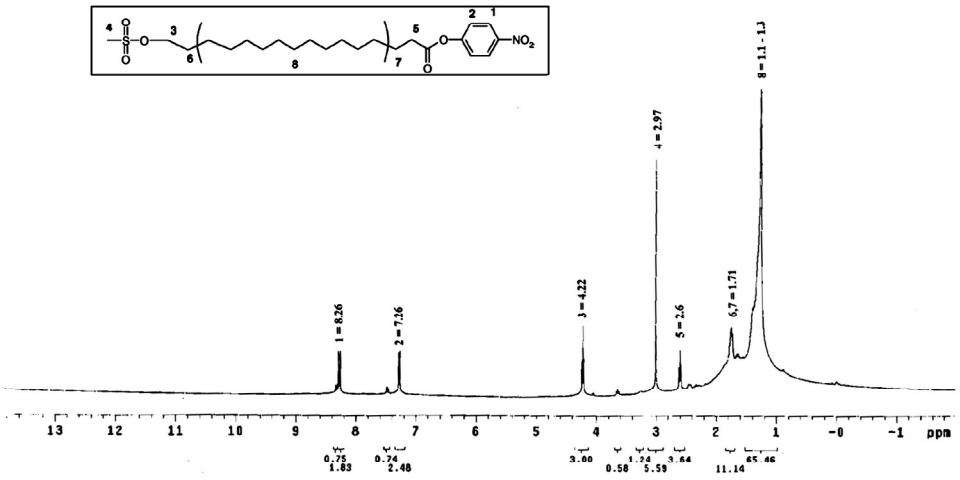

Fig. 3. NMR spectra of p-nitrophenyl (16-methyl sulphone ester) hexadecanoate

affinity for short-chain fatty acid esters (Fig. 4a and 4b). In these microorganisms, Tween 80 induces the esterase production rather than lipase. Surprisingly, four microorganisms *viz.,* two isolated strains (O1 and O2), *P. fluorescens* NRRL B 3178 and *P. geniculata* NRRL B 1606 have shown higher activity with p-NPP when Tween 80 was used as inducer in the medium. The highest p-NPPase (lipase) activity was found to be 34.64 U/mg and 27.72 U/mg for two isolates O1 and O2, respectively in the medium containing Tween 80. In these four microorganisms, lipase production was induced by Tween 80 rather than esterase. In the literature, reports are available on the production of lipase using Tween 80 as an inducer[26]. Tween 80[26] and olive oil[27] induced lipase/esterase production from different microorganisms. The probable dual role of Tween 80 to induce lipase from some microrganisms and esterase from others due to the presence of ester bond with fatty acid of C_{12} carbon chain length in it. The lipase was induced due to its long chain fatty acid part of the synthetic ester (Tween 80), whereas esterase was induced when microorganisms needed to hydrolyse the ester bond present in Tween 80. Though, Tween 80 induced p-NPP hydrolase activity (lipase) of two isolates, olive oil or cutin (Fig. 4c and 4d) did not show any induction of either of p-NPBase (esterase

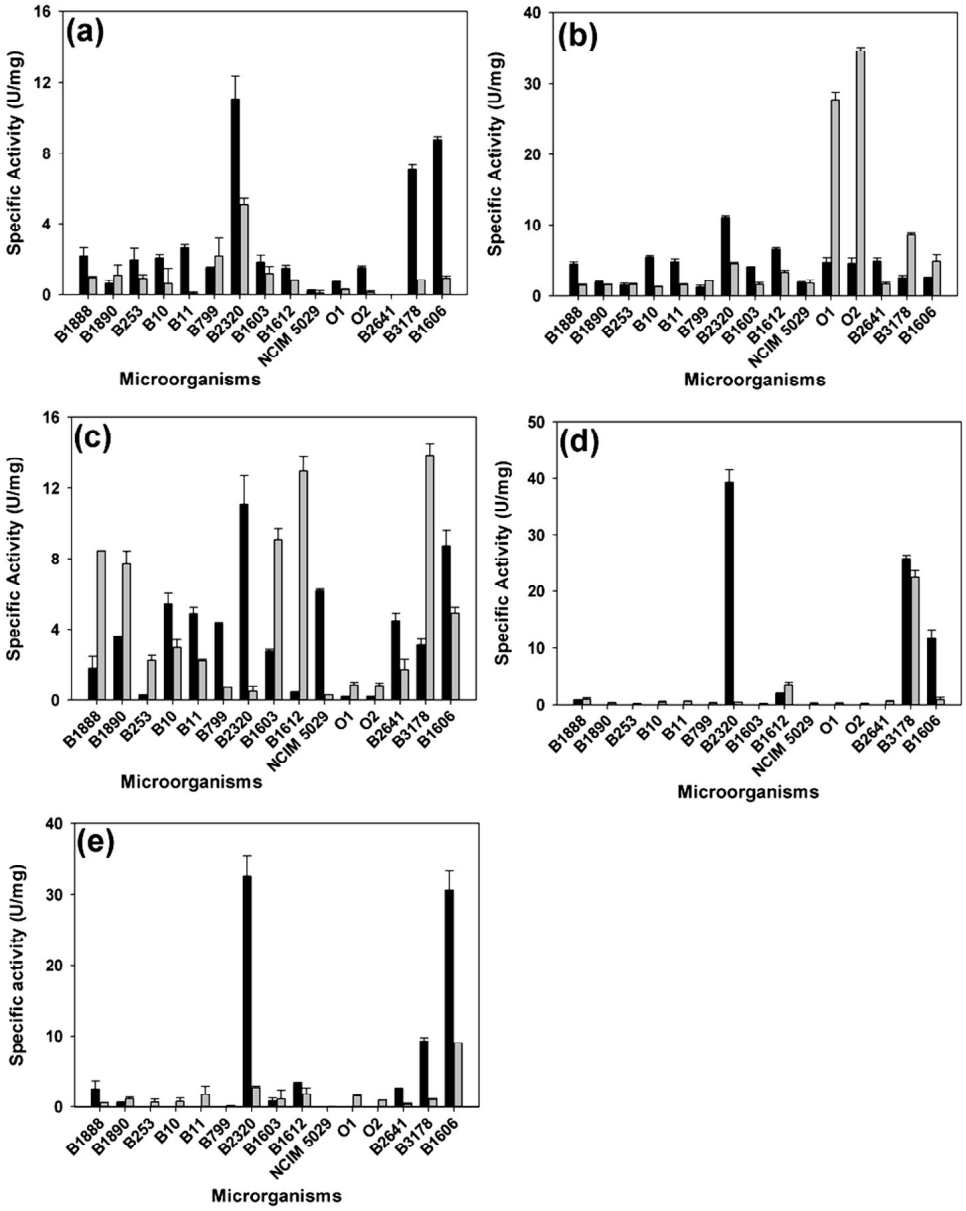

Fig. 4. Effect of different carbon sources on pNPBase (black bar) and p-NPPase (grey bar) activity of different microorganisms. (a) Glucose (b) glucose + Tween 80 (c) glucose + olive oil (d) cutin (e) glucose + cutin

or/ and cutinase) or p-NPPase (lipase) activity of these two isolates. This is due to the fact that Tween 80 is a synthetic ester of oleic acid, whereas olive oil is the mixture of different fatty acids with high content of oleic acid, which could not be utilized by these isolates. Also, cutin is the polymer of long chain hydroxy and epoxy fatty acids. Hence, cutin or olive oil failed to induce the enzymes from the isolates. Interestingly, *P. cepacia* NRRL B 2320, *P. fluorescens* NRRL B 3178 and *P. geniculata* NRRL B 1606 have shown p-NPBase (cutinase) activity in the medium containing cutin as an inducer (Fig. 4e). Among these, *P. cepacia* NRRL B 2320 was observed to show highest cutinase (p-NPBase) activity of 40 U/mg and 32 U/mg when grown on medium containing cutin and cutin with

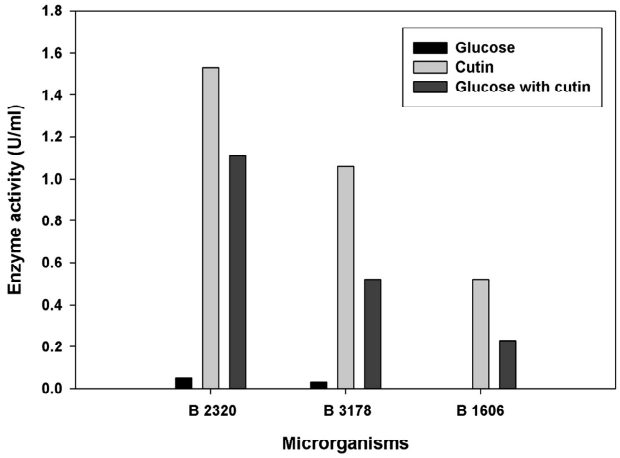

Fig. 5. Activity with P-NMSH of *P. cepacia* NRRL B2320, *P. geniculata* NRRL B1606 and *P. fluorescens* NRRL B3178 in glucose, cutin and glucose with cutin containing media

glucose, respectively (Fig. 4d and 4e). *P. fluorescens* NRRL B 3178 and *P. geniculata* NRRL B 1606 have shown cutinase (p-NPBase) activity of 25.9 U/mg and 11.68 U/mg, respectively in presence of cutin. However, cutin was unable to induced p-NPPase activity in *P. geniculata* NRRL B 1606 and *P. cepacia* NRRL B 2320, but enhanced p-NPPase activity from *P. fluorescens* NRRL B 3178. This was due to the presence of some long chain fatty acids along with hydroxy or dihydroxy fatty acids in the cutin, which induced some other lipases along with cutinase. Glucose has shown diverse effect on the cutinase production in the medium containing glucose and cutin for these three microorganisms. In presence of glucose and cutin, the p-NPBase activity (cutinase) of *P. cepacia* NRRL B 2320 and *P. fluorescens* NRRL B 3178 was repressed (Fig. 4e), whereas induction of the same was observed in *P. geniculata* NRRL B 1606. This was due to the presence or absence of catabolic repressor or inducer in different microorganisms. The lack of catabolic repression was also reported in the *P. putida*[15]. The cutinase activity of these three microorganisms was further confirmed by cutinase specific substrate, p-NMSH. Among these three microorganisms, *P. cepacia* NRRL B 2320 was found to be the best producer of cutinase (Fig. 5). *P. cepacia* NRRL B 2320 have shown p-NPBase, p-NPPase and p-NMSHase (cutinase) activity when grown on medium containing cutin. Degani *et al*.[19] showed that p-NMSH was a highly specific substrate for bacterial cutinase. Purdy and Kolattukudy[6] concluded that the non-specific esterase hydrolyses only soluble substrates, whereas cutinases prefer insoluble substrates. As p-NMSH is indeed insoluble and this could be one of the reasons for the lack of reactivity with nonspecific esterases and specificity towards cutinase. Lipases were also not able to hydrolyse p-NMSH, because lipases require interfacial activation for its activity whereas cutinases can hydrolyse p-NMSH without interfacial activation.

Effect of carbon sources/inducers on the production of cutinase

The effect of different inducers and carbon sources on the production of cutinase is presented in Table. 1. It was observed that the oils *viz*., mustard, sunflower and soybean induced p-NPBase activity much higher than p-NMSH hydrolase activity. But cutin monomer, 16-hydroxyhexadecanoic acid was unable to induce esterase or cutinase production from *P. cepacia*. In presence of 16-hydroxyhexadecanoic acid, *P. cepacia* showed only 9.74 U/mg and $5.8x10^{-2}$ U/mg activity towards p-NPB and p-NMSH, respectively. In our present study, 16-hydroxyhexadecanoic acid, monomer of cutin was also unable to induce cutinase production. This may be due to the polymeric structure of cutin, which is absent in 16-hydroxyhexadecanoic acid. In case of other carbon sources, it was found that the production of cutinase is very less in the medium without cutin. It was also observed that the production of cutinase was much lower level in the medium

containing glucose or other carbohydrate as the sole source of carbon than cutin in the medium. In medium containing only cutin as carbon source the activity of *P. cepacia* towards p-NMSH was found to be $41.2x10^{-2}$ U/mg, whereas in medium containing other carbon sources (*viz*., glucose, lactose, fructose, maltose, sucrose, xylose, galactose, starch) the activity towards p-NMSH was < $7x10^{-2}$ U/mg. Hence, the production of cuinase is favored in the presence of cutin as essential carbon source. Though cutin along with other carbon sources found to induce the p-NPBase activity in some cases, but unable to induce enzyme catalyzing hydrolysis of p-NMSH further. This is due to the presence of carbon sources other than cutin would enhance some esterases but fail to induce the cutinase production any more.

CONCLUSION

In our present study, various microorganisms have shown diverse affinity towards different substrates (p-NPB and p-NPP). The initial screening was done with Tween 80 and olive oil to identify the efficient lipase and esterase producers, which were further screened for the best cutinase producer using tomato cutin as a source of carbon and inducer. *Pseudomonas cepacia* NRRL B2320 was found to be the best cutinase producer. Highest activity was found to be 40 U/mg and 41.2 $x10^{-2}$ U/mg towards p-NPB and p-NMSH, respectively, when grown on the medium containing cutin. The other carbon sources (except cutin) were unable to enhance the cutinase production further. We have also prepared and characterized tomato cutin and p-NMSH.

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