Molecular basis of Tributyl phosphate biodegradation in *Sphingomonas*

By

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Shyam Sunder

List of Publications arising from the thesis

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Conferences and Symposia

1) Molecular basis of TBP biodegradation in *Sphingobium* sp. strain RSMS, Shyam Sunder Rangu, Bhakti Basu, Bindu Muralidharan, S. C. Tripathi & Shree Kumar Apte, Life Science symposium on Advances in Microbiology of Food, Agriculture, Health and Environment, LSS-2015, Bhabha Atomic Research Centre, Mumbai, P64, Pg 160.

2) A *Sphingomonas* capable of degrading Tributyl phosphate; Shyam Sunder R, A. S. Rao and S. K. Apte: Abstracts of 49th annual conference of "Association of Microbiologists of India",Page.P-232,Association of Microbiologists of India, 2008(AMI-2008).

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Dedicated to my Family

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SYNOPSIS

Nuclear fuel reprocessing is a very important link between the three stages of India's nuclear power program. The products {Uranium (U), Plutonium (Pu), and Thorium (Th)} generated in one stage serve as the feed material for the next stage of the program. The organic solvent, tributyl phosphate (TBP) is used for the extraction of U, Pu and Th from spent fuel obtained in each of the aforesaid 3 stages, using PUREX (plutonium uranium reduction extraction) or THOREX (Thorium extraction process) processes. During repeated usage in the PUREX or THOREX process, TBP undergoes chemical/radiolytic degradation (1). This makes it inefficient for further extraction process. At this stage, TBP needs to be treated and discarded as waste. Chemical degradation or incineration is generally used for the treatment of such TBP waste (2). Alkali treatment of TBP at high temperatures results in the formation of dibutyl phosphate (DBP) and butanol (3, 4), while incineration of TBP leads to formation of P_2O_5, a corrosive product (5). Therefore management of the TBP byproducts formed by both the above processes is rather difficult. Since, the chemical or physical processes for TBP treatment are not satisfactory, bioremediation has always been considered an attractive alternative for the TBP and other organic waste management. Microorganisms which could degrade and use TBP for their growth would be very useful, so that no harmful organic by- products remain as happens in alkali hydrolysis process.

A TBP degrading, promising bacterial strain was isolated from Radioactive Solid waste Management Site (RSMS) in Bhabha Atomic Research Centre (BARC) earlier. The strain tolerated high concentrations of TBP, degraded TBP and utilized it for its own growth. The strain was investigated in detail in respect of the following objectives.

1) Identification of RSMS strain using biochemical and molecular approaches.

2) Characterization of RSMS strain for its ability to tolerate, degrade and utilize TBP.

3) Evaluation of the factors regulating TBP utilization /degradation, if any.

4) Elucidation of biochemical pathway of TBP degradation in RSMS strain, and

5) Exploring the possibility to couple TBP degradation with uranium precipitation.

The work carried out is presented in the thesis as following chapters;

Chapter 1: General Introduction. This chapter presents brief survey of the background literature on microbial degradation of TBP. Bacterial strains have been reported in the past to degrade TBP, either as mixed cultures or as single isolates (6-14). For example, mixed cultures containing *Pseudomonas* were shown to degrade 0.532 g/L of TBP (2 mM^e)* in ~3 d, and utilize TBP both as carbon and phosphorous source (13). But, the corresponding *Pseudomonas* strain irreversibly lost the ability to degrade or utilize TBP after eight serial subcultures (13). Strains of *Serratia odorifera* or *Rhodopseudomonas palustris* reportedly degraded 0.6 mM TBP within 8 h or 1.6 mM^e of TBP in 3 weeks. However, these strains could not mineralize or utilize TBP as carbon or phosphorous source for growth (7, 8). Another study described mixed cultures of 11 *Acinetobacter* strains which degraded < 1 mM TBP in ~4 weeks from mixtures of organic waste. The study also developed a bioreactor based technology using the same principle, which was filed as a patent in United States of America (USA) (15). Recently, fifteen bacterial strains were reported for their ability to degrade and utilize TBP as the sole source of carbon and phosphorous

* Since TBP is only sparingly soluble in water (<1.5 mM), the concentration of TBP is described as "TBP equivalent" and specified as "mM^e", throughout the synopsis and the thesis

(6), but showed rather poor rates of TBP degradation (1 – 3.05 mM^e TBP degradation in 4 days). Another recent study isolated a *Klebsiella pneumonia* strain, which could degrade and use TBP in co-metabolism (16).

Most of the aforesaid strains had major drawbacks, which are detailed in the chapter and include the following: (a) several strains did not use TBP as carbon source or phosphorous source, (b) most strains degraded/utilized TBP with very poor efficiency, (c) the concentrations of TBP degraded were very low (<2 mM), (d) some of the strains simply sequestered TBP, but could not degrade or mineralize it, (e) the strains could tolerate TBP only at low concentrations (<5 mM^e).

The present work isolated, identified and characterized in detail, a stable, tolerant and efficient TBP degrading strain, superior to previously reported strains in many respects. Specific objectives of the work are delineated in this chapter. The methods used for the study are detailed in chapter 2, and the results obtained are described in chapter 3-5 and as an annexure.

Chapter 2: Materials and methods. The TBP degrading bacterial strain was isolated from the TBP waste storage tank located at the RSMS, BARC, Mumbai. This chapter describes the detailed protocols used for isolation, enrichment, purification and growth of the strain. The assortment of biochemical tests and standard 16s *rRNA* gene sequencing methods used for identification of the RSMS strain are detailed. The optimized growth conditions and methods used for the estimation of TBP tolerance and growth in terms of increase in turbidity (Absorbance at 600nm) or by scoring colony forming units are specified. The ability of the strain to utilize TBP and/or dibutyl phosphate (DBP) was assessed by inoculating the strain in to mineral medium (MM) (13) modified by supplementing with 100 mM 3-(N-morpholino) propane sulfonic acid (MOPS), and referred as modified mineral medium or MMM. The MMM was supplemented with TBP/DBP as the sole source of carbon and phosphorous. TBP is only

sparingly soluble (<1.5 mM). The ability of the RSMS strain to degrade or tolerate TBP was assessed in immiscible suspensions containing high amounts of TBP, which would give 30-100 mM TBP concentrations when dissolved. TBP concentration in such suspensions are referred to as TBP concentration equivalent denoted as superscript i.e., "mM^e". The TBP/DBP degradation by the RSMS strain was routinely assessed spectrophotometrically as inorganic phosphate released in to the medium (17). The TBP degradation products were extracted by developing suitable protocols and analyzed using gas chromatography. The TBP degradation was also visualized on MM/MMM/LB agar plates, as the zone of TBP degradation around the colony. The tolerance of the strain was assessed by growing the RSMS strain in MMM supplemented with various concentration of TBP/DBP/butanol. Regulation of TBP/DBP degradation by glucose was studied by inoculating the strain in to MMM containing 10 mM^e TBP or 10 mM DBP, supplemented with different concentrations of glucose. TBP non-degrading mutants were isolated using Tn5 mutagenesis, by visualizing zone of TBP degradation around the colony. The colonies which did not show the zone of clearance around them were considered as putative TBP no-degrading mutants, and one of the mutants (mutant SS22) was studied further. Proteomic differences between mutant SS22 and RSMS were identified using two dimensional electrophoretic (2D) resolution of proteins followed by peptide mass finger printing using Matrix Assisted Laser Desorption Ionization (MALDI) mass spectroscopy. Phospho-di and monoesterase assays were carried out by incubating whole cell protein extracts of RSMS/mutant SS22 with Bis-para nitro phenol phosphate (Bis-pNPP) and para-nitrophenol phosphate (pNPP), respectively, and released para-nitrophenol (pNP) was estimated spectrophotometrically. DBP degradation assay was carried out with whole cell protein extracts and released inorganic phosphate was estimated spectrophotometrically.

Similarity searches DNA sequence alignments were carried out using the BLAST algorithm and multalign software (18, 19).

Chapter 3: Characterization of TBP degrading RSMS strain. A single monoxenic bacterial strain capable of degrading and utilizing TBP as the sole source of carbon and phosphorous was isolated and tentatively named as RSMS strain. The strain degraded 10 mM^e TBP present in Luria Bertani agar medium (LBA) or MMM agar (MMMA) and showed a clear transparent TBP degradation zone around the colony. Such phenotype has not been reported earlier for any of TBP degrading strains. Fatty acid methyl ester (FAME) analysis and 16s rRNA gene sequence analysis revealed that the strain belonged to the genus *Sphingobium*.

Bacterial strains which could slowly degrade a maximum of 2 mM^e TBP (6-8, 13, 16, 20) have been reported earlier. The RSMS strain could degrade TBP from non-homogeneous liquid suspensions containing 30 mM^e TBP in 3 days, and utilize it for its own growth as the sole source of carbon and phosphorous. Growth of the strain increased with increasing amounts of TBP up to 30 mM^e TBP. The RSMS strain showed > 25 times increase in CFUs, when incubated with MMM containing 30 mM^e TBP for 2 days. The efficiency of TBP utilization by RSMS strain was found to be much superior to the previously reported strains (6-11, 13, 15, 16, 20-22). The strain also efficiently degraded DBP, and utilized it as the sole source of carbon and phosphorous for growth. The RSMS strain could also utilize butanol as the sole source of carbon. TBP at 10 to 400 μM concentration is known to inhibit cell division in most bacteria (23). The previously reported TBP degrading strain could tolerate up to 5 mM^e TBP (6). The RSMS strain was found to tolerate ~100 mM TBP when supplemented in solid MMM/LB agar media. Such high tolerance is needed when dealing with the actual waste where the concentrations of TBP are very high (4). The strain also tolerated up to 75 mM of DBP and 50 mM of butanol.

The strain also degraded 20 mM^e Tri-iso amyl alcohol phosphate (TAP), in 4d, and utilized it as a sole source of carbon and phosphate. TAP is a higher homologue of TBP and a likely future replacement of TBP.

Chapter 4: Investigations of the molecular basis of TBP biodegradation by RSMS strain. Inorganic phosphate, which is a product of TBP degradation, was found to affect TBP degradation at higher concentrations. Inorganic phosphate at 30 mM and above inhibited TBP degradation and the growth of the strain in MMM supplemented with TBP and KH_2PO_4. The growth inhibition was a consequence of the unavailability of carbon source due to the inhibition of TBP degradation by inorganic phosphate. Glucose at low concentration (0.2-0.55%) supported better TBP dependent growth of RSMS strain in 10 mM^e TBP suspension. However, decreased TBP degradation and corresponding growth inhibition (due to unavailability of phosphorous source) were observed at high concentrations of glucose, suggesting the inhibition of TBP degradation due to glucose. No degradation of TBP, and correspondingly no growth, were observed in cultures supplemented with 5% or 10% glucose. Similarly, DBP degradation was found to be completely inhibited by 10% glucose. Thus, glucose appeared to exert a strong catabolite repression of TBP degradation at higher concentration. Protein profile studies indicated that about 8 proteins were found to be suppressed in the cultures supplemented with 10% glucose and 10 mM^e TBP, in comparison to the cultures grown in MMM containing 10 mM^e TBP. However, these proteins were found to be related to carbohydrate metabolism, and none seemed likely to be related to TBP degradation. Several TBP non degrading spontaneous mutants were isolated during repeated subcultures of RSMS strain. One of the mutants (termed SS22) was characterized in detail. Mutant SS22 could not degrade TBP (as ascertained by the inability to form a zone of clearance around the colony on solid media supplemented with TBP)

or DBP. The mutant SS22 also failed to use TBP or DBP as a sole source of carbon or phosphorous. Both wild type and mutant SS22 showed growth in MMM supplemented with 20 mM butanol and inorganic phosphate (5 mM KH_2PO_4). The results suggest that whole pathway of TBP degradation was affected due to mutation. The protein profiles of wild type and mutant SS22 were found to be identical except for three proteins (1 of ~45 kDa and 2 of ~100 kDa each), which were absent in the mutant as compared to wild type. Both the ~100 kDa proteins showed similarity with the phosphohydrolase of *Alivibrio fischeri,* and were absent in the mutant. Phosphohydrolase is a phosphodiesterase (PDE), known to degrade several phosphodiester compounds such as c-AMP, dimethyl phosphate (DMP), diethyl phosphate (DEP) Bis-pNPP, dementon (24-29), though so far it has not been reported to degrade TBP/DBP. Most of the phosphohydrolases also show non-specific hydrolytic activity for phosphomonoesters (24, 25) or phospho triesters (24). DBP is similar to and a higher homologue to the DMP and DEP. The ~45 kDa protein showed similarity with Exopolyphosphatase-like protein (EPP) of *Marinomonas mediterranea* MMB-1. The enzyme degrades polyphosphates with phosphomonoesterase activity. Though the link between 45 and 100 kDa proteins of RSMS strain and its TBP/DBP degradation capabilities is not fully established, the data suggests that phosphomono-di or tri esterases may be involved in TBP/DBP degradation by RSMS strain.

Chapter 5: Elucidation of biochemical pathway of TBP degradation in RSMS strain. New protocols for extraction and gas chromatographic analysis of the probable intermediates (DBP) and products (butanol) of the TBP degradation were developed and implemented. Gas chromatography in combination with spectrophotometric methods revealed that the RSMS strain completely degraded 30 mM^e TBP in 3 d when inoculated at a cell density of 3 $OD_{600nm.}$ Both DBP, and butanol and phosphate were detected as intermediates and products of TBP

degradation, respectively. After 3 d, neither TBP or DBP, nor butanol were detected in the culture medium suggesting their complete degradation/utilization as carbon source by the cultured cells. The study also showed that the strain completely utilized the butanol (~90 mM) released from the degradation of 30 mM[e] TBP. Such efficient utilization can prevent the accumulation of toxic butanol formed due to TBP degradation in the medium. Independent measurements showed that the phosphate released increased linearly between 0 to 72 h before reaching a peak value (~27 mM) in 3 days. The RSMS strain also completely degraded 20 mM DBP in 2 d with 50% degradation occurring in the first 24 h. Butanol and inorganic phosphate were detected as the end products of DBP degradation also. Based on the gas chromatography and spectrophotometric analyses, a biochemical pathway for the TBP degradation has been proposed. The pathway involves the release of butanol and DBP in the first step of TBP degradation. DBP could be subsequently degraded in two possible ways, (a) it may be degraded in a single step to release two molecules of butanol along with one molecule of inorganic phosphate as the final products, or (b) the DBP could be degraded in two steps, first step resulting in the release of monobutyl phosphate (MBP) and butanol from DBP, followed by the degradation of MBP to butanol and inorganic phosphate in the second step. The involvement of MBP as an intermediate could not be established or ruled out, because of the unavailability of MBP in pure form. The butanol released in each step supports the growth by acting as the sole source of carbon and inorganic phosphate released in the last step acts as the sole source of phosphorus. The release of butanol in each step of the pathway strongly suggests the involvement of phosphoesterases in the TBP/DBP degradation. This is further corroborated by proteomic data indicating absence of phosphoesterases in the mutant SS22. The phosphoesterases of the RSMS strain were partially characterized therefore. Whole cell protein

extracts of the wild type strain showed ~13 μmoles of pNP released/mg protein/h, from Bis-pNPP (phospho diesterase/PDE activity), which was nearly absent in mutant SS22 strain. The RSMS strain also showed >10 Phosphomonoesterase (PME) activity, in comparison to the mutant SS22. Whole cell protein extracts of RSMS strain also showed ~5 times more DBP degradation (PDE activity) in comparison to mutant SS22. Caffeine (a known phosphodiesterase inhibitor) completely inhibited DBP degradation at 1 mM concentration. Whole cell protein extracts of wild type displayed >2 fold higher MBP degradation than the mutant. Decreased PME activity and reduced MBP degradation by the mutant do suggest likely involvement of MBP as an intermediate of TBP degradation.

Annexure: TBP degradation coupled to uranium precipitation. Since uranium can be easily precipitated as phosphate, studies were carried out to couple the excess inorganic phosphate released in the TBP/DBP degradation by the RSMS strain for the precipitation of uranium. The RSMS strain precipitated >90% of uranyl carbonate as uranyl phosphate in 17 h, when inoculated at 1 OD_{600nm} cells in to MMM supplemented with 1 mM uranyl carbonate and 5 mM^e TBP. Though precipitation of uranium could be achieved using free cells of RSMS, recovery of precipitated uranium was rather difficult. Also, the free cells could not tolerate the combined toxic effects of more than 5 mM^e TBP and 1 mM uranyl carbonate. To overcome this problem, *Sphingobium* sp. RSMS cells were immobilized in calcium alginate beads. It was observed that at any given concentration of TBP, the rate of TBP degradation with immobilized cells was less than half in comparison to the free cells. But the immobilized RSMS strain could tolerate 1.5 mM of uranyl carbonate along with 30 mM^e TBP. Uranyl phosphate formed under such conditions exhibited green fluorescence upon UV illumination. The immobilized RSMS cells incubated with 30 mM^e TBP contained ≥95% of 1.5 mM uranium in the beads in 24 h,

facilitating easy removal and recovery of the metal.

Summary. A *Sphingobium* sp. strain RSMS was found to completely degrade up to 30 mM^e TBP from immiscible suspensions, and 50 mM DBP from solutions in 3 and 4 days, respectively. The strain also efficiently utilized TBP and DBP as the sole source of carbon and phosphorous for its growth, and tolerated ~100 mM^e TBP, 75 mM DBP and 50 mM butanol. The RSMS strain also degraded Tri-iso amyl alcohol phosphate (TAP), which is a higher homologue of TBP. High concentrations of inorganic phosphate (final product of TBP degradation) and glucose were found to negatively regulate TBP and DBP degradation, and TBP/DBP dependent growth of the RSMS strain. Gas chromatography and spectrophotometric analyses revealed the involvement of DBP as an intermediate, and butanol and inorganic phosphate as the end products of TBP degradation. Based on these results a biochemical pathway for TBP degradation was proposed. The involvement of monobutyl phosphate (MBP) as an intermediate downstream of DBP was strongly suggested by several results, but could not be ascertained due to unavailability of pure MBP. The RSMS strain exhibited a zone of TBP degradation around the colony, on solid media supplemented with TBP. This was used as a screen for the isolation of >50 TBP non- degrading mutants. One of the mutants (named mutant SS22) showed loss of two proteins from its proteome. These proteins were similar to Phosphohydrolase and Exopolyphosphatase like proteins from other bacteria, and are known to exhibit Phosphodiesterase and Phosphomonoesterase activities. The whole cell protein extracts of mutant SS22 also showed significantly reduced degradation activity towards phosphodiesters and phosphomonoesters, in comparison to the RSMS strain. The whole cell protein extracts of mutant similarly showed ~5 times lesser DBP (phosphodiester) degradation in comparison to the RSMS strain. Gas chromatography analysis revealed that the mutant SS22 cells did not generate any of the

intermediates or products of TBP/ DBP degradation, suggesting that the whole pathway for the TBP degradation was blocked in the mutant SS22. The phosphoesterase like proteins, absent in the mutant appear to be potential candidates for further molecular investigation of TBP degradation. The excess inorganic phosphate released in the TBP degradation could be coupled to uranium precipitation. The RSMS strain, immobilized in alginate beads, could precipitate ≥ 95% of 1.5 mM uranium, in 24 h using phosphate released from degradation of 5 mM[e] TBP.

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Publications.

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2	Dr. S.K. Apte	Guide/Convener		
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4	Dr. J.S. Melo	Member		
5	Dr. Anand Ballal	Member		

List of abbreviations

Bis-pNPP	Bis-para-nitrophenol phosphate
CFU	Colony forming units
DBP	Dibutyl phosphate
MBP	Monobutyl phosphate
mM	Milli molar
MM	Mineral medium
MMA	Mineral medium agar
mM^e	Milli molar equivalent
MMM	Modified mineral medium
MMMA	Modified mineral medium agar
OD	Optical density
PDE	Phosphodiesterase
Pi	Inorganic phosphate
PME	Phosphomonoesterase
pNP	para-nitrophenol
pNPP	para-nitrophenol phosphate
PTE	Phosphotriesterase
RSMS	Radioactive Solid waste Management Site
TAP	Tri-isoamylalcohol phosphate
TBP	Tributyl phosphate

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Chapter I
General Introduction

Energy is absolutely essential for the development of human civilization. There is a constant effort to use different fuel sources for the production of energy. Since, non-renewable energy resources are diminishing and the technological progress in the renewable energy sectors has been limited, the nuclear energy appears to be the most promising immediate solution to the impending energy crisis. Nuclear fission is known to be very energy efficient besides adding less or no pollution to the environment. For example, 1 kg of coal can power a 100-W bulb for ~4 days, and similar amount of natural gas can power it for 6 days, whereas similar amounts of uranium can do the same for almost 140 years [1].

Nuclear industrial operations, while meeting the primary objective of generating power, generate different types of wastes. Depending on their origin in nuclear fuel cycle, they exhibit wide variation in their radioactivity levels and radiochemical composition [1-5]. The main stages of the nuclear fuel cycle which generate radioactive waste include mining and milling and extraction of uranium ore, fuel fabrication, reactor operation and spent fuel reprocessing [1-5].

Nuclear spent fuel reprocessing is most important for India's three stage nuclear programme. The products (uranium, plutonium present in the spent fuel) generated in each of these stages serve as the feeding material for the next stage of the programme. Tributyl phosphate is used for the extraction of uranium and plutonium from such spent fuel obtained in each of these stages of the programme. Repeated large-scale usage of TBP results in generation of large volumes of TBP waste. This study focused on an eco-friendly approach for biodegradation of TBP waste, using a bacterial strain isolated from nuclear waste storage site.

1.1. Physical and chemical properties of tributyl phosphate.

Tributyl phosphate (TBP) is a organophosphorous compound, which is an ester of phosphoric acid and butanol. The physico chemical properties of TBP and its constituents are listed in Tables 1.1, 1.2 and 1.3. Structurally TBP is a phosphotriester, wherein three butyl groups are attached to a phosphate moiety through three phosphoester bonds (Fig. 1.1). Worldwide, about 3000-5000 tons of TBP is manufactured every year, using phosphorus oxychloride and n-butanol as the reactants [2,4,6,7]. TBP is colourless, viscous, non-flammable, non-explosive, odourless liquid and can very well mix with air. It is readily soluble in organic solvents but only sparingly soluble in water (400 mg/L) [7]. TBP hydrolyses rapidly in acidic, neutral, or alkaline solution, forming phosphoric acid and butanol.

Table 1.1. Tributyl phosphate: physical and chemical properties

Molecular formula	$(CH_3CH_2CH_2CH_2O)_3PO$
Molar mass	266.31 g mol^{-1}
Density	0.9727 g/mL
Melting point	−80 °C
Boiling point	289 °C
Flash point	146.1 °C
Solubility in water	400 mg/L (~1.50 mM), at 20°C
Vapour pressure	9 Pa at 25°C
Reaction for manufacture of TBP	$POCl_3 + 3\ C_4H_9OH \rightarrow PO(OC_4H_9)_3 + 3\ HCl$
Synonyms	tri-*n*-butyl phosphate; phosphoric acid, tri-*n*-butyl ester

Source: Adapted from Nakamura, 1991 and Schulz & Navartil, 1984.

Table 1.2. Dibutyl phosphate: physical and chemical properties

Molecular formula	C8H19O4P
Molar mass	210.21 g mol^{-1}
Density	1.06 g/mL
Melting point	-13 °C
Boiling point	250 °C
Flash point	188 °C
Solubility in water	17 g/L at 25 °C
Vapour pressure	< 7.4 x 10^{-3} Pa at 100 °C
PKa	2.32 at 25 °C

Source: UNEP Publications (http://www.inchem.org/documents/sids/sids/107-66-4.pdf)

Table 1.3. Physical and chemical properties of n-butanol

Molecular formula	$C_4H_{10}O$
Molar mass	74.12 g mol^{-1}
Density	0.81 g cm^{-3}
Melting point	−89.8 °C
Boiling point	117.7 °C
Flash point	35 °C (95 °F; 308 K)
Solubility in water	73 g L^{-1} at 25°C

Source: http://pubchem.ncbi.nlm.nih.gov

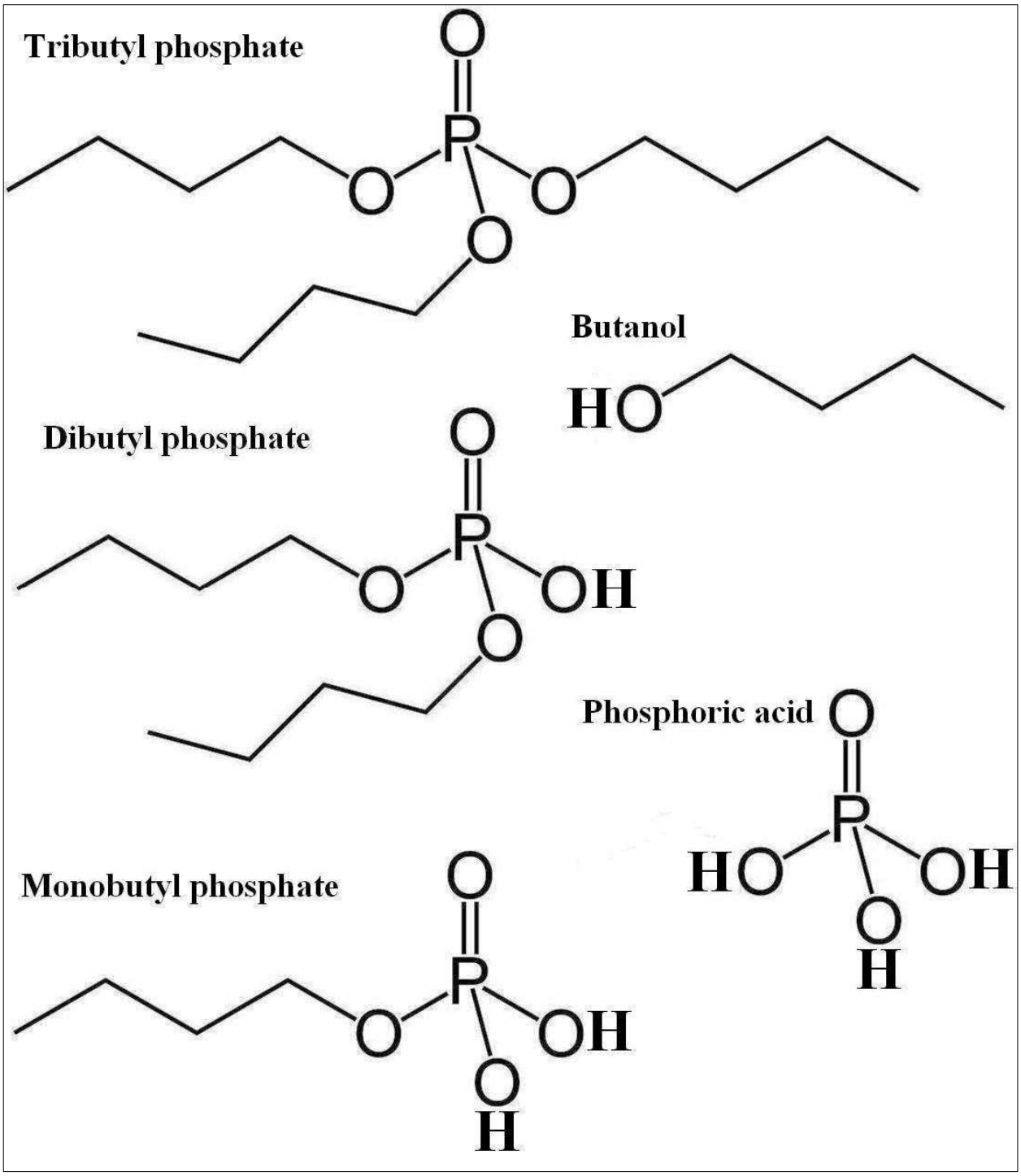

Fig. 1.1. Structure of tributyl phosphate and likely products of its degradation

1.2. Uses of tributyl phosphate.

TBP is mainly used in nuclear industry for the recovery of uranium and plutonium from spent fuel [2,4,6-10]. TBP is also used as a component of aircraft hydraulic fluid. Minor uses of TBP include as an antifoaming agent, as a plasticizer in the manufacture of cellulose esters such as nitrocellulose and cellulose acetate, and in the manufacturing of herbicides [9]. TBP is also used as a solvent in synthetic resins, gums, adhesives etc. [9].

1.3. Role of TBP in India's three stage nuclear program.

Nuclear fuel reprocessing is very important in the closed fuel cycle and also an important link between the three stages of Indian nuclear program. As the name implies the program consist of three stages (Fig. 1.2). The first stage uses naturally occurring uranium with a composition of U^{235} (0.7%) and U^{238} (>99%), in which U^{235} acts as a fissile material while the U^{238} is converted in to Pu^{239} in the pressurized heavy water reactors (PHWR) (Fig.1.2). The spent fuel from the first stage contains trace amounts of U^{235} (depleted uranium) and majority of Pu^{239} (fissile material). In the second stage, fast breeder reactors (FBR) use mixed oxide (MOX) fuel made from the Pu^{239} (which is recovered by reprocessing spent fuel from the first stage) and natural uranium. In FBR Pu^{239} undergoes fission to produce energy. FBR also uses Th^{232}, which on neutron capture is converted to U^{233}. The third stage of nuclear programme uses thermal breed reactors in which the U^{233} produced in the second stage serves as fuel (Fig. 1.2).

Tributyl phosphate is used for the extraction of uranium and plutonium from the spent fuel obtained in each stage of the programme. PUREX (plutonium uranium reduction extraction) process is used for the extraction of U^{235}, U^{238} and Pu^{239} from spent fuel rods. THOREX (Thorium extraction process) is used for the recovery of U^{233} alone or both U^{233} and thorium [11]. In both these processes TBP is used as solvent for the extraction [11].

1.4. Why TBP for the extraction of uranium and plutonium?

TBP has several properties which makes it a good solvent for the extraction of uranium and plutonium: (1) it resists thermal, radiation and chemical degradation, (2) it has physical properties which are useful for the extraction, (3) it is selective for uranium and plutonium over fission products, (4) show good phase separation in the extraction process, (5) it is amenable to stripping with dilute nitric acid, and (6) it is economical and easily available [12,13].

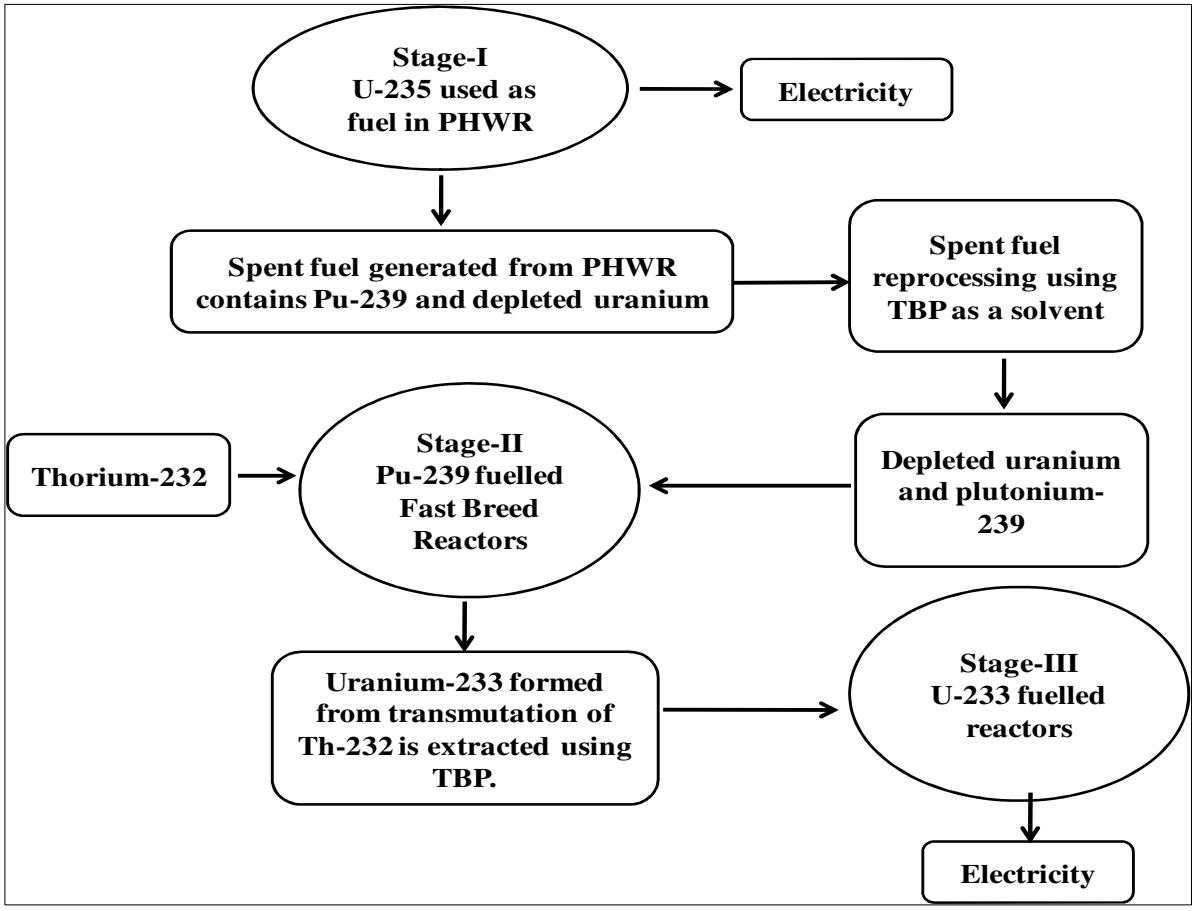

Fig. 1.2. Three stage nuclear program of India. Source: http://barc.gov.in/about/anushakti_sne.html

1.5. PUREX process and waste generation.

The spent irradiated fuel rods are removed from the reactor, and are cooled for long periods before reprocessing. PUREX (plutonium uranium reduction extraction) is a liquid–liquid

extraction method employed for the reprocessing. The irradiated fuel rods are first dissolved in ~ 10 M nitric acid, in which the uranium and plutonium are converted into their nitrate form, ($Pu(NO_3)_4$ and $UO_2(NO_3)_2$. Tributyl phosphate (30%) in dodecane, is added to the above acidic solution. Addition of TBP forms two phases, in which $UO_2(NO_3)_2.2TBP$ and $Pu(NO_3)_4.2$ TBP are extracted in to the organic phase, and the fission products remain in the aqueous phase.

$$Pu(NO_3)_{4(aq)} + 2TBP_{(org)} \longrightarrow Pu(NO_3)_4 .2TBP_{(org)}$$

$$UO_2(NO_3)_{2(aq)} + 2TBP_{(org)} \longrightarrow UO_2(NO_3)_2 .2TBP_{(org)}$$

The TBP phase is separated from the aqueous phase and is further processed for the separation of uranium and plutonium. Uranium and plutonium present in the TBP phase are separated by selective reduction of plutonium which directs it in to the aqueous phase leaving uranium in the organic TBP phase. Plutonium and uranium are further purified to respective oxides. The overall process is depicted in Fig.1.3.

During repeated usage in the PUREX process, TBP undergoes chemical/radiolytic degradation [1]. This makes it inefficient for the extraction process and so should be treated and discarded as waste. The radiochemical composition of spent fuel solvent is tabulated in Table 1.4.

Incineration, wet-oxidation, acid hydrolysis, cementation and alkaline hydrolysis are the methods generally employed for the treatment of TBP waste. Among these, alkaline hydrolysis is simple and used in India for the treatment of TBP waste [8,14]. Alkaline hydrolysis involves treating the TBP waste with concentrated sodium hydroxide solution at 110°C which results in the formation of sodium salt of dibutyl phosphate (DBP), and minor amounts of sodium salt of monobutyl phosphate (MBP), butanol and phosphoric acid [1,8,14].

Dodecane present in the spent solvent doesn't undergo alkali hydrolysis and appears as a clear phase, which is separated and subjected to incineration [1].

A plant based on indigenous technology is used to treat the spent solvent by the 'alkaline hydrolysis' process at the Effluent Treatment Plant (ETP), BARC, Trombay [1]. The process is depicted in Fig.1.4 [14].

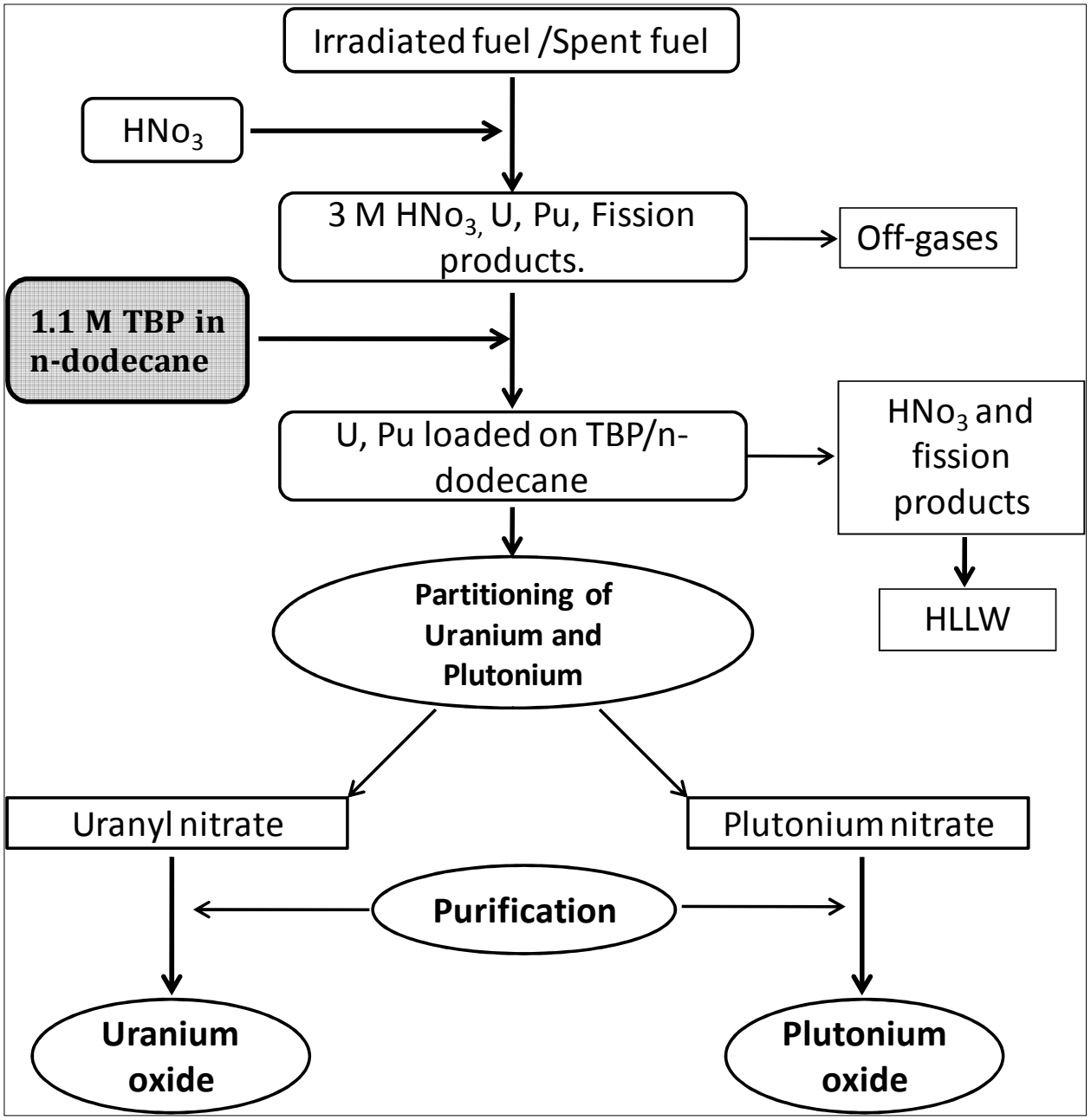

Fig. 1.3. Extraction of uranium and plutonium from spent fuel rods using PUREX process [Source: http://www.ieer.org/purex.gif]

Table 1.4. Radiochemical composition of spent fuel solvent

Radioactivity	Concentration(Bq/ml)
Gross alpha (Pu+Am; Pu/Am= 10)	740 - 1200
Gross beta	28,000 - 40,000
Major fission products	
Ru-106	27,000-37,000
Cs-137	30-90

Source: Manohar et al., 1999b

Disadvantages of alkaline hydrolysis are as follows:

1) Complete mineralization is not possible

2) Process leads to the formation of dibutyl phosphate, which cannot be discarded as such.

3) Management of the byproducts formed in the process is difficult.

4) Process produces large volumes of secondary waste, whose management is difficult

Since, chemical or physical processes for the treatment are not satisfactory, bioremediation has always been considered an attractive alternative for the organic waste management. The development of an eco-friendly process which could allow complete degradation of TBP is highly desirable. For example, it would be desirable to use bacteria which could use TBP as a carbon and phosphorous source for its growth, so that the process will not leave any toxic organic by-products, as happens in alkali hydrolysis process. And the products that could be generated by the process should be easily manageable.

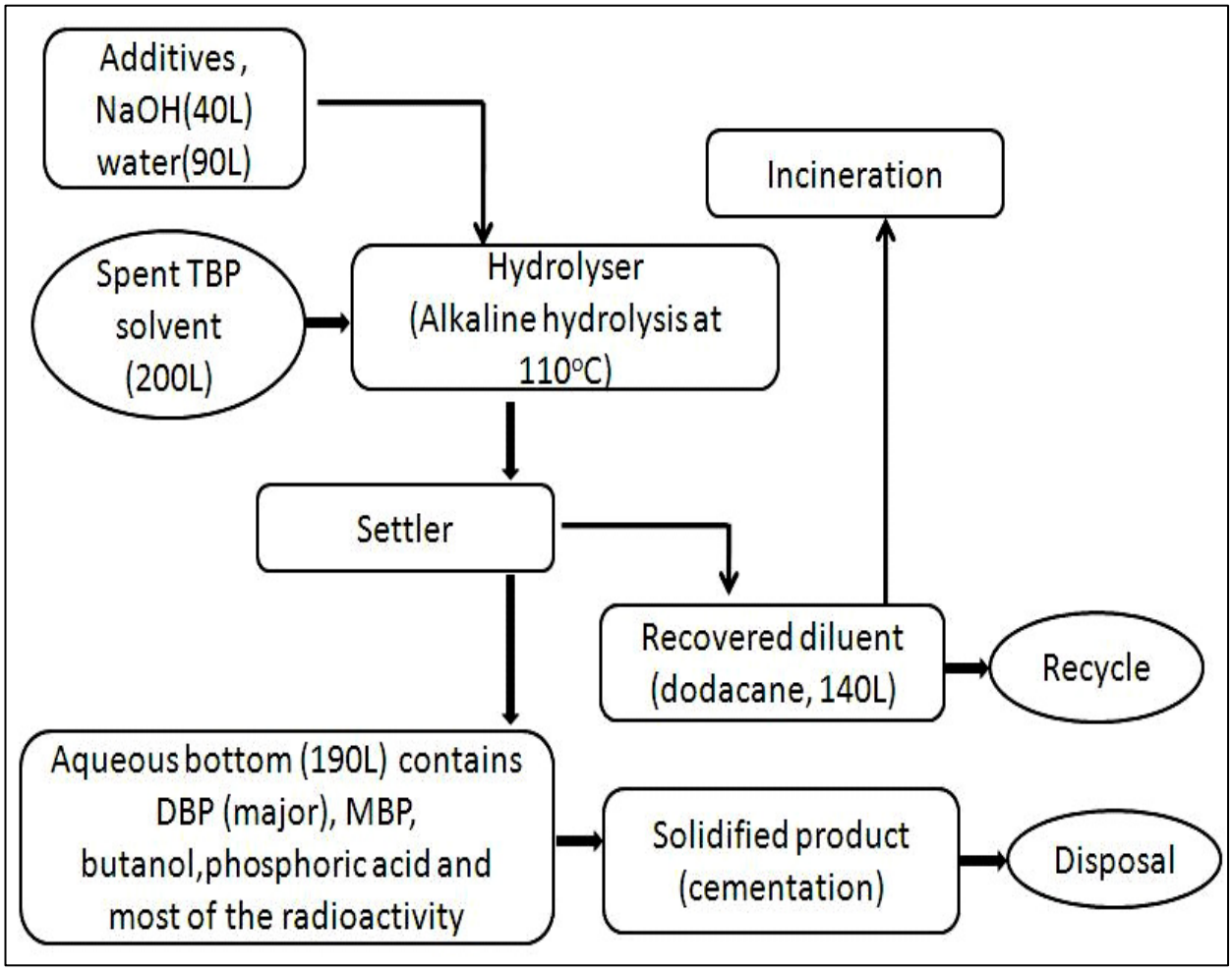

Fig. 1.4. Alkaline hydrolysis of spent TBP solvent (Source: http://www.iaea.org/inis/collection/NCLCollectionStore/Public/32/041/32041334.pdf)

1.6. Toxic effects of TBP.

Tributyl phosphate has irritative effects on the skin, eyes and respiratory tract. TBP was listed as one of the chemicals produced in large volumes by the European Union [15]. Varied applications of TBP result in its spreading into the environment by volatilization, leaching, and abrasion [15]. TBP was detected in samples of 17 domestic and occupational environments, at concentrations ranging from 3.8 pg/L to 0.12 ng/L [16]. TBP was also frequently detected in the aquatic environment [15]. For example, the concentration of TBP in German river waters was found to be 17–1,510 ng/L in the Rhine, Elbe, Main, Oder, Nidda, and Schwarzbach Rivers [17]. It was

suggested that the waste water treatment plants discharge TBP into the aquatic environment [18,19]. The presence of TBP in the environment may have a direct adverse impact on the aquatic organisms and poses a serious threat to human beings through the contamination of drinking water supplies [15].

TBP at 10 to 400 μM concentration inhibits cell division in most bacteria [9]. The LC50 of TBP for other organisms, such as , *Daphnia magna* is TBP 5.5 mg/L. Experiments were carried out in rat/mice using radioactive [^{14}C] labeled TBP and its absorption and metabolism was studied [20-22]. Independent of the route of administration, the highest concentration of TBP was found in muscle, skin and adipose tissues (1 week later), and the residual radioactivity in all tissues was less than 1%. After dermal exposure to TBP at doses of 10 mg/kg body weight for 6 h, rats absorbed 40% of the radioactivity, and the maximum levels in plasma were reached after 4 hours. About 24 % of the applied doses could be washed off the skin, and the rest of the radioactivity detected in the urine, faeces and in air exhaled during the 7 days, accounting for a total of 65% [20,21]. Intra peritoneal injection of TBP in Wistar rats, given at doses of 250 mg/kg body weight, yielded 11 oxidized and partially dealkylated metabolites in the urine in 24-hour (Fig. 1.5). TBP was metabolized almost completely, and unchanged tributyl phosphate accounts for less than 1 % of the ^{14}C-radioactivity excreted in urine and faeces. The main metabolite of tributyl phosphate was dibutyl phosphate (DBP) and other metabolites were, (1) dibutyl 3-hydroxybutyl phosphate, (2) butyl bis(3-hydroxybutyl) phosphate, (3) butyl dihydrogen phosphate,(4) butyl 2-hydroxybutyl hydrogen phosphate, (5) butyl 3-hydroxybutyl hydrogen phosphate, (6) butyl 4-butanoic acid hydrogen phosphate, (7), dibutyl 4-butanoic acid phosphate, (8) butyl 3-hydroxybutyl 4-butanoic acid phosphate, (9) butyl bis(4-butanoic acid) phosphate, and (10) 3-hydroxybutyl dihydrogen phosphate. Based on these metabolites a pathway was

described for TBP metabolism in mammals (Fig. 1.5). Tributyl phosphate was not found to have genotoxic effects in various test systems either *in vitro* or *in vivo* [22].

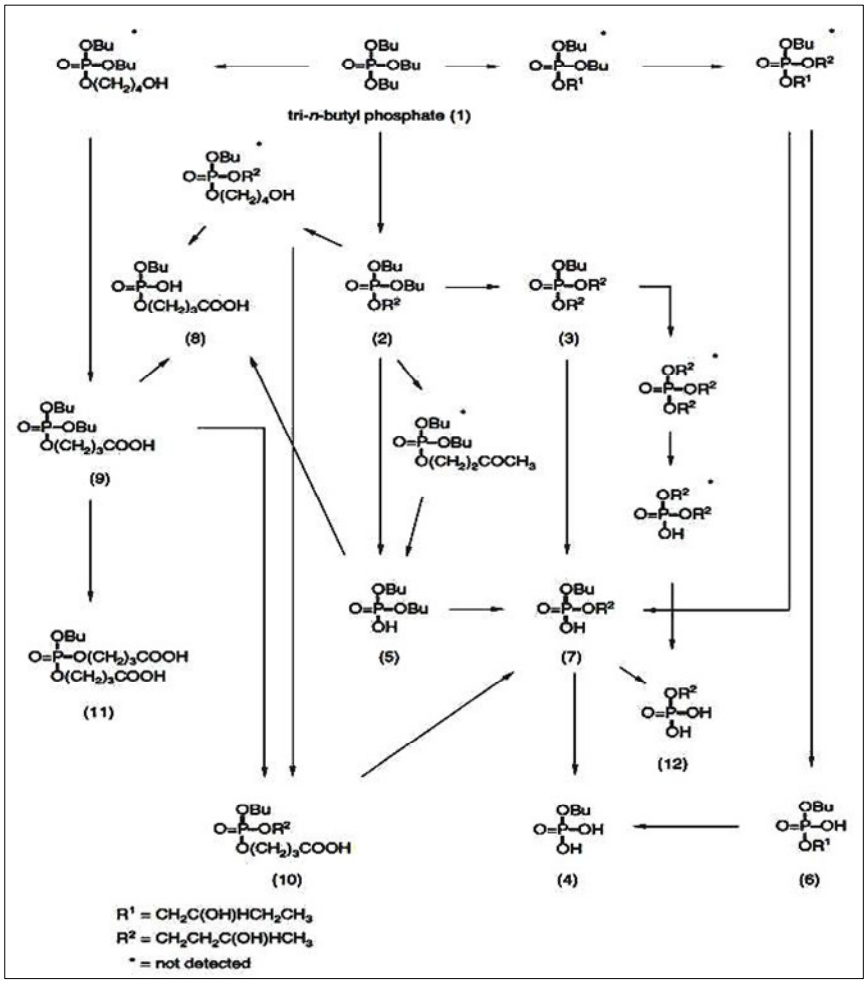

Fig. 1.5. Metabolites formed after administration of TBP to rats (Source: Suzuki et al., 1984b [21])

1.7. Bioremediation.

The observed prevalence and toxicity of TBP make its remediation necessary from the environment. Bioremediation is a technology that utilizes the metabolic capabilities of organisms or products therefrom to clean up contaminated environments. It uses naturally occurring bacteria or fungi or plants to degrade or detoxify substances hazardous to environment. Organic pollutant biodegradation primarily uses microorganisms. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the

contaminated site. Industrialization and extensive use of chemical substances such as petroleum oil, hydrocarbons, benzene, toluene, ethylbenzene, xylenes, chlorinated hydrocarbons, trichloroethylene, perchloroethylene, nitroaromatic compounds, organophosphorus compound, pesticides, and heavy metals contribute significantly to environmental pollution. The microbial communities degrade such compounds for their energy/nutrition needs, or detoxification could be fortuitous in nature (co-metabolism) [23]. A larger number of the xenobiotics and organic compounds are known to be degraded by strains of the genus *Pseudomonas* and strains of Sphingomonads.

1.8. Previous studies on TBP biodegradation

More than 50 bacterial strains have been reported to degrade TBP [24-36]. These studies used either single pure isolates or mixed cultures for TBP degradation, in which few strains exhibited the ability to utilize TBP as carbon and phosphorous for their growth. A strain of *Pseudomonas* was reported to degrade and use TBP as the sole source of phosphorous [34]. A mixed culture of *Pseudomonas* isolated from Mersey River at Warrington (U.K.), degraded 2 mM TBP in 3 days, and showed growth at the expense of TBP as the sole carbon and phosphorus source [36]. However, this ability was unstable and poor growth was seen intermittently. During periods of poor growth, cultures degraded ~ 0.5 mM TBP. Seven isolates were obtained from the mixed culture, but each of the isolates spontaneously and irreversibly lost the ability to degrade TBP and to grow after eight serial subcultures [36]. In the same study, the stability of the strains was maintained using ampicillin in the medium [37]. Though these results suggested that the genes related to TBP degradation could be plasmid born in *Pseudomonas*, no further studies were reported by the same group [37] subsequently.

In another study, 11 strains of *Acinetobacteria* were used for the degradation of TBP from

mixtures of organic waste containing TBP and one or more organic compounds. The mixed cultures of these strains could not use TBP for their growth, but, could degrade <1 mM TBP (200 ppm) in ~4 weeks. The study also developed a bio-reactor based technology using the same principle, which was filed as a patent in United States of America (USA) [28].

About 14 strains of purple non-sulfur photosynthetic bacteria and *Rhodopseudomonas palustris* were found to display the ability to degrade TBP [25]. The study reported disappearance of TBP from their culture medium. Under dark aerobiosis, when cultures of purple non-sulfur photosynthetic bacteria were incubated with 2 mM TBP, disappearance of 0.6 mM TBP was noted within 3 weeks. Under photosynthetic conditions, when cultures of *Rhodopseudomonas palustris* were incubated with 2 mM TBP, 1.6 mM TBP disappeared from the medium within 3 weeks. None of the strains from this study degraded TBP to release phosphate, but only demonstrated the disappearance of TBP from the medium. The stains could not use TBP either as carbon or phosphorous source [25].

The same research group investigated TBP degradation using 8 heterotrophic strains, isolated from a soil sample, which included 5 strains of *Pseudomonas*, *Thauera aromatic*, *Stenotrophomonas maltophilia* and *Serratia odorifera* [26]. In this study also, strains could not mineralize TBP, but could remove it from the medium, and none of the strains could utilize TBP either as carbon or phosphorous source. Among these strains, *Serratia odorifera* was found to be superior in sequestration of TBP from the medium, and removed 0.6 mM TBP from medium within 8 h [26]. In another study, a strain was isolated (mentioned as isolate R1) and could degrade and utilize TBP as a sole source of carbon and phosphorous [30]. The isolate R1 could degrade 1 mM TBP in 120 h. Degradation of low concentrations of TBP was also reported using free or immobilized cells of *Pseudomonas pseudoalcaligenes* MHF ENV, which was isolated

from a soil sample [27]. The strain could use TBP as sole source of carbon, and could degrade as low as 35 μM of TBP concentration in 6 d. When the cells were immobilized the degradation rate increased 2.8 times, which was claimed to be due to enhanced growth of the strain in immobilized matrix [27].

In a recent study, fifteen bacterial strains were reported to use TBP as a sole carbon and phosphorus source [24]. These strains could tolerate 5 mM TBP, which is the first report of the strains which tolerated more than 2 mM of TBP. All the isolates were found to be members of genera *Alcaligenes, Providencia, Delftia, Ralstonia*, and *Bacillus*. Among these, *Providencia* sp. BGW4 and *Delftia* sp. BGW1 were found to be superior, and could show disappearance of ~3 mM of TBP from the medium containing 5 mM TBP. Though, 3 mM TBP disappeared from the medium, only ~0.6 mM of inorganic phosphate was detected in the medium, suggesting very low amount of TBP mineralization [24]. A very recent study isolated *Klebsiella pneumonia*, which could degrade and use TBP in co-metabolism [32].

A TBP degrading bacterial strain was recently isolated from TBP waste storage site (Radioactive Solid waste Management Site) in our laboratory earlier. Preliminary investigations suggested that it could be a *Sphingomonas* strain.

1.9. Sphingomonads

Sphingomonads comprise of four closely related genera, namely, *Sphingomonas*, *Sphingobium*, *Sphingopyxis* and *Novosphingobium* (Table 1.5). The strains of Sphingomonads are strictly aerobic, gram negative, yellow-pigmented, rod shaped, chemo heterotrophic, and contain ubiquinone 10 as the major respiratory quinone [38,39]. They contain glycosphingolipids (GSLs) instead of lipopolysaccharide in their cell envelopes, which is a unique feature of Sphingomonads that distinguishes them from other classes of proteobacteria [38,39].

The Sphingomonads are widely distributed in nature, and have been isolated from many different aqueous and terrestrial habitats, chemically contaminated water sediments [40], sea water [40], plant root systems [41], clinical specimens [41,42], and other sources [38,39]. Several Sphingomonads were reportedly isolated from sites which were heavily contaminated with pesticides, herbicides, and other xenobiotics [43-45]. The phylogenetic relationship of Sphingomonads with other bacteria is depicted in Fig. 1.6

1.10. Sphingomonads as biodegraders.

A unique feature of many Sphingomonads is their ability to degrade a wide range of refractory organic compounds (Table 1.6) such as aromatic compounds, polysaccharides, pesticides and herbicides [43]. Sphingomonads have been shown to mineralize diphenyl ethers [44,46], dioxins [47], polythene glycols [48] , dibenzofurans [49,50], and many other aromatic and chloroaromatic compounds [45,51]. From several studies, it is clear that Sphingomonads play a crucial role in degradation of several contaminants/pollutants in the environment. Several researchers have been interested in elucidating the corresponding metabolic pathways and in characterizing the enzymes involved in such metabolism. Analysis of 26 genomes of Sphingomonads suggested that the ability of these strains to degrade varied range of organic compounds did not relate to different strategies in the pathways for the mineralization of xenobiotic compounds [52]. Previous studies on the physiology and enzymology of a number of mineralization pathways (e.g., for naphthalene (s) or biphenyl) did not demonstrate any significant differences between Sphingomonads and other bacteria [52]. Several studies suggest that Sphingomonads have the ability to adapt more quickly to the degradation of new compounds in the environment than members of other bacterial genera [53]. Many of the organic pollutant degradation enzymes are known to be present on the megaplasmids found in Sphingomonads.

Table 1.5. Differences in characteristic features of 4 clustures of Sphingomonads

Characteristic	*Sphingomonas* (clusture I)	*Sphingobium* (clusture II)	*Novosphingobium* (clusture III)	*Sphingopyxis* (clusture IV)
Gram staining	Gram - ve	Gram - ve	Gram - ve	Gram + ve
Nitrate reductase	+	-	+	-
Major fatty acids	18:1, 16:1 17:1	18:1	18:1	18:1
2-OH fatty acids	14:0 and/ 15:01	14:0	14:0	14:0
Polyamine	Symspermidine	Spermidine	Spermidine	Spermidine

Source: Takeuchi et al., 2001b [41]

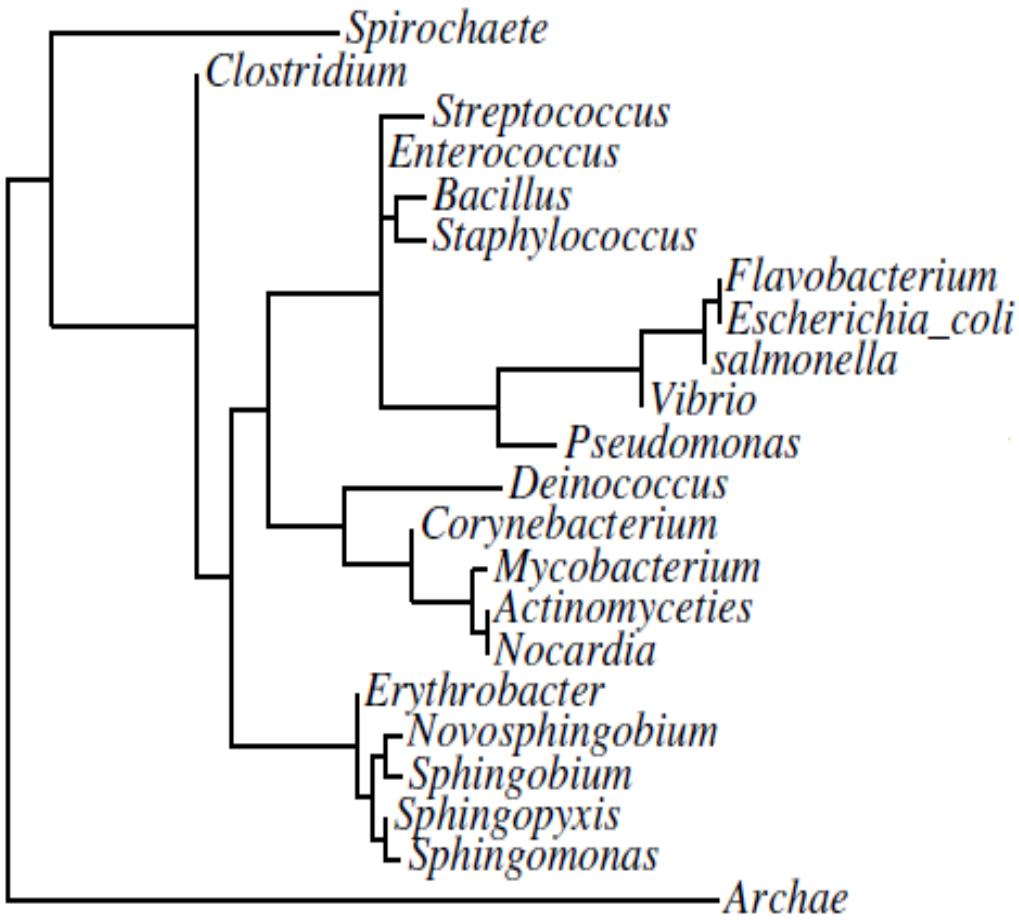

Fig. 1.6. Phylogenetic relationship of Sphingomonads with other bacteria

Table 1.6. Organic compounds metabolized by Sphingomonads.

Compound class or compound	Strain	Reference
Nonylphenol	*Sphingomonas cloacae*	[54]
Dibenzo-*p*-dioxin, dibenxofuran	*Sphingomonas wittichii* strain RW1	[47]
Diphenyl ethers	*Sphingomonas* sp. strain SS3	[46]
Polychlorinated hydrocarbons		
Pentachlorophenol	*Sphingomonas* sp. strain ATCC 39723	[55]
Polychlorinated	*S. chlorophenolica* RA2	[56]
biphenyls	*S. paucimobilis* BPSI-3	[57]
Monoaromatics		
toluene, xylene, cresol	*S. aromaticivorans* F199	[38]
	S. yanoikuyae B1	[58]
Polyaromatic hydrocarbons		
naphthalene, anthracene	*S. yanoikuyae* B1	[59]
phenanthrene	*S. paucimobilis* EPA 505	[60]
fluoranthene	*S. paucimobilis* 2322	[61]
substituted naphthalenes	*Sphingomonas* sp. strain BN6	[62]
Pesticides		
lindane	*S. paucimobilis* UT26	[63]
diclofop-methyl	*S. paucimobilis*)	[64]
carbofuran	*Sphingomonas* sp. strain CF05	[65]
isoproturon	*Sphingomonas* sp. strain SRS2	[66]
Lignin monomers & dimers		
Triterpenoids (resin acids): dehydroabietic acid, abietic acid, palustric acid, dichlorodehydroabietic acid	*S. paucimobilis* SYK-6 *S.* spp. DhA-33	[67,68]
Polymers		
Polyethers (polyethylene glycol)	*S. macrogoltabidus* strain 203, *S. terrae* E-1-A	[69,70]
Tri-alkyl phosphates		
Tributyl phosphate	*Sphingobium.* sp. strain RSMS	This study

Source: Balkwill et al., 2006[38].

Table 1.7. Number and size of megaplasmids detected in various Sphingomonad strains

Strain	Compounds degraded	No. of plasmids	Size (s) of plasmids (kb)
S. yanoikuyae B1	Toluene, biphenyl, naphthalene, anthracene, phenanthrene	1	240
S. herbicidovorans	2-(2,4-Dichlorophenoxy) propioniate,2,4-dichloro phenoxypropionic acid, mecoprop	2	300, 160
S. chlorophenolica ATCC 33790	Pentachlorophenol, 2,4,6-trichlorophenol	4	200, 60, 50, < 50
S. paucimobilis	Q1 Toluene, xylene, naphthalene, biphenyl,anthracene	3	240, 80, < 50
S. wittichii RW1	(Chlorinated) dibenzo-*p*-dioxin(s), dibenzofuran(s)	2	340, 240
Sphingomonas sp. HH69	(Acetoxy-,hydroxy-) dibenzofuran(s)	5	230, 50, 70,50,< 50
Sphingomonas sp. SS3	(4-Chloro-,4-fluoro-) diphenylether	3	340, 230, < 50
S. paucimobilis EPA 505	Fluoranthene,(substituted) naphthalene(s), phenanthrene, anthracene	3	200, 160, 50
Sphingomonas sp. A175	Benzene, 1,4-dichlorobenzene	2	100, _50
Sphingomonas sp. K39	2,3,4,6-Tetrachlorophenol	4	290, 280, 180, 120
S. xenophaga BN6	(Substituted) naphthalene-2-sulfonate(s)	4	260, 180, 100, 50
S. macrogoltabidus	Polyethylene glycol l4000	2	450, 150
S. subterranea	Naphthalene, toluene, biphenyl, dibenzothiophene, fluorene	3	450, 220, 150
S. aromaticivorans F199	Naphthalene, toluene, cresoles, biphenyl, dibenzothiophene, fluorene	2	500, 180
S. aromaticivorans B0695	2-Methylnaphthalene, acenaphthene, anthracene, fluoranthene, phenanthrene	2	195, < 50
S. subarctica KF1	2,4,6-Trichlorophenol, 2,3,4,6-tetrachlorophenol	2	300, 220
*S. stygia*e	Toluene, biphenyl, dibenzothiophene, fluorene	2	290, 120

Source: Basta at al., 2004 [53].

Megaplasmids are present in almost all the Sphingomonads (Table 1.7) and are readily transferred between the species of Sphingomonads [52], which could be one of the reasons for such quick adaptations to environments contaminated with organic pollutant environments [52].

1.11. The present study

Though several strains have been reported to degrade TBP, most of these strains had the following problems: (1) several strains could not use TBP as a source of carbon or phosphorous, (2) few strains degraded and/or utilized TBP with very poor efficiency, (3) the levels of TBP degradation was low (not more than 2 mM), (4) few strains could just remove TBP, but could not mineralize it, (5) few strains could not sustain the ability to degrade and utilize TBP, and (6) most strains could not tolerate high amounts of TBP (maximum reported 5 mM). Besides, the intermediates or the products of the TBP degradation were not known, and no clue was available about the possibly mechanisms responsible for the degradation of TBP.

With a view to develop an efficient system for TBP biodegradation, a bacterial isolate was obtained from TBP waste storage site, BARC and was shown to degrade TBP. The isolate was named as the RSMS strain. It used TBP both as carbon and phosphorous source. The partial 16s *rRNA* gene sequencing indicated that the strain belonged to the genus *Sphingomonas.* Detailed studies were carried using the RSMS strain with the following objectives.

Objectives:

1) Complete identification of RSMS strain using biochemical and molecular approaches.

2) Characterization of RSMS strain for its ability to tolerate, degrade and utilize TBP.

3) Evaluation of the factors regulating TBP utilization /degradation, if any.

4) Elucidation of biochemical pathway of TBP degradation in RSMS strain, and

5) Exploring the possibility to couple TBP degradation with uranium precipitation.

The work carried out for above objectives is presented in the thesis in following chapters:

Chapter 1. General Introduction

Chapter 2. Materials and methods

Chapter 3. Characterization of tributyl phosphate degrading RSMS strain

Chapter 4. Investigations on the molecular basis of TBP biodegradation by RSMS strain

Chapter 5. Elucidation of biochemical pathway of TBP degradation in RSMS strain

Annexure. TBP degradation coupled to uranium precipitation

Chapter 6. Summary

Chapter II

Materials and Methods

2.1. Media, Organism and growth conditions.

A mineral medium (MM) described earlier [36] was used as the basal medium for the growth of RSMS strain, with minor modifications. The mineral medium was buffered with 100-200 mM of 3-(N-morpholino) propane sulfonic acid (MOPS), at pH 7.0. The buffered mineral medium is referred to as modified mineral medium (MMM). The modified mineral medium did not contain any carbon or phosphorous source. Glucose and 5 mM KH_2PO_4, or required amounts of dibutyl phosphate (DBP) or tributyl phosphate (TBP) were added to MMM as the source of carbon and phosphorous. Composition of the MMM is described in Table 2.1. When required, the strain was also grown in Luria Bertani (LB) medium (Tryptone, 10 g/L; Yeast extract, 5 g/L; NaCl, 10 g/L, pH 7). Solid media were prepared by supplementing 1.5% Difco Bacto agar to MMM or LB media, and are respectively referred to as MMMA or LBA. TBP or DBP supplemented solid media plates were prepared by adding required amount of TBP or DBP, shaken thoroughly, poured in glass petri dishes and dried under sterile conditions in laminar hood. The RSMS strain was grown either in liquid MMM at 30^{o}C under shaking (180 rpm) or on solid media. All other strains used in the study were also grown in glucose (1%) and 5 mM KH_2PO_4 supplemented MMM, or LB media.

2.1.1. Phosphate-free glassware.

Special precautions were taken to avoid phosphate contamination, for conducting experiments where TBP and DBP were used as the sole source of phosphorous and carbon. All the glassware were soaked in 30% (v/v) of nitric acid for 12 h and then washed 2 times with distilled water, to remove inorganic phosphate.

Table 2.1. Composition of Macaskie's mineral medium [Thomas et al., 1997]

S.no	Ingredients	Final concentration (g/L)	Master stocks	
1	$CaCl_2$	0.025	0.25 gm in 100 ml	[100X]
Stock II				
2	$MgSO_4.7H_2O$	0.2	2 gm	Dissolved in 100
3	NaCl	0.1	1 gm	ml
4	$(NH_4)_2SO_4$	0.5	5 gm	[100X]
Stock III				
5	Na_2EDTA	0.015	1.5 gm	
6	$ZnSO_4.7H_2O$	0.007	0.7 gm	
7	MnCl2.4H2O	0.0018	0.18 gm	Dissolved in 500
8	$(NH4)_2Fe(SO)_2$	0.003	0.3 gm	ml
9	$CoCl_2.6H_2O$	0.00125	0.05 gm	[200X]
10	$CuSO_4.5H_2O$	0.00125	0.05 gm	
11	$NaMoO_4.2H_2O$	0.00125	0.05 gm	
Buffer stock				
12	MOPS	5.22 (25 mM)	10.44 gm in 20 ml	[100X]
KCl stock				
13	KCl	0.2	2 gm in 100 ml	[100X]
Sodium hydroxide				
14	NaOH	To adjust pH to 7.0	8 gm in 100 ml	[2N]

Master Stocks were prepared separately and autoclaved at 15 lbs/sq.in for 15 min. For 1X working stock, master stocks (stock II, stock III and MOPS) were added in required amounts, pH was adjusted to 7.0 with 2N NaOH, and media were autoclaved at 15 lbs/sq.in for 15 min. $CaCl_2$ master stock was added after cooling and mixed well. The medium was modified by adding 100 or 200 mM MOPS, as specified.

2.1.2. Isolation of TBP degrading strain.

A TBP degrading bacterial strain was isolated from the TBP waste storage tank located at the Radioactive Solid waste Management Site (RSMS) in Bhabha Atomic Research Centre (BARC), Mumbai. Swabs were taken from the side walls of the TBP waste storage tank with sterile cotton and immersed in 10 ml sterile 0.85% NaCl solution, shaken well and added to 250 ml conical flask containing 50 ml Luria Bertani medium, supplemented with 2 mM^e TBP*. The flasks were incubated under shaking (150 rpm) at 30^oC for 3 days. The cells were harvested, washed with saline and directly plated on to mineral medium [36] agar plates supplemented with 2 mM^e TBP, as the sole source of carbon and phosphorous. The plates were incubated at 30^oC for 3 days. The colonies which grew and showed clearance of TBP around them were considered as putative TBP degraders. Such colonies were isolated and purified. The purified colonies were inoculated in to mineral medium supplemented with 2 mM^e TBP, as the sole source of carbon and phosphorous, and grown under shaking (180 rpm) at 30^oC. Cells from grown culture were washed with sterile saline and stored in 15% glycerol at -70^oC. The strain isolated was tentatively named as RSMS strain and identified later as a species of *Sphingobium*.

2.1.3. Visualisation of TBP degradation on solid agar medium

The TBP degradation by the strain was visualized by spotting 20 µl aliquots of 20 OD_{600nm}/ml cells on Luria Bertani agar or MMM agar plates supplemented with required amount of TBP. The plates were incubated at 30^oC for 7 d or till a clearly visible zone of clearance of TBP appeared around the culture spot. When required, *Flavobacterium*, BSAR-1 and Kn20 (Table 2.2) strains were used as controls.

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* Since TBP is sparingly soluble in water (<1.5 mM), the concentration of TBP is described as "TBP equivalent" and specified as "mM^e", throughout the thesis. The "mM^e" is the concentration that would be attained, when TBP is completely dissolved.

Table 2.2. Bacterial strains and oligonucleotide primers used in this study

Strain	Description	Source
RSMS	TBP degrading strain of *Sphingobium*	This study
Sphingomonas sp.BSAR-1	Alkaline phosphatase (PhoK) producing strain	[71]
KN-20	Tn5 mutant of *Sphingomonas* sp. BSAR in alkaline phosphatase (*phoK* gene)	[71]
E. coli DH5α	*F⁻, recA41 endA1 gyrA96 thi-1 hsdr17 (r_k^- m_k) supE44 relAλ lacU169*	Lab collection
E. coli ANU 1041 (pACYC184-Mob)::*Tn*5	E coli ANU1041 strain containing pACYC184-Mob::*Tn5* plasmid (Kan^r)	MTCC, IMTECH, Chandigarh
Flavo bacterium sp. MTCC 2495	Organophosphorous (phosphotriesters) degrading strain	MTCC, IMTECH, Chandigarh
Oligonucleotide primers used in the study		
Primer	**Description**	**source**
BG1 primer (8F)	AGAGTTTGATCA/CTGGCTC, for 16s rRNA gene amplification and sequencing	Lab collection
BG2 primer (1409R)	GGGCGGA/TGTGTACAAGGC, for 16s *rRNA* gene amplification and sequencing	Lab collection
Beu2 primer (517F)	GCCAGCAGCCGCGGTAA for16s *rRNA* gene sequencing	Lab collection
Beu5 primer (926R)	CCGTCAATTCA/C TTTA/G AGTTT, for 16s *rRNA* gene sequencing	Lab collection
npt II forward primer	CGCGGATCCGCCAGTCCGCAGAAACGG	Lab collection
npt II reverse primer	GTCAGATCTTCCCGCTCAGAAGAACTC	Lab collection

2.1.4. Growth measurement of RSMS strain.

Growth was generally measured as colony forming units (CFUs) at 30^{o}C after 48 h growth on LBA plates or MMMA plates supplemented either with 1 % glucose and 5 mM KH_2PO_4, or with TBP/DBP. Growth was also assessed spectrophotometrically as increase in culture turbidity (OD at 600nm)

2.2. Identification of RSMS strain.

2.2.1. Biochemical tests

Ability of the RSMS strain to utilize various carbohydrate sources were assessed using HiCarbohydrate Kit KB009 for 35 carbohydrates (HIMedia Laboratories Pvt.Ltd, India). The kit uses solid agar media supplemented with different individual carbohydrate sources and a pH indicator. Upon usage of the carbohydrate source the pH of the medium changes and can be visualized by the change in colour of the pH indicator. Other biochemical tests, such as lysine utilization, citrate utilization, ornithine utilization, urease detection, phenylamine deamination, nitrate reduction and H_2S production were conducted using a HiAssorted Biochemical test kit KB002 (HiMedia Laboratories Pvt. Ltd., India).

2.2.2. Whole cell fatty acid analysis

RSMS strain was grown in Difco R2 medium, which consists of 0.05% peptone, 0.05% casamino acids, 0.05% yeast extract, 0.05% dextrose, 0.05% soluble starch, 0.03% dipotassium phosphate, 0.03% sodium pyruate, and 0.005% magnesium sulfate at pH 7. Whole cell fatty acids from the culture were extracted and esterified by alkylation. The methylated fatty acids (fatty acid methyl esters) were quantified using gas chromatography. The process was conducted at the Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.2.3. 16s *rRNA* gene sequencing.

Partial amplification of 16s *rRNA* gene was carried out in stringent aseptic conditions. Colony PCR (polymerase chain reaction) was carried out using a pure, single and isolated colony of RSMS strain. The 16s *rRNA* gene amplification was carried out using BGI (5'-AGAGTTTGATCCTGGCTCAG-3') [72] and BGII (5'-GGGCGGA/TGTGTACAAGGC-3') [73] primers. The gene was amplified in thermocycler (Eppendorf, Germany) with the following steps: (1) initial denaturation at 94°C for 3 minutes, (2) annealing at 94°C for 30 sec, (3) renaturation at 55°C for 30 sec, (4) polymerization at 72°C for 45 sec, and (5) final extension at 72°C for 10 minutes. The reaction was performed for 30 cycles (step 2 to step 4). The purified product (1.4 kb) obtained by PCR amplification was visualized by gel electrophoresis and sequenced by dideoxy method using Big Dye terminator cycle sequencing kit v3.1 (Applied Biosystems, USA) and an automated ABI prism DNA sequencer. The sequencing PCR was carried out by using BGI, BGII, and two internal primers, Beu2 (517f) GCCAGCAGCCGCGGTAA and Beu5 (926r) CCGTCAATTCA/CTTTA/GAGTTT. The overlapping sequences obtained by all these 4 primers were aligned using Multalin program (http://multalin.toulouse.inra.fr/multalin/) [74], and consensus sequence was considered. Similarity searches for the obtained 16s *rRNA* gene sequence (1248 nt) were carried out using the BLAST algorithm available at http://www.ncbi.nlm.nlh.gov [75]. The nucleotide sequence (1248 nt) was submitted to GenBank (Accession. No. EU629211.2). The phylogentic relationship of the RSMS strain was deduced by the maximum likelyhood method (http://phylogeny.lirmm.fr/phylocgi/ simplephylogeny.cgi) [76]. The RSMS strain was deposited with Microbial Type Culture Collection and Gen Bank, Chandigarh, India (http://mtcc.imtech.res.in) under the accession number 11630.

2.3. Assessment of TBP degradation and TBP dependent growth of RSMS strain.

2.3.1. Preinoculum preparation. A single colony of RSMS strain was inoculated in MMM supplemented with 1% glucose and 5 mM KH_2PO_4. Exponentially grown cells were harvested by centrifugation at 10,000 rpm for 5 min. The cell pellet was washed three times, with equal volumes of MMM to remove residual glucose and phosphate, and resuspended in fresh MMM. This cell suspension was used as preinoculum for all the growth and TBP degradation related experiments.

2.3.2. Growth and TBP degradation by RSMS strain

All growth related experiments in liquid MMM were started with an initial inoculum density of 0.1 OD_{600nm} (~ $5x10^7$ CFU/ml). The ability of the RSMS strain to utilize TBP or DBP was assessed by inoculating the strain in to MMM containing desired amounts of TBP or DBP (British Drug Houses, UK) as the sole source of carbon and phosphorous. Following controls were also included: TBP supplemented MMM without cells, glucose supplemented culture in MMM without added TBP, or KH_2PO_4 supplemented culture in MMM without added TBP.

Cell density required for the optimal rate of TBP degradation was assessed by inoculating RSMS culture at various cell densities (OD_{600nm}) in to MMM supplemented with 20 mM DBP or 30 mM^e TBP. Growth was assessed as CFUs or as increase in cell density (at 600nm) (as mentioned in section 2.14, above) and TBP/DBP degradation was assessed as phosphate released by phosphomolybdic acid method (as mentioned in section 2.3.5, below).

2.3.3. Tolerance of the RSMS strain to TBP/DBP and butanol

The ability of the RSMS strain to tolerate TBP/DBP/butanol was assessed by plating the RSMS culture on to the MMM agar supplemented with different concentrations of TBP/DBP/butanol. The plates were incubated at 30^oC and CFUs were counted after 2 days. The concentration of

TBP/DBP/butanol at which no growth was observed was considered as the MIC for the respective compound for RSMS strain. In these experiments, TBP/DBP served as the source of both carbon and phosphorous, while in case of butanol 5 mM KH_2PO_4 was added to the medium as phosphorous source.

2.3.4. Suppression of TBP degradation in the presence of glucose/inorganic phosphate

Suppression of TBP degradation by glucose was studied by inoculating the RSMS strain [at $5X10^7$ CFUs/ml (0.1 OD_{600nm})] in to MMM containing 10 mM^e TBP and supplemented with different concentrations of glucose. Following controls were also included: RSMS culture in MMM, RSMS culture in MMM supplemented with 10 % glucose, or RSMS culture in MMM supplemented with 10% glucose and 5 mMKH_2PO_4. Growth was assessed as colony forming units (CFUs) and TBP degradation was spectrophotometrically estimated as phosphate released upon TBP degradation. Suppression of TBP/DBP degradation was also assessed on solid MMM agar containing 10 mM^e TBP or 10 mM DBP supplemented with 10% glucose, and incubated at 30°C. The MMM agar containing 10 mM^e TBP or 10 mM DBP alone were used as controls. Growth was assessed as CFUs formed after 3 days of incubation at 30^oC.

The reversal of suppression of TBP degradation was assessed by transferring the cultures grown in 5% glucose + 5 mM^e TBP to MMM supplemented with 5 mM^e TBP. RSMS culture transferred from 5 mM^e TBP to the similar medium was used as control for the comparison. The lag period and the time required for the revival of growth in both cases were noted. The suppression or induction of DBP degradation was also assessed by estimating DBP degradation in cell freee extracts of the cultures.

Suppression of TBP degradation in presence of inorganic phosphate was studied by inoculating the RSMS strain (at 0.1 OD_{600nm}/ml) in to MMM containing 10 mM^e TBP and supplemented

with/without various concentration of KH_2PO_4. The growth was assessed as increase in cell density at 600 nm. The degradation of TBP could not be measured (in terms of inorganic phosphate) in this experiment due to interference from the added excess phosphate (KH_2PO_4).

2.3.5. Routine analysis of TBP degradation

TBP and DBP degradation were routinely assessed spectrophotometrically by measuring phosphate released in the medium by phosphomolybdic acid method [77]. For the estimation of phosphorous, 700 µl of reagent mixture (10% ascorbic acid and 0.42% ammonium molybdate, 1:6, v/v) was added to 300 µl sample. The reaction mixture was mixed properly and incubated at 45^{o}C for 20 min. The amount of phosphomolybdate formed in the reaction was spectrophotometrically estimated at 820 nm. Aqueous solution of KH_2PO_4 was used to obtain standard curve for phosphorous estimation.

2.4. Isolation of TBP non degrading mutants of RSMS strain.

2.4.1. Tn5 mutagenesis.

TBP non degrading mutants were isolated by conjugation between RSMS (Str^r, recipient cells) and *E. coli* ANU1041 strain (Kan^r donor strain) (Table 2.2). The donor strain contained pACYC184-Mob::Tn5 plasmid with *nptII* gene (Kan^r) as a selected marker. Donor and recipient cells were mixed at 1:10 ratio and spread on a sterile 0.22 µ pore sized membrane filter (Millipore, India). The membrane filter was placed on LB agar medium and incubated overnight at 30^{o}C. Individual controls of RSMS or *E. coli* ANU1041 were incubated similarly on different membrane filters. After overnight incubation, the cells were collected from the membrane and resuspended in 0.85% NaCl solution. The suspension was appropriately diluted and plated on LB agar supplemented with 10 µg/ml of kanamycin and 50 µg/ml sptreptomycin and incubated at 30^{o}C for 2 days. The kanamycin and streptomycin resistant colonies were patched on to LB agar supplemented with 10 mM^e TBP for the screening of TBP non-degrading mutants.

The colonies which did not show zone of TBP degradation around them were considered as putative TBP non- degrading mutants. These were tested further for their inability to degrade and utilize TBP as the sole source of carbon and phosphorous. The TBP non degrading mutants were stored at -70^{o}C as glycerol stocks.

One of the TBP non degrading mutants (mutant SS22) was studied further. The 16s *rRNA* gene sequencing and 41 biochemical tests were done to identity the mutant SS22 and to assess that it derived from the parent RSMS strain.

2.4.2. Probing of Tn5 transposon in TBP non degrading mutant SS22

Attempts were made to probe the Tn5 transposon in mutant SS22 by Southern blotting followed by hybridization with DIG-labeled *nptII* probe. The *nptII* gene from *E. coli* ANU1041::Tn5 was PCR amplified as a 1.2 kb DNA fragment which was purified and subjected to denaturation at 100^{o}C for 10 min followed by instant chilling on ice. Hexanucleotide random primers and digoxygenin (DIG) labeling mix were added to the 1.2 kb denatured DNA to DIG-label the probe using the protocol given in the random priming DIG-labeling kit (Roche Biochemicals GmBH, Germany) and incubated at 37^{o}C for 18 h. The DIG-labeled probe DNA was purified using Sephadex G-50 spin columns (Roche Biochemicals GmBH, Germany) to remove free nucleotides.

The genomic DNA of both mutant SS22 and wild type RSMS strain were digested with restriction enzymes (XhoI, Bam HI, Hind III). Digested DNA fragments were separated by agarose gel electrophoresis. The separated DNA fragments (in gel) were subjected to depurination in 0.25 N HCl for 10 min, and was washed twice with denaturation solution (Table 2.3) for 15 min each. The gel was neutralized with neutralization solution (Table 2.3) for 15 min. The DNA present in the gel was blotted on to a negatively charged nylon membrane (Roche

Biocehmicals GmBH, Germany) by capillary transfer using 20X SSC solution (Table 2.3). Overnight DNA-DNA hybridization was carried out with DIG labeled *nptII* probe at 50^{o}C in hybridization buffer (Table 2.3). The blot was washed and incubated with blocking buffer containing anti DIG antibody. The blot was developed using chemiluminiscent substrate, CDP star (Roche chemicals GmBH, Germany) and the results were recorded on a X-ray film.

Dot blot based hybridization was carried out similarly by cross linking intact genomic DNA on nitrocellulose membrane followed by denaturation, hybridization and detection. The blots were developed either with NBT/BCIP (nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate) or with CDP star substrate. The experiment included positive controls [purified *nptII* gene fragment and genomic DNA of Kn20 (a Tn5 transposon based *phoK*-mutant of *Sphingomonas* sp. strain BSAR-1)].

Table 2.3. Solutions used for Southern blotting and hybridization of DNA

Solution	Composition
DNA denaturation solution	0.5 N NaOH, 1.5 M NaCl
DNA neutralization solution	0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl
20X SSC	3 M NaCl, 0.3 M sodium citrate, pH7.0
Hybridisation buffer	5X SSC, 0.1% (w/v) N-lauroyl sarcosine, 0.02% (w/v) SDS, 1% blocking reagent

2.4.3. Isolation of spontaneous TBP non-degrading mutants

The zone of TBP degradation was used as the method of screening for the isolation of spontaneous TBP non-degrading mutants. The RSMS strain was subjected to repeated subculturing in MMM supplemented with 1% glucose and 5 mM KH_2PO_4. The cells obtained in

5th subculture were pelleted using centrifugation, washed twice and resuspended in 0.85% NaCl solution. The suspension was appropriately diluted and plated on to LB agar medium supplemented with 5 mM[e] TBP, and incubated at 30°C for 3 days. The colonies which did not show zone of TBP degradation around them were considered as putative TBP non-degrading spontaneous mutants. These colonies were purified and tested for their inability to degrade and utilize TBP as the sole source of carbon and phosphorous.

2.5. Isolation, estimation, resolution and identification of protein.

2.5.1. Protein extraction from cells.

Exponentially growing bacterial cultures were subjected to centrifugation (7000 rpm, for 5 min), and resuspended in 1mM Tris-HCl buffer, pH 8 to obtain a cell density of 20 OD_{600nm}/ml. A protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) was used at 1 mM to inhibit the protein degradation. The cells were broken using sonication (Branson, USA) at 25-30Hz with following settings: the amplitude was set at 25-30Hz, pulse was on and off alternatively for 3 sec each, and the cells were effectively subjected to 3 min pulse. The cell debris was separated by centrifugation at 14000 rpm for 30 min to obtain the cell free extract. The protein estimation was routinely carried out by standard Lowry method using a commercial kit (Genei™ protein estimation kit, Bangalore, India).

2.5.2. Two dimensional electrophoretic separation of proteins.

Two dimensional electrophoresis was carried out as reported earlier [78]. About 500-750 µg of cell free extract was subjected to DNase and RNase enzyme treatment (at a final concentration of 1ug/ml) for 1 h on ice to remove the nucleic acids. The protein extract was concentrated to 5 µl by vacuum centrifugation (using Speed Vac) and resuspended in 50 µl rehydration buffer [6% urea, 2% thiourea, 2% CHAPS (3-((3-Cholamidopropyl) dimethyl ammonio)-1-propane

sulfonate)), 0.2% Dithio thretol (DTT) and immobilized pH gradient (IPG) buffer (10 µl/ml], incubated at room temperature (RT) for 1 h, and then stored at -70^{o}C. The 17 cm IPG (immobilized pH gradient) strips in 3-10NL pH range were rehydrated using 330 µl of rehydration buffer, and incubated at RT for 1 h. The rehydrated strips were covered with mineral oil to avoid the drying of the gel due to evaporation and rehydration was continued overnight at RT. Iso- electric focusing (IEF) followed by 14% SDS-PAGE was carried out exactly as described earlier [78,79].

2.5.3. Protein identification by MALDI mass spectrometry

The gel pieces containing desired protein for identification were excised from the 2D gel. The protein present in the gel plug was subjected sequentially to destaining, reduction, alkylation, and in-gel trypsin digestion as described earlier [78]. The oligopeptides generated in trypsin digestion were subjected to three sequential elution steps. First elution step involved 0.1 % trifluoroacetic acid (TFA), followed by 0.1% TFA+Acetonitrile (1:1, v/v). The last elution step involved 100% acetonitrile, which elutes all the hydrophobic peptides. The oligopeptides from all three elution steps were pooled and subjected to mass spectrometry (Ultra Flex III MALDI-ToF/ToF mass spectrometer, Bruker Daltonics, Germany) [78]. Mass spectra generated for oligopeptides were searched for similarity in MASCOT database. MASCOT searches were conducted using the NCBI database with the following settings: (1) Number of maximum permitted mis-cleavages as 1, (2) fixed modifications of cystein with Carbamidomethylation, (3) variable modification of methionine residues by oxidation, (4) peptide tolerance as 100 ppm, and (5) peptide charge as +1. A Mascot score of >65 with a minimum of 6 peptide matches was considered to be a significant identification (p <0.05) when sequence coverage was at least 25%.

2.6. Analysis of TBP degradation products

The protocol developed for sampling, extraction of TBP degradation products and their quantitative determination by gas chromatography was as follows:

For the analysis of TBP degradation products by RSMS strain or Mutant SS22, freshly prepared preinoculum was inoculated at a cell density of 3 OD_{600nm} in to 5 ml of MMM supplemented with 30 mM^e TBP or 20 mM DBP. The cultures were incubated at 30^oC under shaking (180 rpm). Samples were removed each day and subjected to centrifugation at 10000 rpm for 5 min. A small volume (500 µl) of the supernatant was aliquoted for the analysis of butanol and inorganic phosphate. The remaining ~ 4.5 ml of supernatant was collected and acidified with 1/100 volumes of 10 N H_2SO_4. To the acidified sample, equivolume of 10% benzyl alcohol (in benzene, v/v) (hereafter referred as BBA/Benzene Benzyl Alcohol) was added, shaken well for 15 minutes and allowed to stand for clear separation of layers. The top organic layer (BBA) was subjected to esterification using diazomethane, and the esterified samples were injected in to gas chromatograph in appropriate volumes (1-2 µl). Quantification of butanol was carried out by direct injection of appropriate volume (1-2 µl) of sample. The concentration of each compound was calculated by using the standard curves for each analyte.

For the preparation of standard curve. Predetermined amounts of TBP (1-30 mM^e) or DBP (1-30 mM) were added to MMM and individually acidified with 1/100 volumes of 10 N H_2SO_4. From the acidified samples TBP and DBP were extracted in to BBA and esterified with diazomethane as described above. Esterified samples were injected in to gas chromatograph in appropriate volumes (1-2 µl). Quantification of butanol was carried out by direct injection of MMM containing predetermined amount of butanol. Standard curves for all the analytes were prepared by plotting peak areas against the amount of the analyte injected in to gas chromatograph.

A gas chromatograph (Schimadzu-GC-2014) equipped with a 10% XE-60 column (1.5 m x 0.32 cm) was used for the analysis of TBP, DBP and butanol. Helium was used as carrier gas at a flow rate of 40 ml/min. Column temperature was maintained at 120^{o}C (1 min) to 230^{o}C (10 min) or at 170^{o}C (1 min) to 230^{o}C (10 min) for TBP and DBP detection, and 70^{o}C (1 min) to 230^{o}C (10 min) for butanol detection. The injection port and detector temperatures were maintained at 240^{o}C and 260^{o}C, respectively. Thermal Conductivity Detector (TCD) was used for the quantitation of organophosphates (TBP, DBP) while butanol was estimated using a Flame Ionization Detector (FID).

2.7. Determination of Phosphodiesterase (PDE) and Phosphomonoesterase (PME) activities

2.7.1. PDE and PME assays.

About 100 µg of cell free extracts of mutant/wild type were incubated with the substrates for phosphodiesterase (PDE) or Phosphomonoesterase (PME) at 30^{o}C, for 20 min. Bis-para-nitrophenolphoshate (Bis-pNPP) and para-nitrophenolphosphate (pNPP) were used as the substrates of PDE and PME activities, respectively. The para-nitrophenol (pNP) formed in the PDE and PME reaction was spectrophotometrically estimated at 405 nm. The activity was reported as nmole of para-nitrophenol released/mg of protein/h. Citrate buffer was used to maintain pH 3 to 6, Tris-Cl buffer was used to maintain pH 7 to 9 and carbonate buffer was used to maintain pH 9.5 to 10.8 (Table 2.4). The assay at pH 7 was also carried out in MMM.

2.7.2. In gel PDE and PME assays.

The PDE and PME activities were also investigated by in gel assays. About 1.5 mg of cell free protein extracts from wild type/mutant cells were electrophoretically resolved on to 4 lanes (4 lanes were merged) by 12% native PAGE. The gel was washed twice with MMM for 10 minutes each, and incubated with MMM containing (a) 10 mM Bis-pNPP, or (b) 10 mM pNPP.

The released yellow colored product (pNP) due to PDE or PME activities was recorded after 0.5 h. The para-nitrophenol is soluble and diffusible in the MMM, therefore large amount of protein (1.5 mg) had to be used to visualize PDE and PME activity in short time.

2.8. DBP degradation by cell free protein extracts

DBP degradation was assessed in cell free protein extracts of mutant and wild type cells by incubating ~100 µg protein with 10 mM DBP in 3 ml of buffer (Table 2.4). The reaction mixture was incubated for 24 h at 30^{o}C, and the released inorganic phosphate (due to DBP degradation) was measured using phosphomolybdic acid method. Different buffers were used for maintaining pH between 3-10.8 as described in Table 2.4. DBP degradation was also investigated at high temperature by incubating the reaction mixture at 60^{o}C. The inhibition of DBP degradation by caffeine was studied by incubating the whole cell protein extracts in MMM supplemented with 10 mM DBP and 1 mM caffeine for 24 h, before estimation of phosphate.

Table 2.4. Composition of buffers used for PDE and PME assays

Buffer	Composition	Final pH
Citrate buffer	82 mM citric acid and 18 mM tri-sodium citrate	3
Citrate buffer	59 mM citric acid and 41 mM tri-sodium citrate	4
Citrate buffer	35 mM citric acid and 65 mM tri-sodium citrate	5
Citrate buffer	11.5 mM citric acid and 88.5 mM tri-sodium citrate	6
Tri-HCl buffer	50 mM Tris, pH was adjusted with 0.2 M HCl	7.0
Tri-HCl buffer	50 mM Tris, pH was adjusted with 0.2 M HCl	8
Tri-HCl buffer	50 mM Tris, pH was adjusted with 0.2 M HCl	9.0
Carbonate buffer	80 mM sodium bicarbonate and 20 mM sodium carbonate	9.4
Carbonate buffer	40 mM sodium bicarbonate and 60 mM sodium carbonate	10
Carbonate buffer	10 mM sodium bicarbonate and 90 mM sodium carbonate	10.8
MMM	As described in section 2.1	7

2.9. Monobutyl phosphate degradation using cell free extracts of wild type and mutant.

Monobutyl phosphate is commercially available only as a mixture of DBP and MBP (60:40 w/w) but not in pure form. The MBP degradation assay was carried out by exploiting the inhibition of DBP degradation by caffeine. About 100 µg of cell free extract of RSMS/mutant SS22 was added to 3 ml MMM containing 5 mM MBP (also contains 5.50 mM of DBP) and 1 mM caffeine, and incubated at 30^{o}C for 24 h. Since, caffeine inhibits the DBP degradation, the inorganic phosphate detected in the reaction mixture after 24 h was considered to be solely due to MBP degradation.

Chapter III

Characterization of the tributyl phosphate degrading RSMS strain

Introduction

Several bacterial strains have been reported to degrade TBP, either as mixed cultures or as single isolates [24-26,29,33,34,36,80,81]. For example, mixed cultures containing *Pseudomonas* have been shown to degrade equivalent of 2 mM (2 mM^e)* TBP in ~3 d, and utilize TBP both as carbon and phosphorous source [36]. But, the corresponding *Pseudomonas* strain irreversibly lost the ability to degrade or utilize TBP after eight serial subcultures [36]. In contrast, *Serratia odorifera* or *Rhodopseudomonas palustris* respectively degraded 0.6 mM TBP within 8 h or 1.6 mM^e of TBP in 3 weeks. However, these strains could not mineralize or utilize TBP as carbon or phosphorous source for growth [25,26]. In a study, 11 *Acinetobacteria* strains were used for the degradation of TBP from mixtures of organic waste. The mixed cultures could degrade < 1 mM^e TBP (200 ppm) in ~4 weeks. The study also developed a bioreactor based technology using the same principle, which was filed as a patent in United States of America (USA) [28]. Recently fifteen bacterial strains were reported for their ability to degrade and utilize TBP as the sole source of carbon and phosphorous [24], but showed rather poor rates of TBP degradation (1 – 3.05 mM^e TBP degradation in 4 d). In another study, a strain (isolate R1) was found to degrade and utilize TBP as a sole source of carbon and phosphorous was isolated [30]. The isolate R1 could degrade 1 mM TBP in 120 h. A very recent study isolated *Klebsiella pneumoniae*, which could degrade and use TBP in co-metabolism [32].

Though several strains have been reported to degrade TBP, they had the following problems:

(1) few strains could not use TBP as carbon or phosphorous source, (2) few strains utilized TBP with very poor efficiency, (3) the efficiency of degradation was poor, (4) the amount of TBP degradation was low (< 2 mM^e), (5) few strains could just bind to TBP, but could not mineralize it, (6) few strains lacked stability with respect to TBP degradation and TBP utilization, and

* Since TBP is sparingly soluble in water (<1.5 mM), the concentration of TBP is described as "TBP equivalent" and specified as "mM^e", throughout the thesis

(7) the strains could not tolerate TBP beyond 5 mM^e). Besides, the intermediates and products of TBP degradation have not been explored and no clues are available about the genes/proteins responsible for TBP degradation.

The present work isolated, identified and characterized, a stable, TBP tolerant and efficient TBP degrading bacterial strain, superior to previously reported strains in many respects. These results are detailed below.

3.1. Isolation of TBP degrading strain.

The TBP waste storage tank, located at Radioactive Solid waste Management Site (RSMS), BARC was chosen for the isolation of TBP degrading strain. Enrichment was carried out using LB medium containing 2 mM^e TBP and the isolated culture was grown and spread on to mineral medium containing 2 mM^e TBP. The colonies which showed growth on such plates were considered as putative TBP degraders, since they showed significant growth on mineral medium supplemented with 2 mM^e TBP as the sole source of carbon and phosphorous. The procedure resulted in the isolation of a single monoxenic bacterial isolate capable of degrading and utilizing TBP as the sole source of carbon and phosphorous. The strain was named as RSMS strain.

3.2. Visualisation of TBP degradation on solid agar medium.

The solubility of TBP in aqueous medium is only 400 mg/L [7]. On account of its very low solubility, TBP containing LBA and MMMA solid media were opaque in nature (Fig. 3.1). The isolated RSMS strain formed a clear transparent distinct zone around the culture spot revealing degradation of TBP (Fig. 3.1a and b). The visibility of such transparent zone started 2 days after inoculation and became distinct after ~7 days. When colonies were evenly spread on the MMM or LB agar plates containing TBP, TBP from the medium was completely degraded within ~7 days resulting in clearance of the opacity and eventually resulting in transparency of the medium.

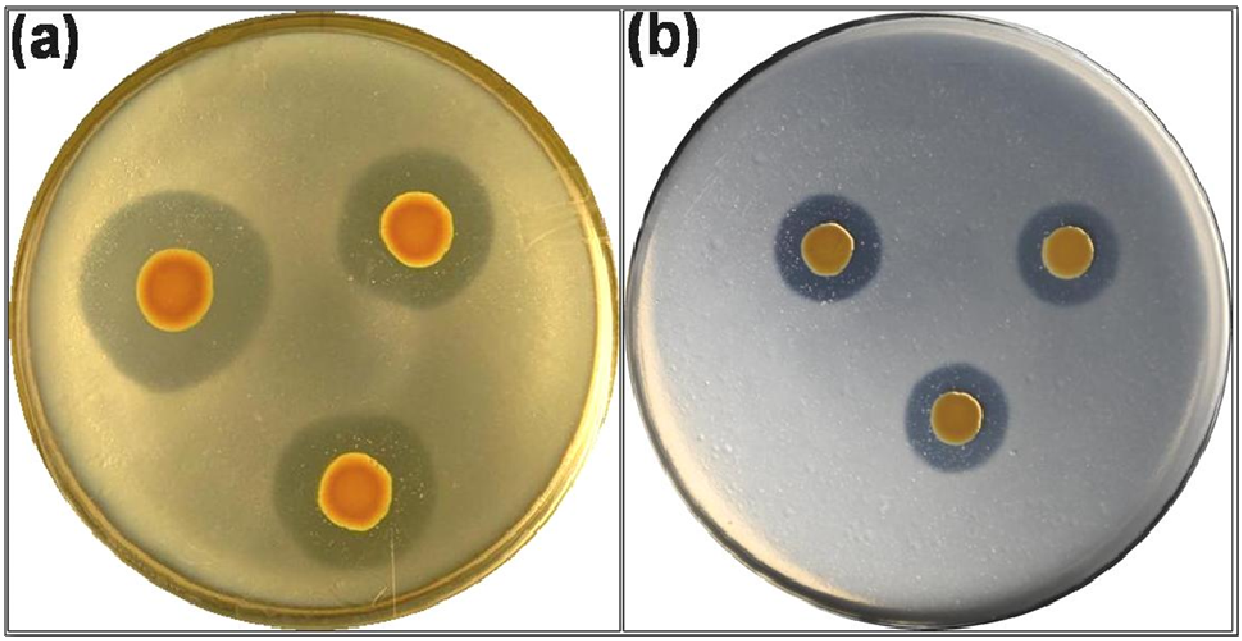

Fig. 3.1. Zone of clearance formed by TBP degradation. Zone around the culture spots of RSMS strain in (a) Luria Bertani Agar (LBA) medium, or (b) MMM agar plates, supplemented with 10 mM^e TBP. About 20 µl of $3OD_{600nm}$/ml culture was spotted and incubated at 30^oC. The zone of clearance was recorded after 1 week of incubation.

3.3. General features of RSMS strain.

RSMS strain showed optimum growth in rich media such as LB or TGY at 30°C. The colonies grew round in shape, reaching up to a size 2 mm in 3 days, with yellow coloured pigmentation (Fig. 3.2a). In MMM agar containing TBP, the colonies did not show pigmentation up to 1 week, after which they developed in to yellow coloured colonies (Fig. 3.2b). The cells appear to be rod shaped under microscope (Fig. 3.2c). The strain exhibited resistance to streptomycin, spectinomycin, carbanicellin, and chloramphenicol (Table 3.1).

3.4. Identification of the RSMS strain.

Standard methods were used for the identification of the RSMS strain, including 16s *rRNA* gene sequencing and total fatty acid analysis. Further, the strain was characterized for its ability to use several carbohydrate sources for growth, and presence of several enzymes was also assessed.

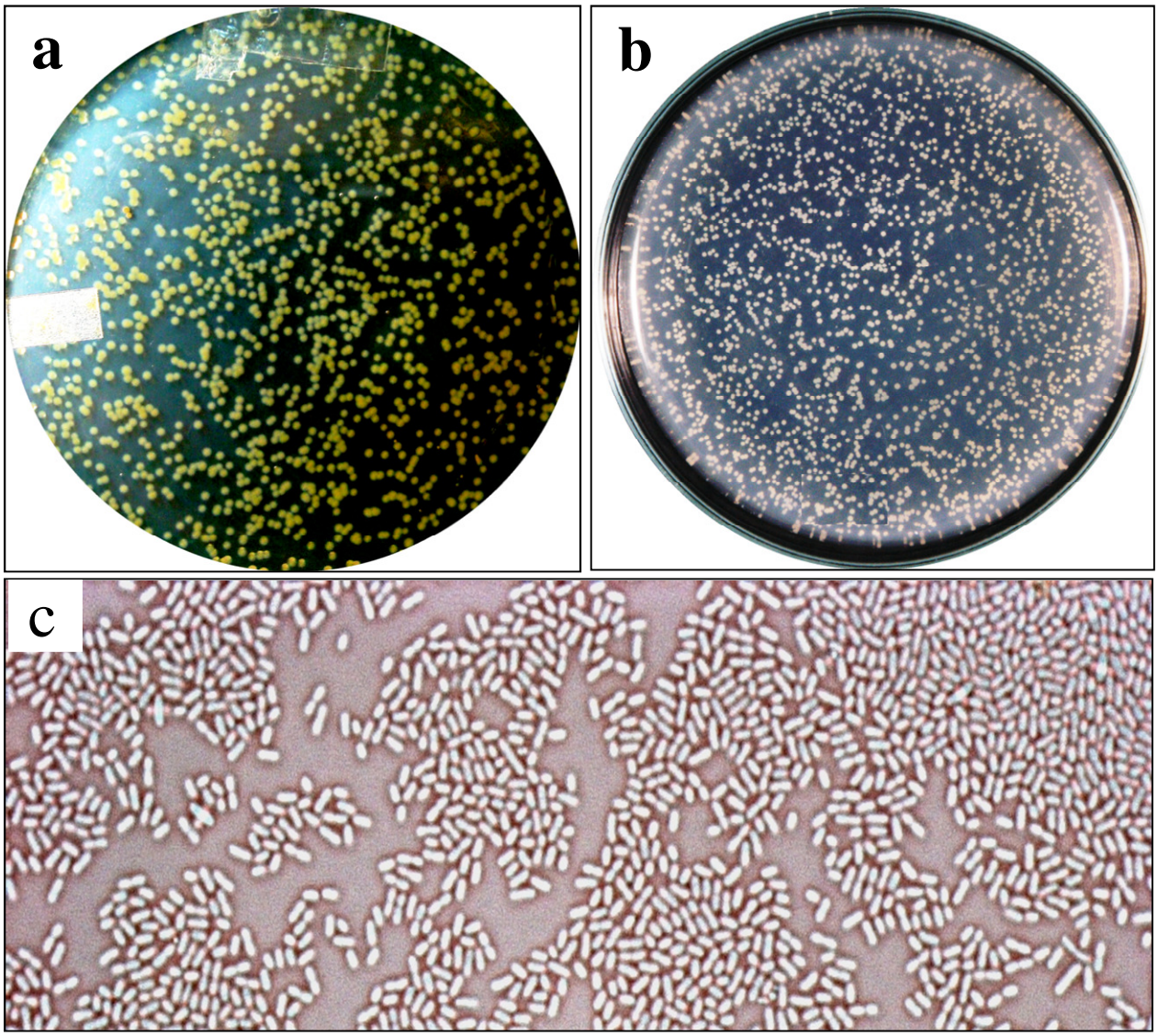

Fig. 3.2. The morphology of RSMS strain. Colonies of RSMS strain after 2 days of incubation at 30°C on (a) LB agar medium or (b) MMM supplemented with 5 mM[e] TBP as sole source of carbon and phosphorous. (c) RSMS strain observed under light microscope at 1500X magnification.

Table. 3.1. Antibiotic resistance profile of RSMS strain

Antibiotic (µg/ml)	Resistance	Antibiotic (µg/ml)	Resistance
Streptomycin (50)	+	Carbanicellin (100)	+
Kanamycin (10)	-	Ampicillin (25)	-
Spectinomycin (50)	+	Chloromphenicol (30)	+

3.4.1. Biochemical characterization of the strain.

RSMS strain could use several carbohydrates, such as dextrose, galactose, xylose, maltose, L-arabinose and glucosamine, but could not use lactose, fructose, raffinose, glycerol and several other carbohydrates (Table 3.2). The strain showed weakly positive reaction for ribose, trehalose, esculin, and citrate. The strain showed lysine decarboxylase and ornithine decarboxylase activities, suggesting that it could utilize lysine and ornithine for its growth. The strain did not produce H_2S suggesting the absence of metabolism related to the reduction of sulfur containing compounds. The strain was negative for urease, deaminase and nitrate reductase.

3.4.2. Fatty acid methyl ester analysis.

The total fatty acid composition of the RSMS strain was analysed at IMTECH, Chandigarh, India, using gas chromatography of fatty acid methyl esters generated by derivatization of fatty acids. The chromatography peaks were identified using retention time and Equivalent Chain Lengths (ECL), in which the retention time is directly proportional to the ECL (Fig. 3.3). The strain contained 18:1 w7c as the major fatty acid, which constituted 63.26% of the total cell fatty acids (Table 3.3). Other major fatty acids included 16:0 and 2-hydroxy myristic acid (2OH-14:0) which constituted 13.71, 7.51 percent of total fatty acids, respectively. Presence of oleic acid (18:1) as the major fatty acid is the characteristic feature of Sphingomonad strains [41]. Further, the strain showed highest similarity to *Sphingomonas echinoides*, with a similarity index of 0.769 (Table 3.4). Other stains which showed similarity to the RSMS strain included *Sphingomonas sanguinis*, *Novosphingobium capsulatum* and *Sphingomonas paucimobilis* with similarity indices of 0.604, 0.504 and 0.676, respectively. Though, FAME analysis identified the RSMS strain as a Sphingomonad, the subsequent 16s *rRNA* gene sequence analysis distinguished the four genera of the Sphingomonads.

Table. 3.2. Biochemical test with RSMS strain

S.No.	Test	Result	S.No.	Test	Result
Carbohydrate utilization tests					
1	Lactose	-	19	Inositol	-
2	Xylose	+	20	Sorbitol	-
3	Maltose	+	21	Mannitol	-
4	Fructose	-	22	Adonitol	-
5	Dextrose	+	23	Alpha Methyl-D-Glucoside	-
6	Galactose	+	24	Ribose	±
7	Raffinose	-	25	Rhamnose	±
8	Trehalose	±	26	Cellobiose	±
9	Melibiose	-	27	Melezitose	-
10	Sucrose	-	28	Alpha Methyl D Mannoside	-
11	L-Arabinose	+	29	Xylitol	-
12	Mannose	-	30	ONPG	-
13	Inulin	-	31	Esculin	±
14	Sodium Gluconate	-	32	D-Arabinose	+
15	Glycerol	-	33	Citrate	±
16	Salicin	-	34	Malonate	-
17	Glucosamine	+	35	Sorbose	-
18	Ducitol	-			
Other biochemical tests					
1	Lysine decarboxylase	+	5	Nitrate reduction	-
2	Ornithine decarboxylase	+	6	H2S production	-
3	Urease	-	7	Oxidase	+
4	Deamination	-	8	Catalase	+

+, - and ± indicates positive, negative and weakly positive reactions respectively

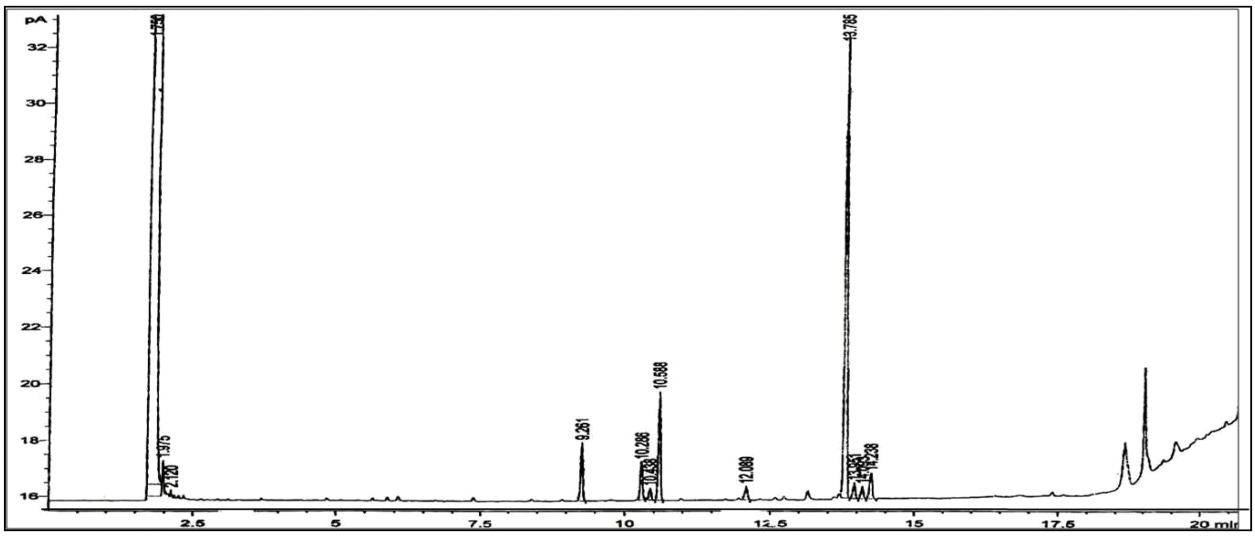

Fig. 3.3. Whole cell fatty acid analysis of RSMS strain. Gas chromatogram of fatty acid methyl esters of whole cells.

Table 3.3. FAME analysis of RSMS strain

RT	Response	Ar/Ht	R Fact	ECL	Peak Name	percent
1.750	3.552E+8	0.028	-	7.014	Solvent Peak	-
9.261	11166	0.043	0.952	15.206	14:0 2OH	7.51
10.438	2147	0.043	0.936	15.910	16:1 w5c	1.42
10.582	20767	0.040	0.935	16.000	16:0	13.71
12.089	2772	0.043	0.920	16.865	17:1 w6c	1.80
13.785	98872	0.048	0.906	17.826	18:1 w7c	63.26
13.951	3192	0.044	0.904	17.920	18:1 w5c	2.04
14.083	26969	0.045	0.903	18.000	18:0	1.72
14.238	5569	0.050	0.902	18.082	11 methyl 18:1 w7c	3.55

RT depicts the retention time of each fatty peak and corresponding Equivalent Chain Length (ECL) is indicated. Based on the area and the height of the peak, percentage of each fatty acid was calculated.

Table 3.4. Comparison of fatty acid profile of RSMS strain with other sphingomonads

Similarity Index	Strain
0.769	*Sphingomonas echinoides*
0.604	*Sphingomonas sanguinis*
0.504	*Novosphingobium capsulatum*
0.676	*Sphingomonas paucimobilis*

Similarity of RSMS strain with respect to the whole cell fatty acid profile indicated in terms of similarity index

3.4.3. Partial 16s *rRNA* gene sequence analysis.

A 1.4 kB PCR amplification product of 16s *rRNA* gene obtained was purified and sequenced using four different primers. Final 16s *rRNA* gene sequence consisted of 1248 nucleotides and was submitted to Genbank (Accession No. EU629211.2). The strain showed highest (>99%) sequence similarity of 1245/1248 nucleotides to *Sphingobium fuliginis* strain PQ-1, *Sphingobium* sp. KK22, *Sphingobium* sp. PHE3, *Sphingobium* sp. PNB and *Sphingobium* sp. YBL1 (Table 3.5). The genus *Sphingobium* belongs to one of the four closely related clusters of Sphingomonads [41]. The 16s *rRNA* gene sequence similarity values between species of different clusters are reportedly lower, ranging from 92±6 to 96±5% [41]. Further, these four clusters are distinguished by signature nucleotides at various positions [41] of 16s *rRNA* gene (Table 3.6), Hence, further analysis was carried out by aligning 16s *rRNA* gene sequences of the strains from four clusters of Sphingomonads. Multiple sequence alignment was carried out using Multalin program available at http://multalin.toulouse.inra.fr/multalin/ [74]. *Sphingobium* contains A, A, T at positions 359, 987, 1218 of the 16s *rRNA* gene, respectively, whereas strains from other three clusters contain G, G, and C at these positions (Table 3.6).

RSMS strain showed signature nucleotides identical to other *Sphingobium* strains (Table 3.6). Strains of Sphingomonads lack a stretch of 25 nucleotides (461 to 487) in comparison to *E.coli* (Fig.3.4), and have several differences at various positions in the gene (data not shown). Phylogenetic relationship analysis corroborated the BLAST analysis, wherein the RSMS strain showed highest similarity with *Sphingobium fuliginis* strain PQ-1, *Sphingobium* sp. KK22, and *Sphingobium* sp. YBL1 (Table 3.5 and Fig. 3.5). All these strains along with RSMS shown to belonged to single branch of phylogenetic tree (Fig.3.5). Hereafter in this thesis the strain will be referred to as *Sphingobium* sp. strain RSMS., or shortly as *Sphingobium* RSMS or RSMS strain.

Table 3.5. Strains showing highest similarity with the 16s *rRNA* gene of the RSMS strain using BLAST analysis.

Bacterial strains	Identity	Identity %	Accession No.
Sphingobium fuliginis strain PQ-1	1245/1248	99	KF145126.1
Sphingobium sp. KK22	1245/1248	99	HQ830159.1
Sphingobium sp. PHE3	1245/1248	99	FJ654290.1
Sphingobium sp. PNB	1245/1248	99	HM367594.1
Sphingobium sp. YBL1	1245/1248	99	EU159274.1
Sphingobium fuliginis strain R6-408	1245/1248	99	JQ659844.1
Sphingobium fuliginis strain R1-704	1245/1248	99	JQ659577.1

Bacterial strain which showed highest similarity, with respect to the 16s *rRNA* gene sequence of RSMS are listed. The analysis was carried out with BLAST programme.

Table 3.6. The signature nucleotides at various positions of 16s *rRNA* gene sequence of Sphingomonads.

Genus	Signature at position				
	52:359	134	593	987:1218	990:1215
RSMS strain	U:A	G	U	A:U	U:G
Sphingobium (Cluster II)	U:A	G	U	A:U	U:G
Sphingomonas (Cluster I)	C:G	G	G	G:C	U:G
Novosphingobium (Cluster III)	C:G	G	U	G:C	U:A
Sphingopyxis (Cluster IV)	C:G	G	U	G:C	U:G
Rhizomonas	C:G	A	A	G:C	U:G
Blastomonas	C:G	G	U	G:C	U:A
Erythrobacter	C:G	G	U/C	G:C	U:A

The signature nucleotides of 16s *rRNA* gene distinguishes the four clusters of Sphingomonads. The nucleotides which underlined are nucleotides present in all the four clusters of Sphingomonads. The numbering is done with reference to *E. coli* 16s *rRNA* gene sequence.

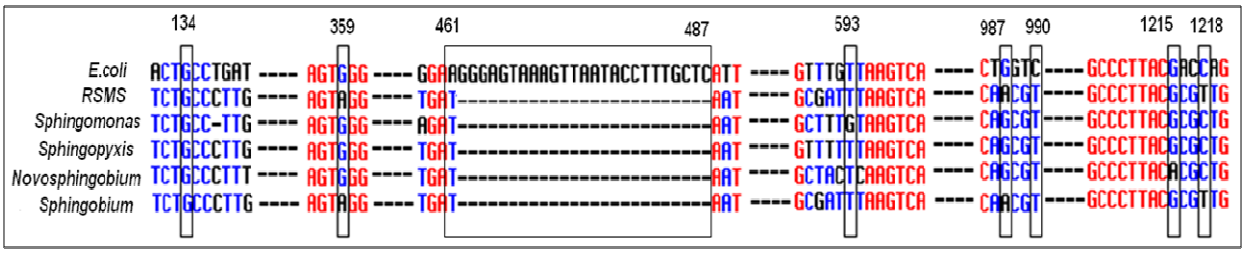

Fig. 3.4. Sequence alignment of 16s *rRNA* genes of four clusters of Sphingomonads and *E.coli*. The multiple sequence alignment of 16s *rRNA* gene was carried out using multialign programme. Signature nucleotides are marked in boxes at 134, 359, 593, 987, 990, 1215 and 1218 positions. The main difference between Sphingomonads and *E. coli* is shown as a box between 461 and 487 positions

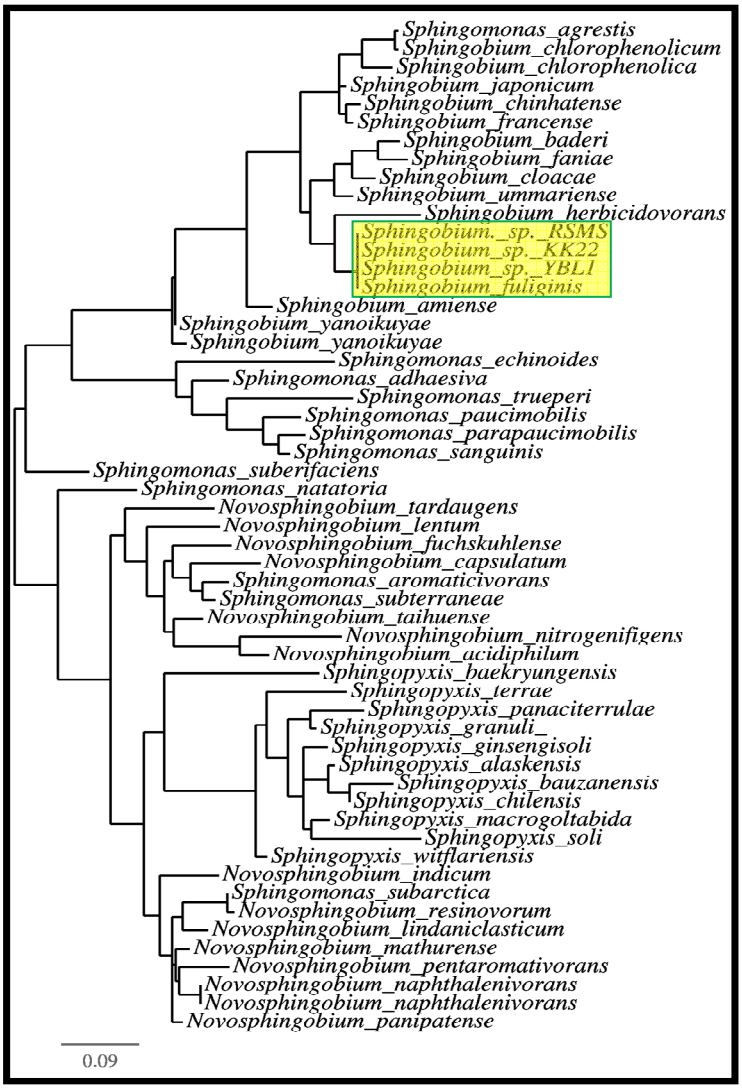

Fig. 3.5. Phylogenetic relationship of RSMS strain based on 16s *rRNA* gene sequence. The phylogenetic tree was constructed based on the maximum likelyhood method (http://phylogeny.lirmm.fr/phylo_cgi/simple_phylogeny.cgi), by taking 16s *rRNA* gene sequences of 55 Shingomonads available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

3.5. Optimization of growth conditions for TBP degradation and utilisation.

3.5.1. Effect of pH and buffering conditions.

The strain showed optimum TBP degradation and growth at pH 7. TBP degradation and growth of the RSMS strain decreased with increasing or decreasing pH (Fig. 3.6). TBP degradation was assessed as phosphate released. TBP degradation was completely inhibited below and above pH 6 and pH 7.8, respectively. Addition of 3-(N-morpholino) propane sulfonic acid (MOPS) buffer affected growth and TBP degradation**.** TBP degradation increased with increasing concentration of MOPS till 100 mM (Fig. 3.7). While 25 mM and 50 mM MOPS supported only ~8 and 16 mM^e of TBP degradation in 8 days, 100-200 mM MOPS supported complete degradation of 30 mM^e TBP in 8 days (Fig. 3.7). The lesser degradation of TBP at lower concentrations of MOPS could be attributed to the acidification of the medium (Table 3.7), due to release of phosphoric acid upon TBP degradation, which decreased the growth/inhibited TBP degradation. Hence, all subsequent experiments were carried out in mineral medium containing 100 mM MOPS, which was named as modified mineral medium (MMM).

3.5.2. Effect of temperature on TBP degradation and growth.

The RSMS strain showed optimum TBP degradation and growth at 30^oC (Fig. 3.8). The growth and TBP degradation decreased above and below 30^oC (Fig. 3.8). No growth or TBP degradation was observed at 40^oC.

3.6. Growth on TBP as the sole source of carbon and phosphorous.

Growth and TBP degradation abilities of the RSMS strain were assessed in MMM containing TBP as the sole source of carbon and phosphorous. RSMS strain could not grow in MMM containing glucose or KH_2PO_4 alone.

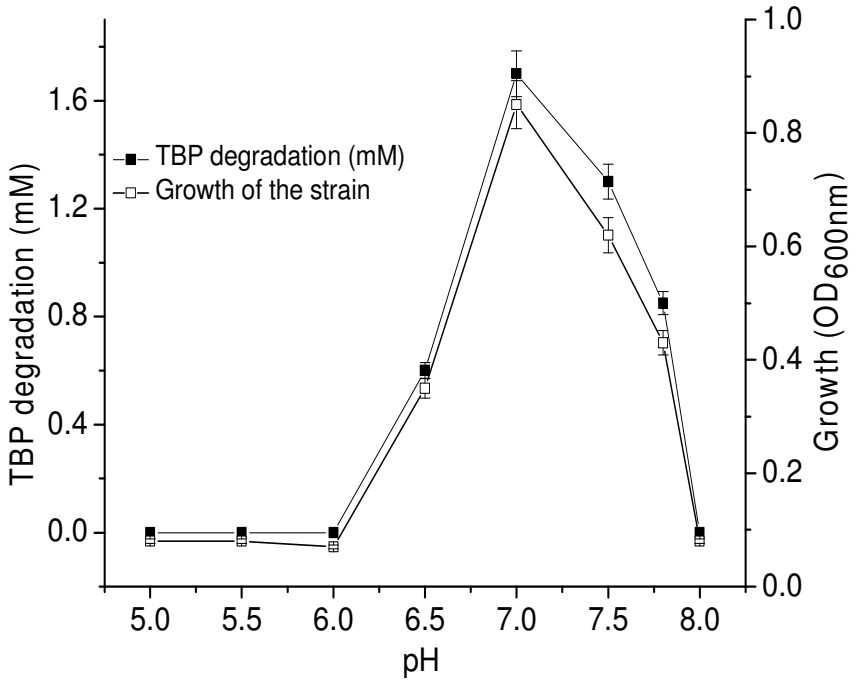

Fig. 3.6. Optimisation of pH and buffering conditions for TBP degradation and growth of RSMS strain. TBP degradation and growth of the strain at different pH values was assessed by inoculating RSMS strain (at 0.1 OD_{600nm}/ml) in to MMM containing 2 mM^e TBP as the sole source of carbon and phosphorous. TBP degradation was spectrophotometrically estimated as inorganic phosphate released and growth was measured as increase in OD at 600 nm after 24 h incubation at 30^oC and 180 rpm.

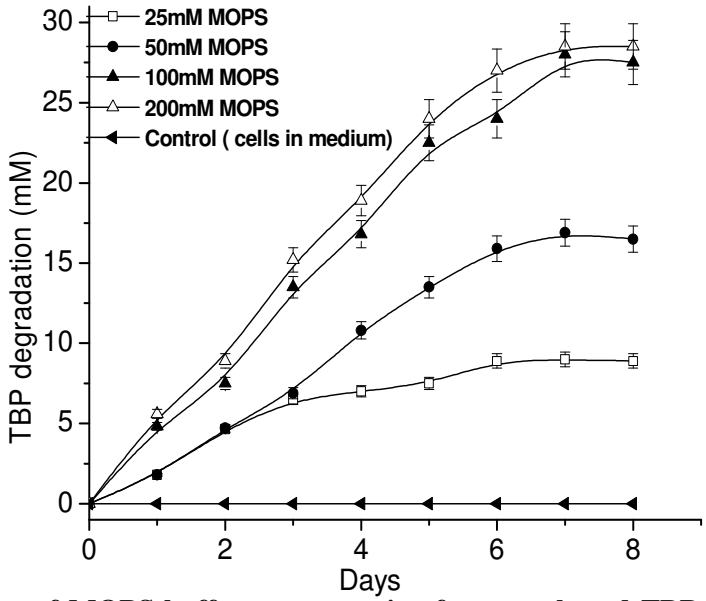

Fig. 3.7. Optimization of MOPS buffer concentration for growth and TBP degradation by RSMS strain. Optimum concentration of MOPS was assessed by inoculating the RSMS strain at 0.1 OD_{600nm}/ml in to mineral medium containing 30 mM^e TBP and various concentrations of MOPS buffer. The initial pH was adjusted to 7. TBP degradation was spectrophotometrically estimated as inorganic phosphate released and growth was measured as increase in OD at 600 nm.

Table 3.7. The pH change in the mineral medium during the growth of the RSMS strain

Days after incubation	pH of the medium in different concentration of MOPS			
	25 mM MOPS	50 mM MOPS	100 mM MOPS	200 mM MOPS
0	7	7	7	7
2	6.7	6.8	7	7
5	6.5	6.7	7	7
8	6.5	6.7	7	7

RSMS strain was inoculated at 0.1 OD_{600nm}/ml, in to mineral medium containing 30 mM^e TBP and different concentrations of MOPS. The pH of the medium was monitored for 8 days.

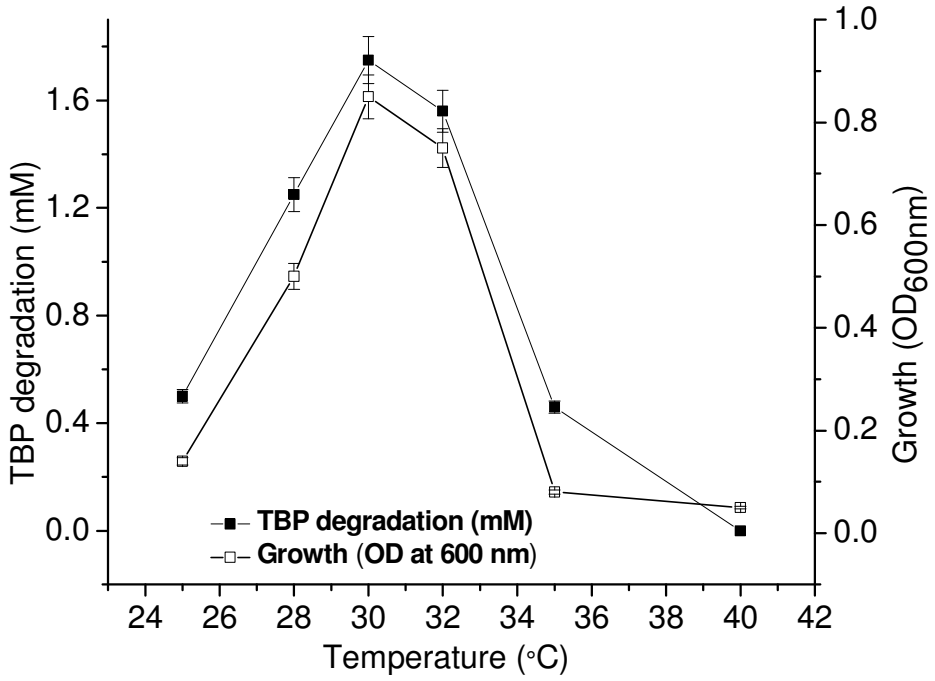

Fig. 3.8. Optimisation of temperature for degradation and growth of RSMS strain in TBP. The temperature dependence of TBP degradation and growth of the strain was assessed by inoculating RSMS at 0.1 OD_{600nm}/ml in to MMM (pH 7) containing 2 mM^e TBP as the sole source of carbon and phosphorous. The flasks were incubated at 180 rpm at different temperatures for 24 h. TBP degradation was measured with respect to the inorganic phosphate released and growth was assessed as increase in OD at 600nm.

However, MMM supplemented with 2 mM^e TBP alone supported active growth of the strain (Fig. 3.9a and 3.10a), suggesting that the strain could utilize TBP as the sole source of carbon and phosphorous. The experiment also used *Flavobacterium* (a phosphotriesterase enzyme containing strain which is phylogenetically close to RSMS), an alkaline phosphatase (*phoK*) positive strain BSAR-1 (a *Sphingomonas* strain, phylogenetically close to RSMS) and its isogenic mutant (*phoK*::Tn5) Kn20 as the controls (Fig. 3.9a and b). Both these strains failed to degrade or use 2 mM^e TBP either as carbon or phosphorous source (Fig. 3.9a and b), whereas RSMS could degrade and use TBP for its growth (Fig. 3.9a and b).

The RSMS strain showed maximum growth in MMM containing 30 mM^e TBP (Fig. 3.10a). Growth of the RSMS strain increased with increasing concentration of TBP up to 30 mM^e TBP (Fig. 3.10a). The number of CFUs in MMM suspensions containing 30 mM^e TBP increased from $5x10^7$/ml at inoculation to $1.3x10^9$/ml after 48h, which is equivalent to > 25 times increase in cell number or 4.5 doublings. The growth was also measured in terms of increasing OD at 600nm, which increased from 0.1 to 2.8, with 1 OD_{600nm} culture being equivalent to $5x10^8$cells/ml (Fig. 3.10b). The deduced doubling time for the strain at this TBP concentration was ~ 11 h. The strain continued to show TBP degradation from TBP suspensions even after attaining stationary phase. During growth phase, the maximum rate of degradation observed was ~9 mM^e of TBP in ~2 days (Fig. 3.10c), and the strain degraded 30 mM^e TBP in 8 days (Fig. 3.7). The strain completely degraded 2 mM^e TBP within 24 h after inoculation, but supported growth only up to 3 generation (0.1 OD_{600nm} to 0.8 OD_{600nm} per ml) (Fig. 3.10), whereas 5-30 mM^e TBP supported 4 to 4.5 generations of growth (0.1 OD_{600nm} to 1.6 OD_{600nm} per ml) (Fig. 3.10).

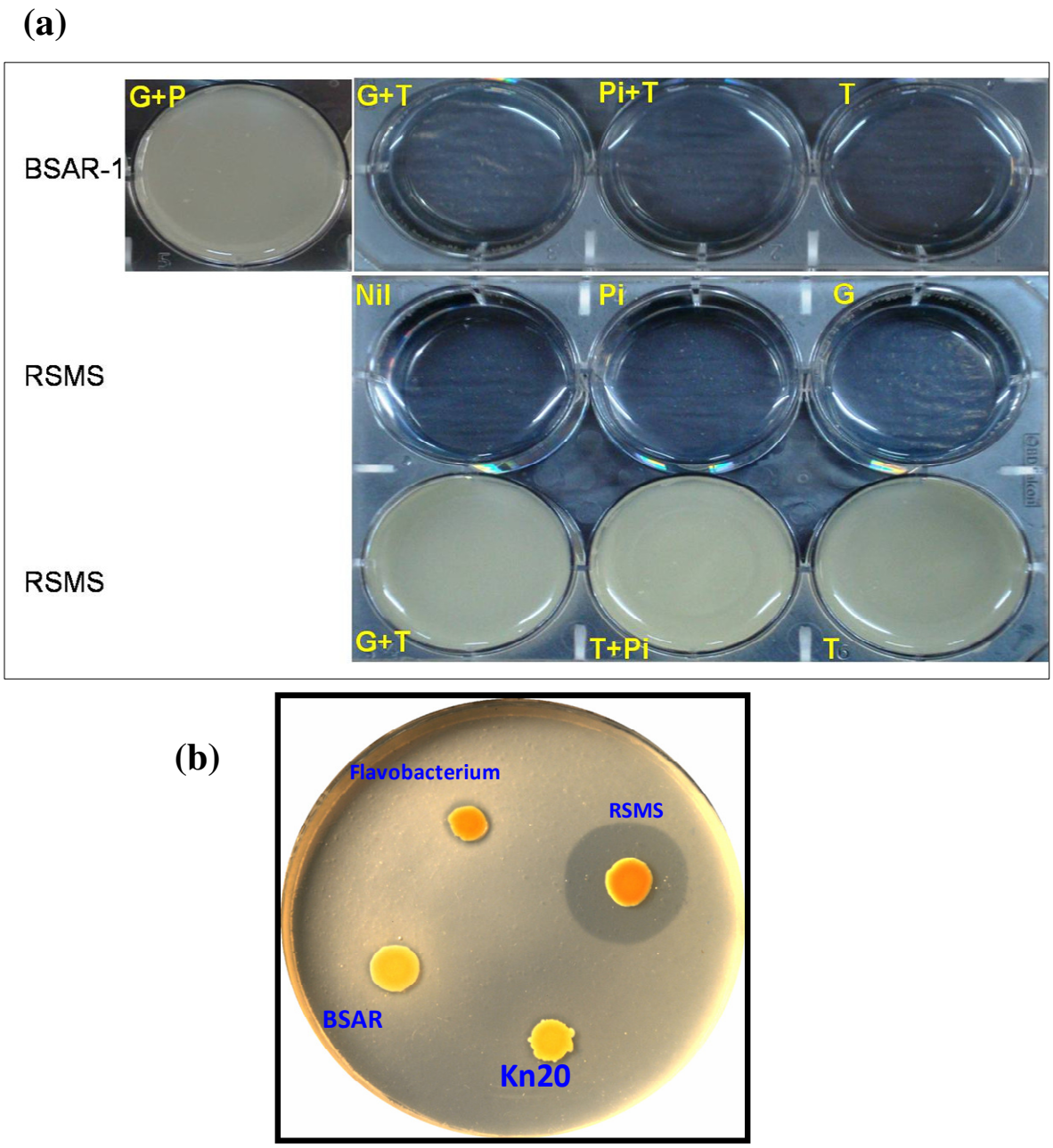

Fig. 3.9. Visualisation of the RSMS strain growth using TBP as the sole source of carbon and phosphorous. (a) The growth of the cultures was visualized after 48 hours incubation in MMM containing specified components. *Sphingomonas* strain BSAR-1 was used as a control under identical growth conditions. G, Pi and T depict 1% glucose, 5 mM KH_2PO_4 and 2 mM^e TBP respectively, while "Nil" indicates that no carbon or phosphorous source was added to the MMM. In case of RSMS 5 mM^e TBP was used. (b) Ability of RSMS strain to degrade TBP when supplemented with 10 mM^e TBP in LB agar medium. The zone of clearance was visualized after 7 days of incubation at 30°C.

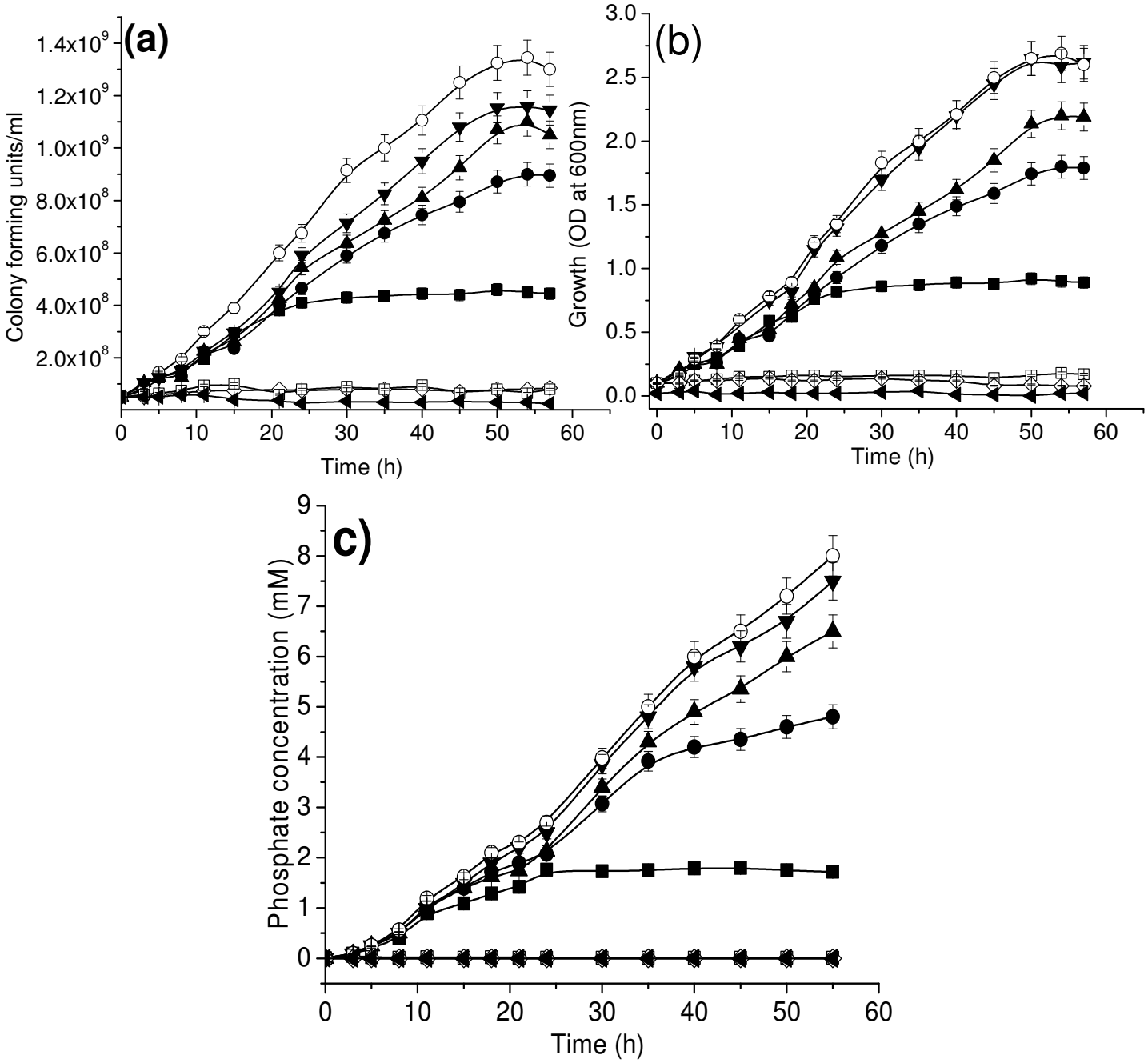

Fig. 3.10. Growth and TBP degradation kinetics of the RSMS strain. Ability of the RSMS strain to use TBP as the sole source of carbon and phosphorous was assessed by inoculating the strain at 0.1 OD_{600nm} (~ $5x10^7$ CFU/ml), in to MMM supplemented with 2 mM^e (■), 5 mM^e (●), 10 mM^e (▲), 30 mM^e (○), or 50 mM^e (▼) of TBP. Growth was assessed as colony forming units (a), or increase in optical density at 600nm (b). TBP degradation (c) was spectrophotometrically estimated as phosphate released upon TBP degradation. Following controls were also included: TBP supplemented MMM without cells (◄), glucose supplemented culture in MMM without added TBP (□), or KH_2PO_4 supplemented culture in MMM without added TBP (◊).

3.7. Optimisation of initial inoculum density and TBP concentration for TBP degradation.

The initial inoculum density determined the rate of TBP degradation by RSMS strain (Fig. 3.11). Cultures inoculated at 3 OD_{600nm}/ml degraded 30 mM^e TBP in 3 days, whereas, cultures inoculated at 0.1 OD_{600nm}/ml degraded only 25 mM^e TBP in 8 days (Fig. 3.11). Cell densities more than 3 OD_{600nm}/ml did not show significant improvement of TBP degradation (Fig. 3.11). Cultures inoculated at 3 OD_{600nm}/ml degraded 2 mM^e TBP (concentration used in previously reported studies) within 8h after inoculation (Table 3.8). No further increase in the rate or amount of TBP degradation was observed beyond 30 mM^e TBP concentration.

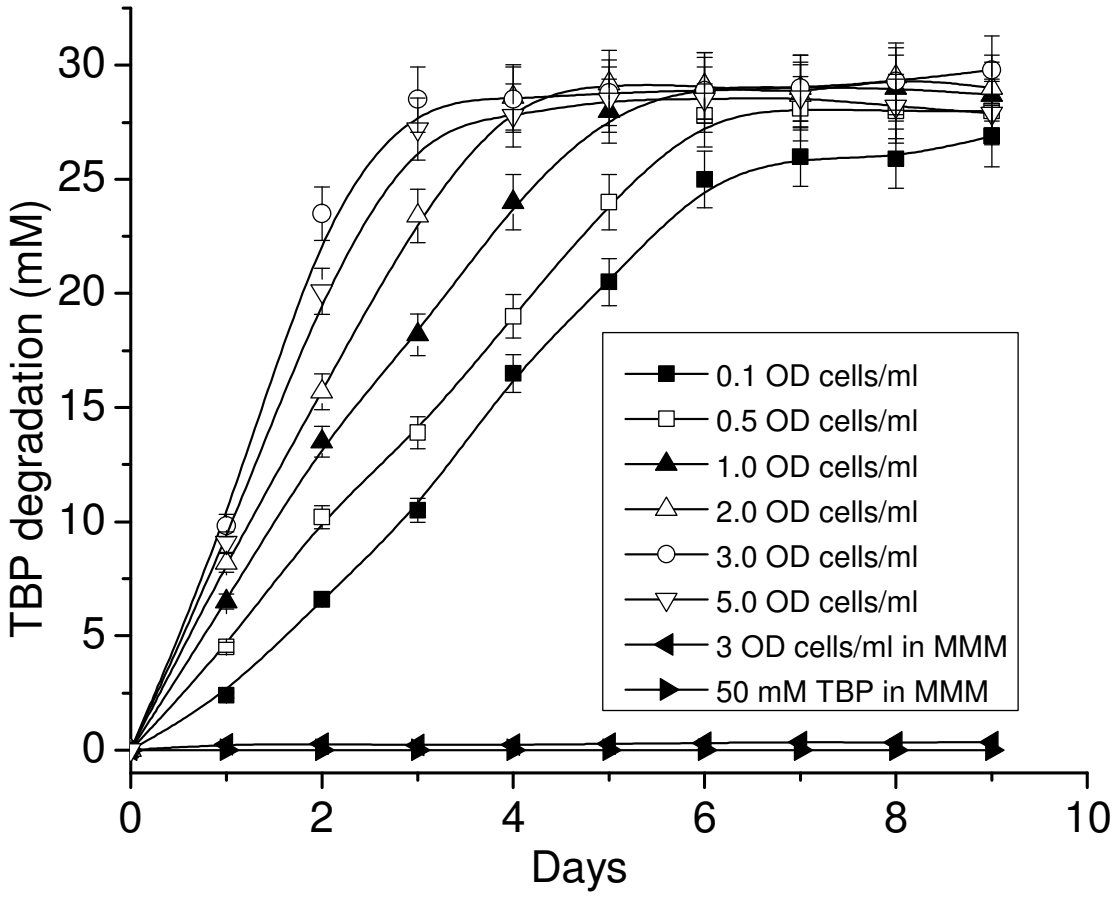

Fig. 3.11. Optimisation of cell density for TBP degradation. Degradation of TBP by RSMS strain inoculated at different cell densities (OD_{600nm}) in MMM supplemented with 30 mM^e TBP as the sole source of carbon and phosphorous. TBP degradation was spectrophotometrically assessed in terms of phosphate released 24 after inoculation.

Table 3.8. Time required for complete degradation of different concentrations of TBP using 3 OD_{600nm} cells/ml.

	Concentration of TBP (mM^e)				
Amounts of TBP added (mM^e) to MMM	2	5	10	20	30
Time (h) taken for the complete degradation of added TBP	8 ± 0.5	18 ± 1	31.5 ± 3	55.5 ± 4.5	73 ± 5.5

Degradation of TBP by RSMS strain inoculated at 3.0 OD_{600nm}/ml in to MMM containing various TBP concentrations. TBP degradation was monitored as phosphate released, at different time intervals. The values reported are average of three replicates.

3.8. TBP degradation and growth under static conditions.

TBP degradation and growth were also observed under static conditions, though at a slower initial rate in comparison to growth under shaking conditions (Table 3.9). The growth and degradation of TBP started very late in comparison to the shaking conditions. When the strain was inoculated at 3.0 OD_{600nm}/ml, under static conditions, the TBP degradation was at least 10 times slower than that under shaking, as observed after 2 days of inoculation (Table 3.10).

3.9. Degradation of dibutyl phosphate.

Degradation of dibutyl phosphate (DBP) was also studied using RSMS strain in a similar way. The strain could efficiently use DBP as sole source of carbon and phosphorous. The growth of the strain (in terms of CFU) using DBP as a sole source of carbon and phosphorous was found to increase with increasing concentrations of DBP up to 50 mM DBP (Fig. 3.12). The number of CFUs in MMM suspensions containing 50 mM DBP increased from $5x10^7$/ml at inoculation to $8x10^8$/ml after 30 h. The increase in CFUs is equivalent to >15 times increase in cell number or 4 doublings. The strain showed DBP degradation even after reaching stationary phase (Fig. 3.12b).

The strain could degrade a maximum of 50 mM DBP within 4 days after inoculation when inoculated at 3 OD_{600nm}/ml (as compared to maximum of 30 mM^e TBP in 3 days) (Fig. 3.12d). The degradation of DBP increased with increasing concentration of DBP. The maximal rate of DBP degradation was found to be ~12 mM/d at 50 mM DBP concentration (Fig. 3.12d).

Table 3.9. TBP degradation and growth of the RSMS strain under static conditions.

	Growth of the strain (OD_{600nm}/ml)				**TBP degradation (mM^e)**			
Time (days)	**1**	**2**	**5**	**6**	**1**	**2**	**5**	**6**
Shaking (180 rpm)	1.05	1.85	1.69	1.59	2.2	4.8	9.5	9.4
Static conditions	0.22	0.33	0.95	1.42	0.048	0.35	6.5	8.2

The strain was inoculated at 0.1 OD_{600nm}/ml in to MMM containing 10 mM^e TBP and incubated at 30°C, either under shaking at1 180 rpm or under static conditions. Growth was measured spectrophotometrically as increase in cell density at 600 nm and TBP degradation was measured spectrophotometrically as inorganic phosphate released

Table 3.10. Degradation of 30 mM^e TBP under static conditions.

	TBP degradation (mM^e)			
Time (days)	**1**	**2**	**5**	**6**
Shaking (180 rpm)	9.5	20.5	27.5	27.8
Static conditions	0.5	2.8	8.5	12.6

Degradation of 30 mM^e TBP with 3 OD_{600nm}/ml cells under static conditions. TBP degradation was measured spectrophotometrically as inorganic phosphate released. The values represent average of three independent experiments. The values showed less than 10% of deviation from the mean value.

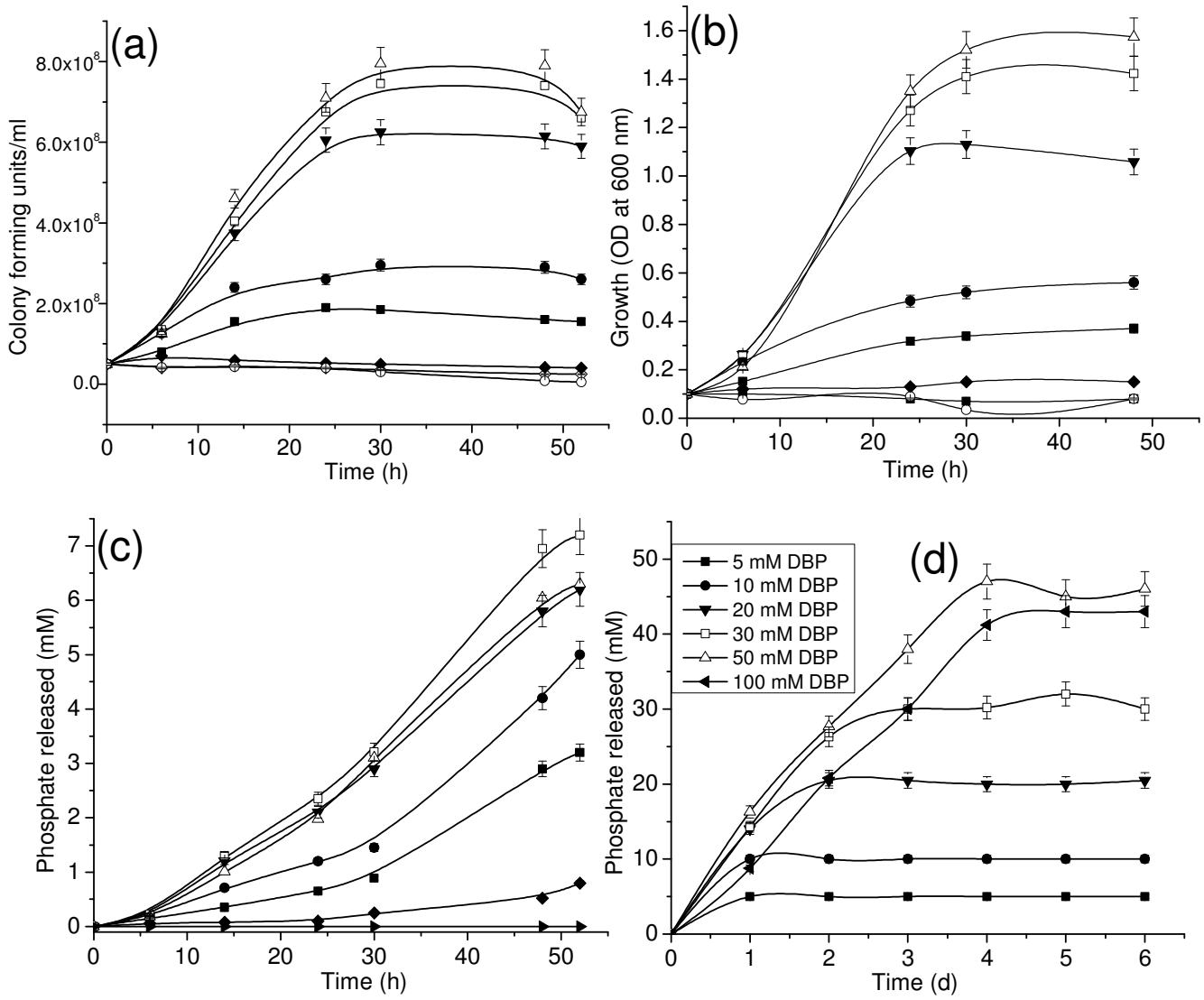

Fig. 3.12. Growth and DBP degradation kinetics of the RSMS strain. Growth was assessed as colony forming units (a), or increase in optical density at 600 nm (b), and DBP degradation was spectrophotometrically estimated as phosphate released upon DBP degradation (c). Growth and DBP degradation were studied in MMM supplemented with 2 mM (♦), 5 mM (■), 10 mM (●), 20 mM (▼), 30 mM (□), or 50 mM (Δ) of DBP, as the sole source of carbon and phosphorous. Initial inoculum density was set at an OD_{600nm} of 0.1 (~ $5x10^7$ CFU/ml). Following controls were also included for comparison: DBP supplemented MMM without cells (◄), glucose supplemented culture in MMM without added DBP (O), or KH_2PO_4 supplemented culture in MMM without added DBP (◊). (d) Degradation of different concentrations of DBP with $3OD_{600nm}$/ml cells.

3.10. Stability of the TBP degradation and utilization by RSMS strain.

Stability of the RSMS strain with respect to TBP degradation and its utilization was assessed in 20 repeated consecutive subcultures of 24 h duration each, in MMM supplemented with 10 mM^e TBP. In 20 repeated subcultures the strain sustained its ability to degrade and utilize TBP as a sole source of carbon and phosphorous (Table 3.11).

Table 3.11. TBP degradation and utilization for 20 repeated subcultures.

subculture	Growth (OD_{600nm}/ml)	TBP degradation	subculture	Growth (OD_{600nm}/ml)	TBP degradation
1	1.06	1.95	**11**	0.79	1.80
2	0.9	1.72	**12**	0.79	1.65
3	0.85	1.65	**13**	0.85	1.72
4	0.825	1.75	**14**	0.92	1.69
5	0.87	1.59	**15**	0.82	1.65
6	0.89	1.8	**16**	0.91	1.59
7	0.81	1.72	**17**	0.82	1.65
8	0.82	1.79	**18**	0.85	1.72
9	0.95	1.69	**19**	0.82	1.8
10	0.82	1.62	**20**	0.81	1.71

Stability of the RSMS strain with respect to TBP degradation and utilization was assessed in MMM containing 10 mM^e TBP as the sole source of carbon and phosphorous. After 24 h in each subculture, cells were harvested, washed three times with equal volume of MMM and resuspended at 0.1 OD_{600nm}/ml in fresh MMM containing 10 mM^e TBP. The experiment was repeated for 20 subcultures. TBP degradation was monitored spectrophotometrically, in terms of inorganic phosphate released, and growth was assessed as increase in optical density at 600 nm.

Average growth and degradation of TBP during 20 subcultures was found to be 0.88±0.027 (OD_{600nm}), and 1.73±0.032 mM, respectively in 24 hours. Cultures of RSMS strain stored as glycerol stocks in -70°C did not lose the ability of TBP degradation and growth even after 6 years (data not shown).

3.11. Butanol as the sole source of carbon for the growth of the RSMS strain.

RSMS strain utilized butanol as the sole source of carbon for its growth (Fig. 3.13). The growth of the strain increased with increasing concentration of butanol till 40 mM. The cultures reached cell densities of 2.5 ± 0.2 OD_{600nm}/ml at 30/40 mM of butanol concentration, which is equivalent to ~4.5 generations or ~25 times increase in the cell number. The growth was, however, found to be reduced above 50 mM concentration.

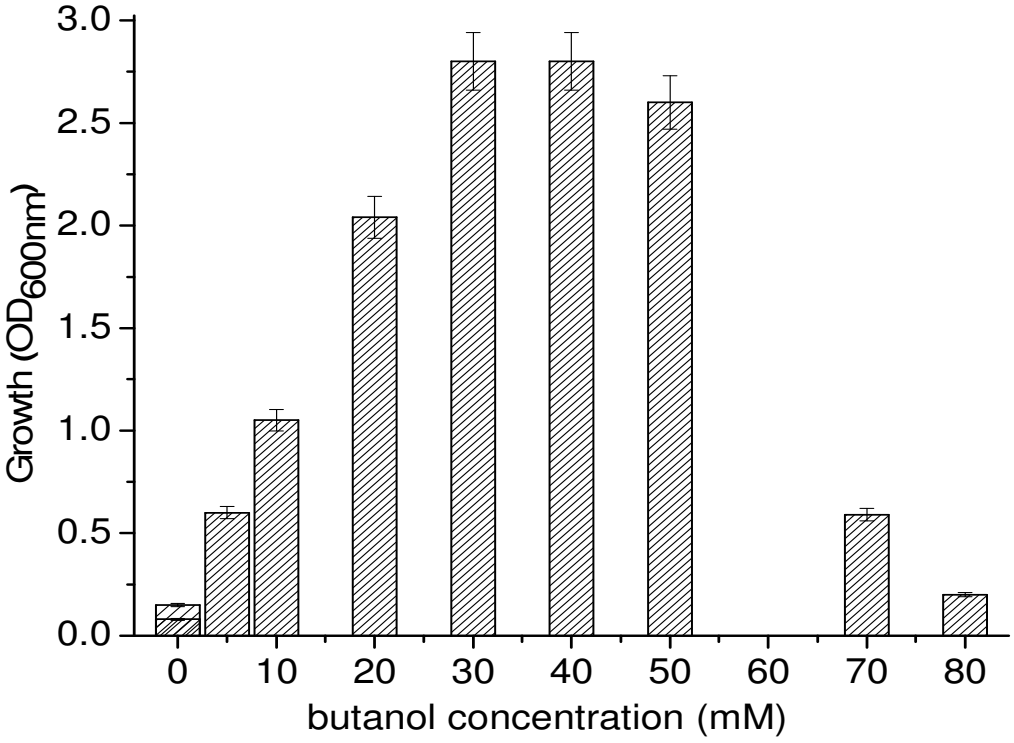

Fig. 3.13. Growth of RSMS strain in MMM supplemented with varied concentrations of butanol and 5 mM KH_2PO_4. The strain was inoculated at a density of $5x10^7$ cells/ml, and incubated at 30°C and 180 rpm. The growth was assessed as increase in optical density at 600nm, after 24 h incubation.

3.12. Tolerance of RSMS strain to TBP, DBP and butanol

3.12.1. Tributyl phosphate

The strain tolerated high concentrations of TBP (Fig. 3.14). There was no change in the number of the CFUs (280 $\pm$ 20) and their morphology even at 100 mM^e TBP concentrations compared to control (1% glucose+ 5 mM KH_2PO_4), except that 100 mM^e TBP showed smaller colonies in comparison to those at lower concentrations of TBP. In comparison, the strain showed lesser tolerance towards TBP in liquid medium (Fig. 3.10).

3.12.2. Dibutyl phosphate.

Strain RSMS also survived high concentrations of DBP (Fig. 3.15a). The number of CFUs remained unaltered in MMMA containing up to 60 mM concentrations of DBP. There was no reduction in the size of the colony and no change in the morphology of the colony even at 60 mM DBP. At 75 mM DBP concentration the number of CFUs reduced significantly, and at 90 mM no colonies were observed. However, when $3OD_{600nm}$/ml cells were used in liquid media, the cells survived 100 mM of DBP and degraded about 45 mM of DBP (Fig. 3.12d).

3.12.3. Butanol.

The RSMS strain used butanol as the sole source of carbon for the growth and gave visible colonies (Fig. 3.15b). The strain survived upto 50 mM of added butanol and the number of CFUs remained unaltered in MMMA containing up to 50 mM concentrations of butanol. However, no colonies were observed in 60 mM of butanol. Thus, MIC of the butanol for RSMS strain was found to be 60 mM.

3.13. Degradation and utilization of Tri-iso amyl alcohol phosphate.

In recent past, several studies have proved Tri-isoamyl alcohol phosphate (TAP) to be a superior solvent in comparison to Tributyl phosphate (TBP), with respect to the efficiency for Uranium and Plutonium extraction [12].

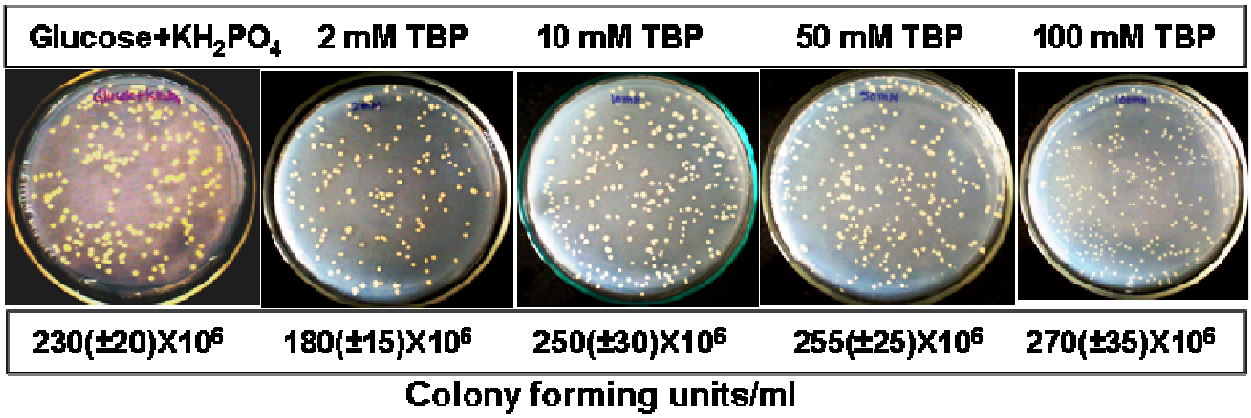

Fig. 3.14. Tolerance of RSMS strain to different concentrations of TBP. Tolerance was assessed by spreading 100 µl of 1 OD_{600nm} cells/ml at $5x10^5$ dilution on MMM agar supplemented with varying concentrations of TBP. MMMA supplemented with 1% glucose and 5 mM KH_2PO_4 was used as control. CFUs in test plates were compared with the control. The plates were incubated at 30°C for three days.

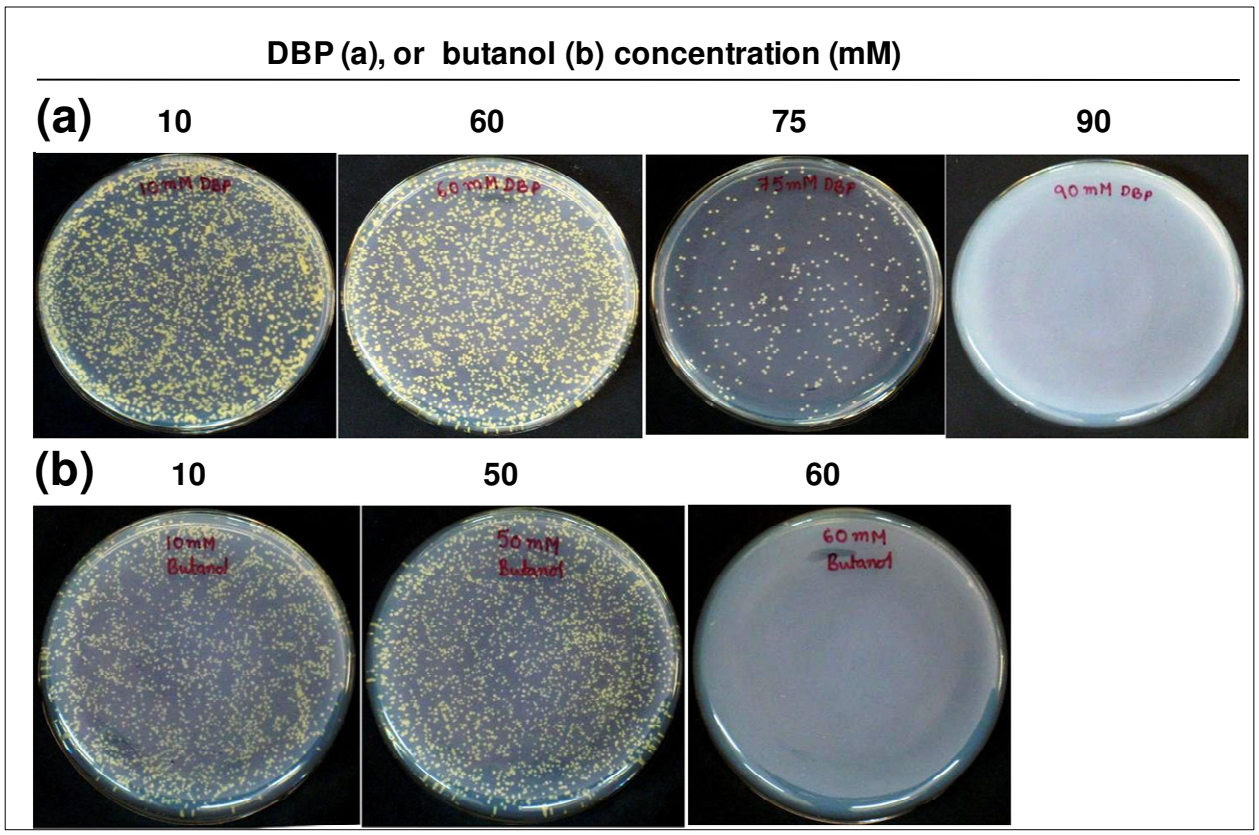

Fig. 3.15. Tolerance of RSMS strain to DBP and butanol. Tolerance of the strain to different concentrations of (a) DBP and (b) butanol were assessed by spreading 100 µl of 4 OD_{600nm} cells/ml at $5x10^5$ dilution on MMM agar supplemented with varying concentrations of DBP or butanol and 5 mM KH_2PO_4. The plates were incubated at 30°C for three days.

Structurally, TAP has three iso-amyl alcohol moieties (where as TBP contains three butanol moieties) covalently attached to three oxygen atoms of a phosphoric acid (Fig. 3.16) Because of the extra methyl group in iso-amyl alcohol, the solubility of TAP is ~20 times lesser than that of the TBP. Low solubility and the presence of branched alkyl chain makes TAP less prone to acidic degradation [12]. The potential of TAP solvent has already been established and the solvent has proved to be very effective and economically more viable than TBP [12]. Usage of TAP will lead to high volumes of nuclear waste whose management would rather be difficult. In view of this, experiments were carried out to investigate the bio-degradation of TAP using RSMS strain.

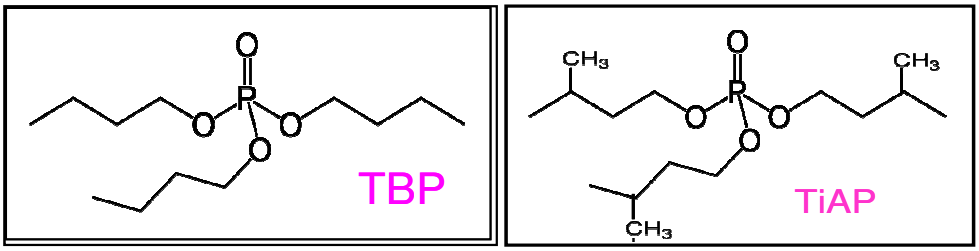

Fig. 3.16. Structural differences between tributyl phosphate and tri-isoamyl alcohol phosphate

MMM containing 1% glucose or KH_2PO_4 alone did not support the growth of the RSMS strain. However, MMM supplemented with 5 mM^e TAP alone supported active growth of the strain, suggesting that the strain utilized TAP as sole source of carbon and phosphorous (Fig. 3.17a). Growth and TAP degradation by the strain increased with increasing amounts of TAP up to 30 mM^e (Fig. 3.17a and b). The number of CFUs increased up to ~12 times in 8 days, through 3.5 doublings. The low rate of TAP degradation and less efficiency of utilization as a carbon source, as compared to TBP could be attributed to the low solubility of the TAP.

When RSMS strain was inoculated at different cell densities in to MMM supplemented with 30 mM^e TAP, the TAP degradation after 24 hours found to be 1 mM^e (± 0.15mM) and 5 mM^e (± 0.5 mM), respectively, for 0.5 and 3 OD_{600nm} cells/ml (Fig. 3.18a).

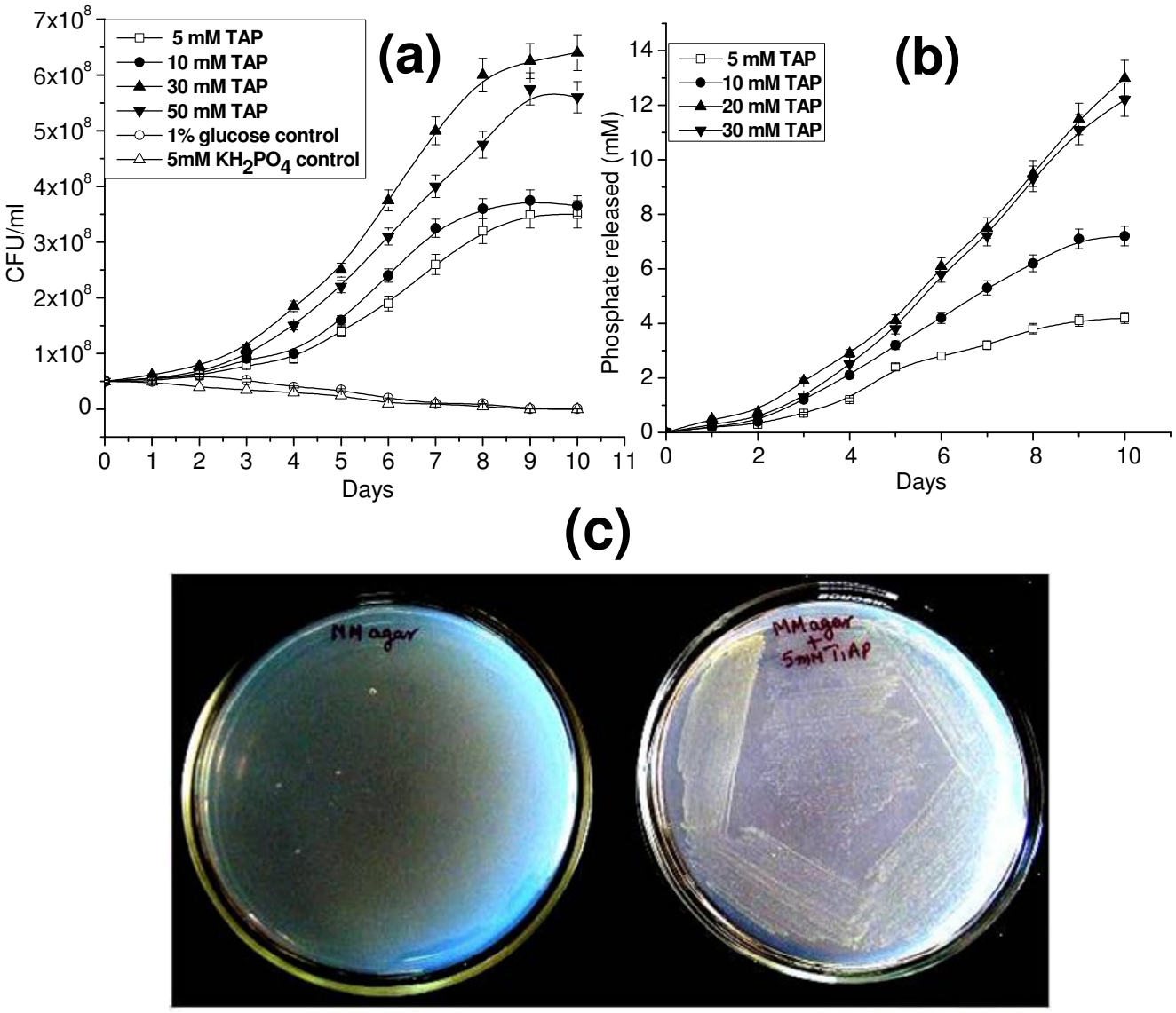

Fig. 3.17. Growth and TAP degradation kinetics of the RSMS strain. (a) Growth and (b) TAP degradation were studied in MMM supplemented with 5 mM^e (□), 10 mM^e (●), 30 mM^e (▲), or 50 mM^e (▼) of TAP, as the sole source of carbon and phosphorous. Initial inoculum density was set at an OD_{600nm} of 0.1 (~ $5x10^7$ CFU/ml). Following controls were also included for comparison: glucose supplemented culture in MMM without added TAP (○), or KH_2PO_4 supplemented culture in MMM without added TAP (Δ). (a) Growth was assessed as colony forming units (CFU), and (b) TAP degradation was spectrophotometrically estimated as phosphate released upon TAP degradation. (c) Growth of RSMS strain on minimal agar medium supplemented with TAP as sole source of carbon.

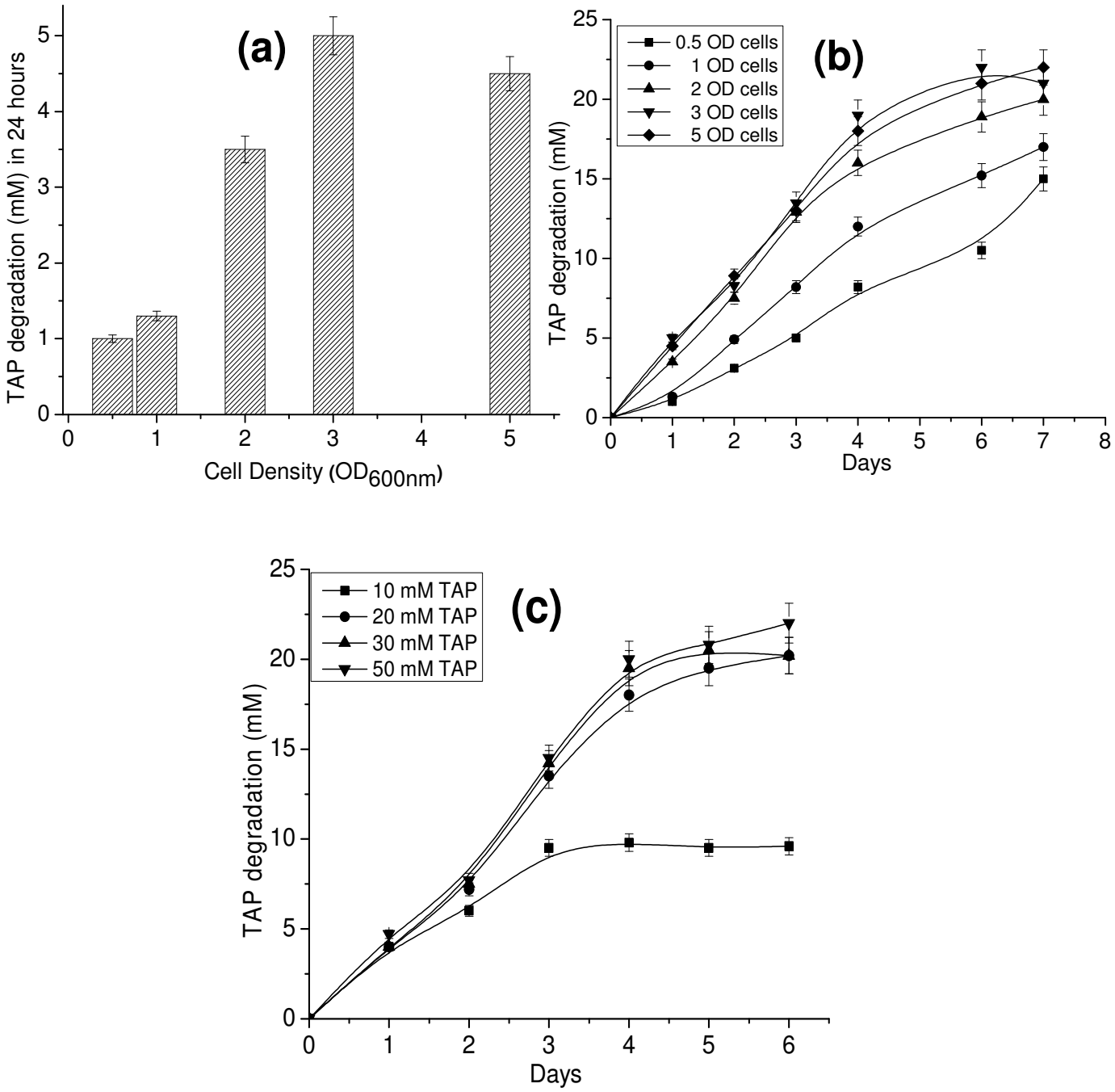

Fig. 3.18. TAP Degradation kinetics in RSMS strain. Degradation of TAP by RSMS strain inoculated at different cell densities (OD_{600nm}/ml) in MMM supplemented with 30 mM^e TAP. The degradation was measured after 24 hours (a), or for 7 days (b). c) Degradation of various concentrations of TAP with 3 OD_{600nm}/ml cells. TAP degradation was spectrophotometrically assessed in terms of phosphate released.

Inoculation of 3 OD_{600nm} cells/ml of RSMS showed 20 mM^e TAP degradation in 4 days (Fig. 3.18b). Cell densities above 3 OD_{600nm}/ml did not show any significant improvement in degradation (Fig. 3.18b).

Experiments were also carried out to see the degradation of various concentrations of TAP with 3 OD_{600nm} cells/ml (optimum cell density). RSMS strain showed incomplete TAP degradation, i.e., only 20 mM^e TAP was degraded in 4 days (Fig. 3.18c).

Discussion

The previous studies randomly isolated TBP degrading strains from river, soil and other samples, and did not specifically choose sites which were exposed to TBP. Such choice allowed isolation of strains which could degrade or tolerate rather low concentrations of TBP. This study chose TBP storage tanks at BARC for the isolation of TBP degrading strain, wherein the TBP concentrations were very high and no other carbon or phosphorous source was available. Such a choice might have facilitated exclusion of organisms incapable of utilizing TBP or unable to survive at such high TBP concentration.

Xenobiotics or organic compound degradation abilities of several strains have been demonstrated using zone of clearance principle. For example, bacterial strains which degrade cellulose, hydrocarbons, polycaprolactones, extracellular protein, crude oil, or chlorothalonil exhibit such zone of clearance, when respective substrate are used in the medium [82-87]. However, such phenotype has not been reported for any of the earlier strain reported to degrade TBP. *Sphingobium* strain RSMS degraded high levels of TBP present in solid LB agar medium or MMM medium, showing a zone of degradation around the colony (Fig. 3.1).

Such phenotype could be very useful as a screen for isolation of TBP non-degrading mutants of RSMS strain or new strains of bacteria capable of degrading TBP.

The RSMS strain was subjected to different biochemical and molecular tests to obtain its definite identity. Presence of the 18:1 as the major fatty acid (Table 3.3) is one of the important characteristics of the strains belonging to Sphingomonads [41]. FAME analysis based 2-hydroxy fatty acid profiles provide valuable information for the differentiation of the four clusters (Genera) of Sphingomonads, namely, *Sphingomonas*, *Sphingopyxis*, *Novosphingobium* and *Sphingobium* [41]. Members of *Sphingomonas* (Cluster I) contain 2-OH 14:0 and/or 2-OH 15:0 as the major fatty acid, whereas those of *Sphingopyxis* (Cluster IV) are characterized by the presence of 2-OH 15:0 and/or 2-OH 16:0 in addition to 2-OH 14:0. Members of *Sphingobium* (Cluster II) and *Novosphingobium* (Cluster III) contain 2-OH 14:0 as the major fatty acid [41]. Strain RSMS contains only one hydroxyl fatty acid, 2-OH 14:0, which constitutes 7.51 percent of total fatty acids (Table 3.3), suggesting that it could belong to either clusture II or III. However, signature nucleotides of 16s *rRNA* gene [41] confirmed that the RSMS strain belongs to the genus *Sphingobium* (Table 3.6). The strain was negative for nitrate reductase activity (Table 3.2), which is also a characteristic feature of genus *Sphingobium*, and distinguishes it from other two clusters (*Sphingomonas* and *Novosphingobium*) of Sphingomonads. Though several bacterial isolates have been reported to degrade TBP, this is the first study to report a Sphingomonad as a TBP degrader. The strains of Sphingomonads are very well known for their ability to degrade a variety of refractory organic compounds or xenobiotics (Table 1.6), suggesting their ability to adapt to the new conditions very rapidly.

TBP at 10 to 400 μM concentration inhibits cell division in most bacteria [9]. Few strains have been reported to tolerate 5 mM^e TBP concentration, which is the highest tolerance to TBP

reported so far [24]. In contrast, the RSMS strain was found to tolerate as high as ~100 mM^e in solid media (Fig. 3.14) and >30 mM^e TBP in suspension (Fig. 3.10) without loss of viability in MMM which is truly remarkable. It could also survive well on 50 mM DBP or 50 mM butanol (Fig. 3.15), which have been proposed as likely intermediates and products of TBP degradation. Such high tolerance is needed when dealing with the actual waste where the concentrations of TBP are very high [4].

One of the most important characteristic features required for a bioremediation proficient strain is to maintain stability. Few of the previously reported strains were found to be unstable over a period of time, even after subculturing them in TBP containing medium. For example, mixed cultures of *Pseudomonas* showed alternative rapid and slow growth periods during serial subcultures, and single strains of *Pseudomonas* cultures irreversibly lost the ability to degrade or grow on TBP after 8 serial subcultures [36]. Stability of the TBP degradation and utilization of RSMS strain was carefully monitored for 20 repeated subcultures of 24 h duration each. RSMS strain showed excellent stability over several repeated subcultures, both with respect to TBP degradation and its utilization as sole source of carbon and phosphorous (Table 3.11).

TBP degradation capabilities of bacterial strains reported earlier range from 1.6 mM^e in 3 weeks (*R. palustris*) and 2 mM^e in 3 days (mixed cultures of *Pseudomonas*) [25,37] at 2 mM^e TBP concentration (Table 3.12). A recent study reported release of ~0.6 mM inorganic phosphate from media suspensions containing equivalent of 5 mM^e TBP in 4 days [24]. In contrast, the RSMS strain was found to be far more efficient, and degraded 2 and 30 mM^e TBP in 8 h or 73 h, respectively (Table 3.8 and Fig. 3.11). This is the highest rate of TBP degradation reported so far.

The RSMS strain was also found to be capable of degrading DBP and TAP, which is a higher

homologue of TBP. Solubility of TAP is far less (~20 times) than TBP, which makes it a very difficult target for biodegradation, but RSMS strain could degrade 20 mM^e TAP in 4 days and also utilize TAP as the sole source of carbon and phosphorous (Fig. 3.17 and 3.18). It also tolerated and utilized high concentration of butanol (Fig. 3.13 and 3.15). While butanol and DBP are expected products and intermediates of TBP degradation, the TAP is a solvent of the future. Isolation of a single strain capable of managing all these toxic solvents can prove to be a boon for nuclear industry. The isolated *Sphingobium* sp. strain RSMS showed distinct novelties and was found to be superior to several previously reported TBP degrading bacteria in many respects (Table 3.12). It exhibited much higher tolerance to TBP/DBP/Butanol, and far better stability and efficiency of TBP/DBP degradation. It could also utilse all of them as sole source of carbon and phosphorous. These abilities make *Sphingobium* sp. strain RSMS an attractive choice for TBP/DBP bioremediation.

Though several strains were reported to degrade TBP earlier (Table 3.12), only a few strains could utilize it as a sole source of carbon and phosphorous, though not efficiently [24,32,36]. The RSMS strain efficiently degraded and used TBP, DBP and TAP for growth. The solubility of TBP in water is 400 mg/L (1.5 mM) [7]. However, TBP concentration at storage sites is 1.1 M, and any real biodegrading strain must function at much higher concentration of TBP than what the solubility permits. We attempted such degradation of TBP from non-homogeneous liquid suspensions and in solid media containing equivalent of up to 30 mM^e TBP. Even from such immiscible suspensions, TBP was rapidly and completely degraded and utilized by RSMS strain (Fig. 3.11). This was evident from, (1) formation of a clear, transparent zone of clearance around cells on TBP containing agar plates (Fig. 3.1), (2) TBP concentration dependent increase in phosphate release (TBP degradation) as well as growth of the RSMS strain (>25 times

increase in CFUs) in TBP supplemented suspensions (Fig. 3.10), and (3) release of ~29 mM inorganic phosphate after complete degradation of 30 mM^e TBP (Fig. 3.11). The strain also utilized DBP (50 mM) and butanol (50 mM) very well, indicating that it can not only remediate TBP but also take care of its degradation products. This type of efficient utilization can prevent the accumulation of toxic byproducts such as butanol formed due to TBP degradation in the medium and is very desirable in environmental bioremediation.

Table 3.12. Comparison of TBP degradation capabilities between RSMS strain and earlier reported strains

Strain and reference	Maximum degradation or sequestration (mM^e)	TBP as sole source of carbon and Pi for growth	Bio-activity reported for TBP*
Mixed culture of *Pseudomonas* [36]	2 mM^e in 3 days	Yes	Mineralization
Mixed culture of *Acenetobacteria* [28]	< 1mM in 4weeks	Yes	Mineralization
Purple non photosynthetic bacteria [25]	0.6 in 3 weeks	No	Disappearance
R. Palustris [25]	1.6 mM^e in 3 weeks	No	Disappearance
Serratia odorifera [26]	0.6 in 8h	No	Disappearance
R1-isolate [30]	1 mM in 5 days	Yes	Mineralization
Pseudomonas pseudoalcaligenes [27]	~0.1 mM in 6 days	Yes	Mineralization
Deilftia sp. BGW1 [24]	~3 mM^e in ~4 days	Yes	Partial mineralization (~0.6 mM)
RSMS (present study)	30 mM^e in 3 days	Yes	Mineralization

*Mineralization refers to complete degradation to inorganic phosphate. Disappearance refers to the sequestration of TBP from the medium, which may or may not result in the release of any product of TBP degradation.

Chapter IV

Investigations on the molecular basis of TBP biodegradation by RSMS strain

TBP is a phoshotriester and complete mineralization of TBP was earlier proposed to be mediated by phosphoesterases, where the tri-, di- and monoesterases would sequentially act on TBP releasing butanol and phosphate as final products [36]. Though several strains capable of degrading TBP have been known, the gene (s) or the proteins required for such pathway of TBP degradation still remain unknown. Similarly, the knowledge about the regulation of TBP degradation is quite limited. TBP degradation is known to be inhibited by inorganic phosphate (final product of TBP degradation) above 10 mM concentration [80]. Possibility of its regulation by catabolite repression has never been explored. Catabolite repression is a mechanism whereby the expression of genes needed for the utilization of secondary carbon sources is prevented in the presence of a preferred carbon source such as glucose. The process was discovered in *E. coli* and was originally referred to as the glucose effect, because, it was found that glucose repressed the synthesis of enzymes required for the utilization of secondary carbon sources (lactose). The reverse of the glucose effect is also known wherein glucose utilization is suppressed by organic acids/aromatic compounds [88-93]. For example, *Pseudomonas putida* strain CSV86 prefers aromatic compounds over glucose [88].

This study, demonstrated negative effects of inorganic phosphate and glucose on TBP degradation by the RSMS strain. A TBP non-degrading mutant was isolated and characterized to reveal potential protein candidates likely to be responsible for TBP/DBP degradation in RSMS strain.

4.1. Phosphate mediated inhibition of TBP degradation

Inorganic phosphate (KH_2PO_4) at low concentrations (5 mM) increased the growth of the strain in presence of TBP (Fig. 4.1). However, inorganic phosphate at 30 mM concentration and above inhibited the growth of the strain. No growth was observed in cultures supplemented with 10 mM^e TBP and 50 mM inorganic phosphate (Fig. 4.1). Degradation of TBP (in terms of inorganic phosphate released) could not be measured in this experiment accurately due to interference by added excess inorganic phosphate.

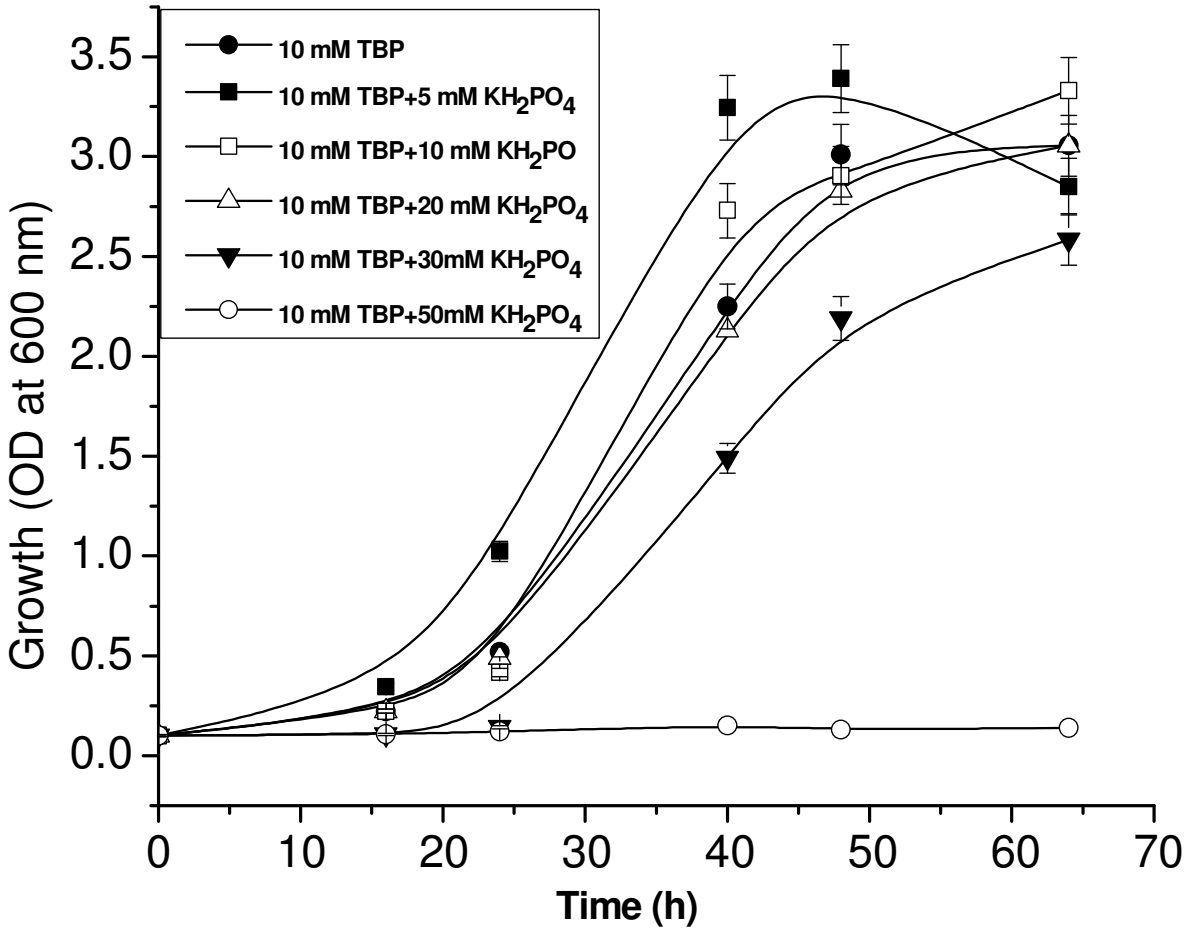

Fig. 4.1. Effect of inorganic phosphate on TBP degradation. RSMS strain was inoculated in to MMM supplemented with 10 mM^e TBP containing various concentrations of KH_2PO_4. Growth was monitored spectrophotometrically at 600 nm.

4.2. Suppression of TBP and DBP degradation in the presence of glucose

Possible catabolic repression by glucose was evaluated (Fig. 4.2) in MMM suspensions containing 10 mM^e TBP, wherein the growth was less than that in 30 mM^e TBP suspension indicating that available carbon and phosphorous were limiting. Cultures grown in 10 mM^e TBP suspensions alone, showed 3 times less growth compared to the cultures grown in same amounts of TBP along with 0.2% or 0.5% glucose (Fig. 4.2a), indicating positive effect of low concentration of carbon source. But, the TBP degradation was observed to be similar in all the above cases (Fig. 4.2b). Decreased TBP degradation and corresponding growth inhibition (due to unavailability of phosphorous source) were observed with increasing concentrations of glucose above 0.5% (Fig. 4.2a and b). No degradation of TBP and correspondingly no growth was observed in cultures supplemented with 5% or 10% glucose (Fig. 4.2a and b). The culture containing 2% glucose and 10 mM^e TBP showed ~24 h of lag in growth, during which no TBP degradation was observed (Fig. 4.2b).

DBP degradation and DBP dependent growth were found to be similarly inhibited by 10% glucose (Table. 4.1). The suppression of TBP or DBP dependent growth in presence of 10% glucose could be clearly visualized on MMM agar plates containing 10 mM TBP or DBP supplemented with or without 10% glucose (Fig. 4.3). However, such repression of TBP/DBP degradation by glucose was reversible (Fig. 4.4), and TBP degradation and TBP dependent growth were restored upon removal of glucose (Fig.4.4a and b). RSMS cultures which were grown in MMM containing 5 mM^e TBP, when transferred to similar medium (MMM with 5 mM^e TBP) did not show any lag with respect to TBP degradation or growth. In contrast, cultures grown in 5 mM^e TBP and 5% glucose when transferred to MMM supplemented with 5 mM^e TBP showed a ~6 h lag in growth (Fig. 4.4).

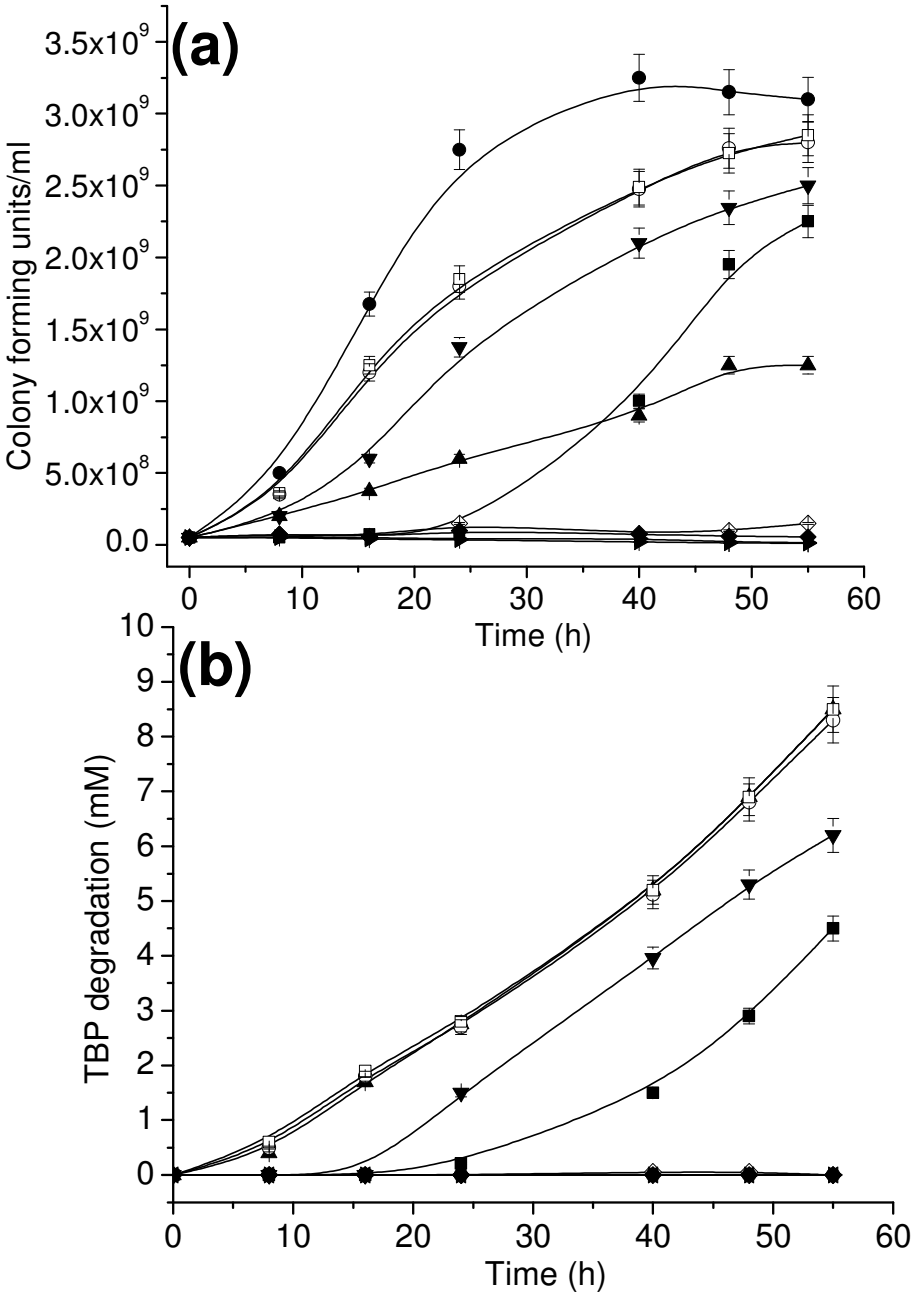

Fig. 4.2. Inhibition of (a) TBP dependent growth, and (b) TBP degradation by glucose. MMM suspension containing 10 mM[e] TBP (▲) was supplemented with glucose either at 0.2 % (o), 0.5 (□) %, 1 % (▼), 2 % (■), 5 % (◊), or 10 % (◄) concentration. Following controls were also included: cells in MMM (►), cells in MMM supplemented with 10 % glucose (♦), or cells in MMM supplemented with 10% glucose and 5 mM KH_2PO_4 (●). Growth was assessed as colony forming units (CFU) and TBP degradation was spectrophotometrically estimated as phosphate released upon TBP degradation.

Table. 4.1. Suppression of TBP and DBP degradation in the presence of 10% Glucose

Additions to the MMM	Growth (OD_{600nm})	Phosphate released (mM)
10 mM^e TBP	1.15 ± 0.09	2.3 ± 0.15
10 mM^e TBP + 10% Glucose	0.08 ± 0.02	Nil
10 mM DBP	0.75 ± 0.05	0.95 ± 0.07
10 mM DBP + 10 % glucose	0.07± 0.02	Nil

RSMS strain was inoculated at 0.1 OD_{600nm} in MMM supplemented with 10 mM^e TBP or 10 mM DBP with or without 10% glucose. TBP or DBP degradation (phosphate released) and growth (increase in OD_{600nm}) were measured 24 h after inoculation. Values reported are averages of three replicates.

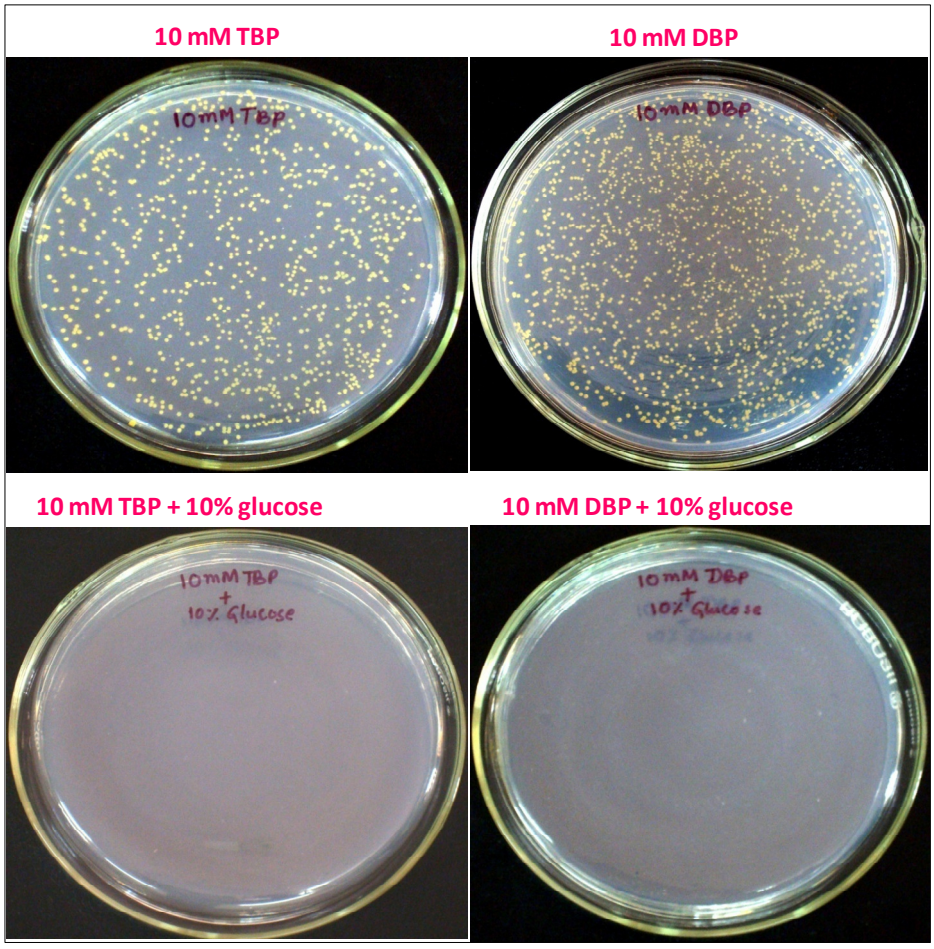

Fig. 4.3. Suppression of TBP or DBP dependent growth of RSMS strain by glucose. Exponentially grown culture of RSMS strain was plated on to the solid MMM agar containing 10 mM^e TBP or 10 mM DBP, supplemented with or without 10% glucose, and incubated at 30°C. Growth was assessed as CFUs formed after 3 days of incubation.

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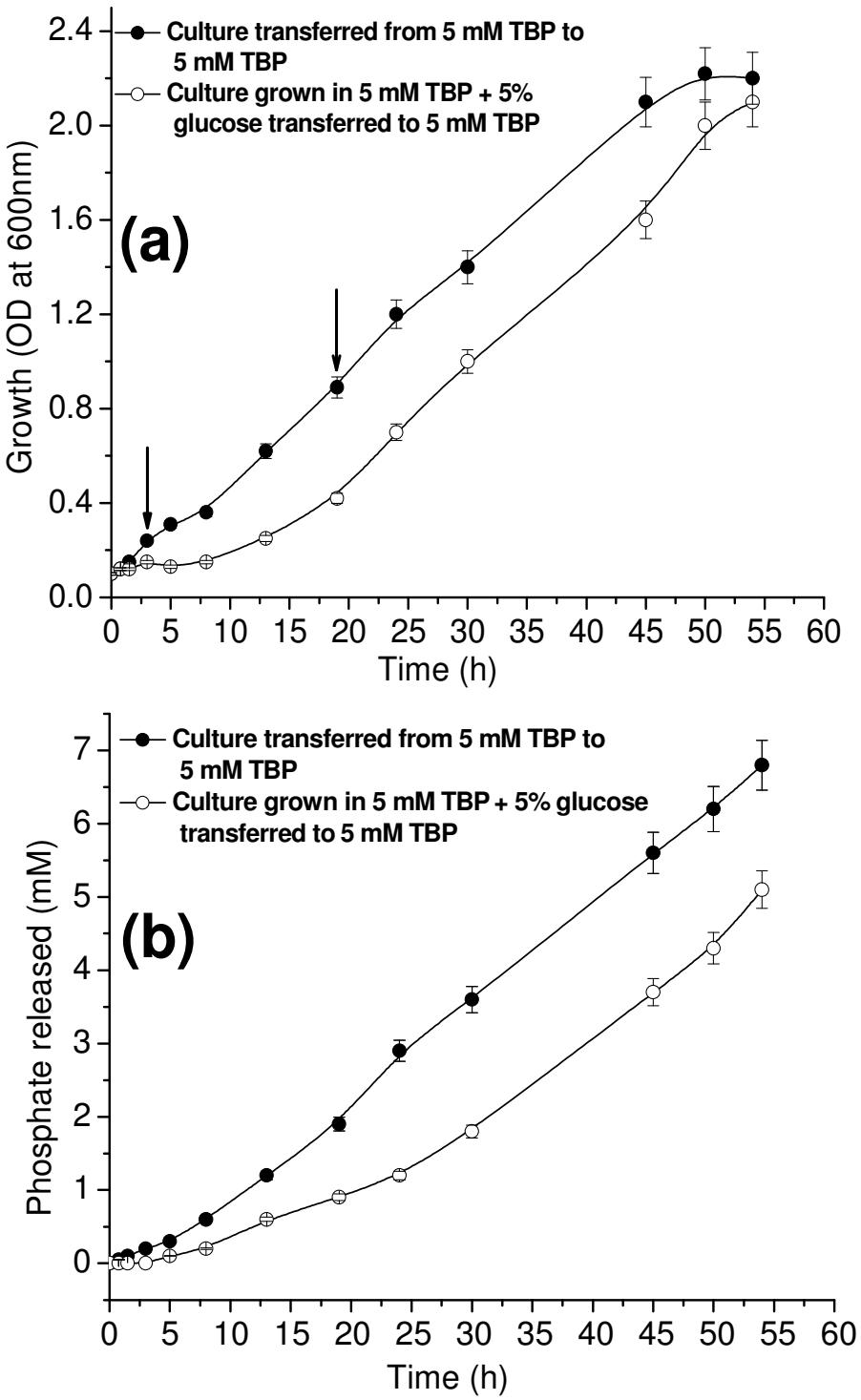

Fig. 4.4. Induction of TBP degradation in RSMS strain. Cells of RSMS strain were inoculated in to MMM containing either 5% glucose + 5 mM[e] TBP or 5 mM[e] TBP alone, and incubated for 2 h at 30^{o}C and 180 rpm to repress TBP degradation. The cells were then harvested, washed with MMM and transferred to MMM containing 5 mM[e] TBP as the sole source of carbon and phosphorous. The growth (a), and TBP degradation (b) were monitored.

DBP degradation measured in cell free extracts of RSMS strain were similarly suppressed in DBP + glucose grown cultures and revived after a lag, upon the removal of glucose (Table 4.2). Cell free extracts of RSMS cultures grown with 5 mM^e TBP/5 mM DBP showed 1.60/1.58 µmole DBP degradation/mg protein/h, respectively, whereas, no DBP degradation was observed in the cell free extracts of the cultures grown with 5% glucose and 5 mM^e TBP or 5 mM DBP (Table 4.2a). However, such repression was reversed, after transferring the cultures to MMM containing 5 mM TBP and no glucose (Table 4.2b).

Table 4.2. Effect of glucose on DBP degradation measured in RSMS cell free extracts

(a) Repression of DBP degradation		
Growth conditions	**Time (h)**	**DBP degradation (µmole Pi/mg protein/h)**
5 mM^e TBP in MMM	2	1.60 ± 0.2
5 mM^e TBP + 5% glucose in MMM	2	0.016 ± 0.005
5 mM DBP in MMM	2	1.58 ± 0.17
5 mM DBP + 5% glucose in MMM	2	0.012 ± 0.005
(b) Induction of DBP degradation after removal of glucose		
Cultures transferred from 5 mM^e TBP + 5% glucose to 5 mM^e TBP in MMM	3	0.02 ± 0.005
	19	1.84 ± 0.005
Cultures transferred from 5 mM^e TBP to 5 mM^e TBP in MMM	3	1.95 ± 0.17
	19	1.90 ± 0.005

(a) RSMS cells grown in MMM supplemented with 5 mM^e of TBP were incubated in 5 mM^e of TBP/DBP, with or without 5% glucose at 30^oC and 180 rpm for 2 h to repress TBP/DBP degradation. Cell free extracts (~100 µg protein) from such cultures were incubated with 10 mM DBP at 30^oC for 5 h. DBP degradation was spectrophotometrically measured as phosphate released. (b) Cell free extracts were prepared from cultures shown in Fig. 4.4. at the time points indicated by arrows, and DBP degradation assays were carried out using 100 µg of protein as described in (a).

Thus glucose, at high concentration, clearly repressed TBP or DBP degradation and suppressed corresponding growth. Both TBP/DBP degradation and TBP/DBP dependent growth could be restored upon removal of glucose. Such differential expression of TBP/DBP degradation in the absence/presence of glucose presented an opportunity to examine proteins which may contribute to TBP/DBP biodegradation.

4.3. Protein profile studies related to glucose based suppression of TBP degradation.

4.3.1. Resolution of proteins using one and two dimensional electrophoresis.

Protein profiles resolved by one dimensional SDS-PAGE revealed suppression of ~7 proteins in the cultures incubated in MMM containing 10% glucose and 10 mM^e TBP (Fig. 4.5) as compared to cultures incubated in MMM containing 10 mM^e TBP alone or TBP with low concentrations of inorganic phosphate which slightly enhanced TBP dependent growth. The protein profiles in TBP and inorganic phosphate (TP) treatments, or TBP (T) alone were identical but glucose grown cultures (10GT) (Fig. 4.5) which showed loss of at least 7 proteins in SDS-PAGE. The protein extracts of cultures grown in 10% glucose and 10 mM^e TBP, or TBP alone were also resolved by two dimensional electrophoresis (Fig. 4.6) and compared. The proteins whose levels were found to be prominently suppressed by glucose in three independent experiments were noted and are marked in Fig. 4.6.

4.3.2. Identification of proteins using peptide mass finger printing.

The proteins whose levels were suppressed in the presence of 10% glucose and 10 mM^e TBP, in comparison to TBP alone, were identified by peptide mass finger printing using MALDI (Matrix Assisted Laser Desorption Ionization) Time of Flight Mass Spectrometry (ToF-MS). Among the 10 suppressed proteins identified, 6 proteins showed similarities with proteins of carbohydrate metabolism, and included Iso-citrate dehydrogenase (Idh), Acetyl-CoA acetyl transferase (Act),

Aspartate semialdehyde dehydrogenase (Asd), Succinate synnthetase (ß sub unit) (Suc C), Malate dehydrogenease (Mdh) and 3-Oxoacyl-ACP-Synthase (3-O-ACPase) (Table 4.3). Other identified proteins included Xre-family transcriptional regulator (Xf-Tr), Arginyl-tRNA synthetatse and ribosomal protein (RPL25). However, none of the identified proteins appeared likely to be related to TBP degradation.

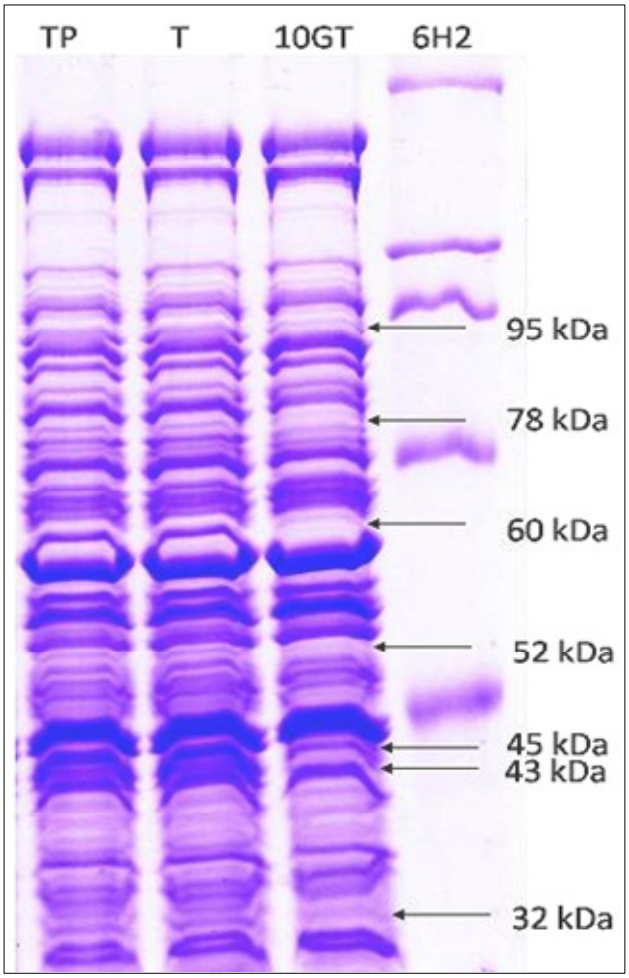

Fig. 4.5. Protein profile differences between TBP, TBP+Pi and TBP+glucose supplemented cultures in one dimensional SDS-PAGE. About 80 µg of protein extracts of the cells grown in MMM supplemented with TBP/TBP+Pi/ TBP+10% glucose were separated on SDS-PAGE. The gel was stained with coomassie brilliant blue (CBB). The proteins which were suppressed in 10GT in comparison to T are marked by arrows. The apparent molecular mass of candidate proteins calculated based on protein standard (Sigma-6H2) co-electrophoresed on the same gel, are shown on the right hand side.

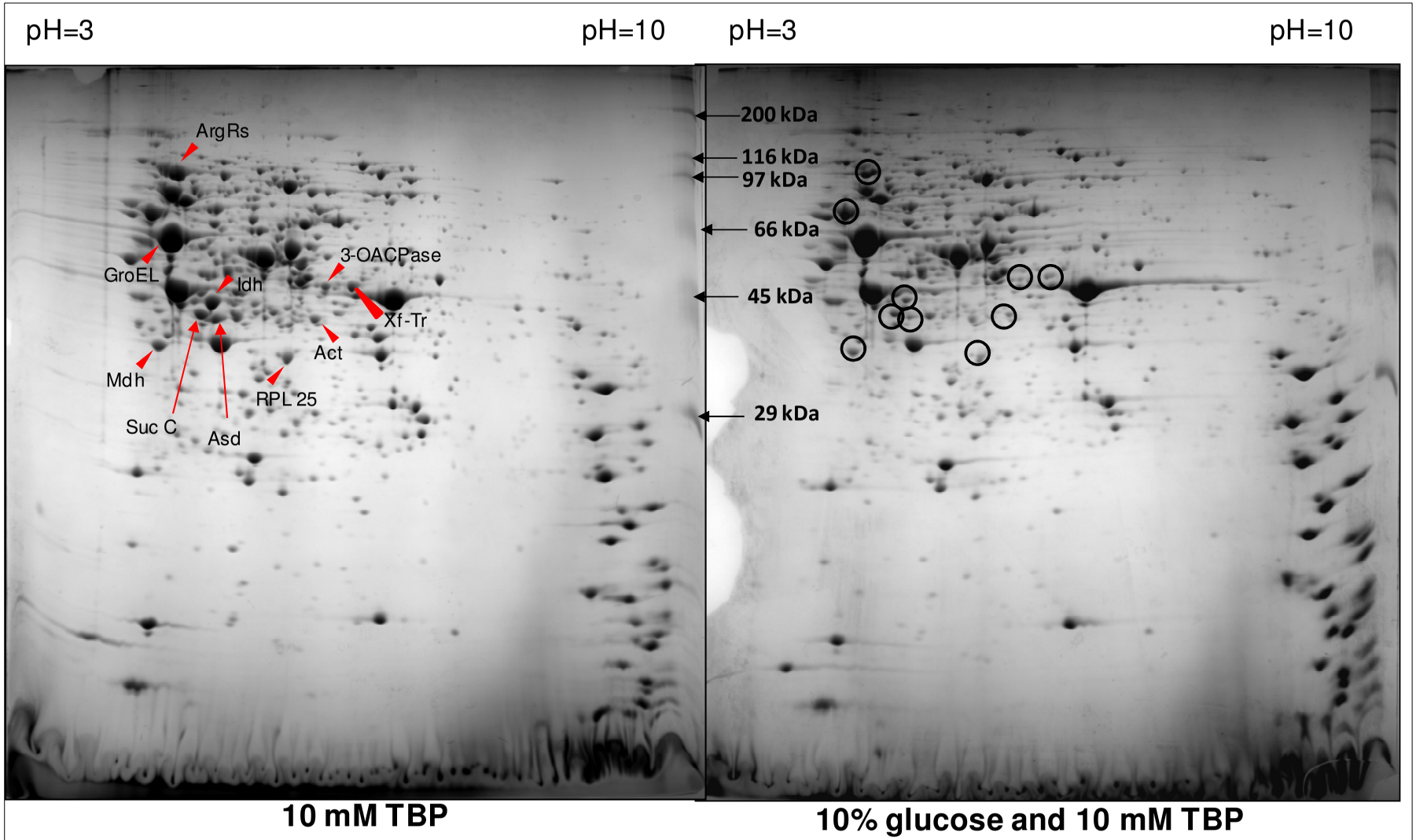

Fig. 4.6. Two dimensional electrophoretic separation of soluble proteins isolated from RSMS strain. Total proteins extracted from TBP or TBP + glucose incubated cultures were iso-electrophoretically separated on 17 cm IPG strips in 3 to 10 pH range. Proteins resolved on IPG strips were further separated by 14% SDS-PAGE in the second dimension. The proteins prominently suppressed in the presence of glucose, with reference to TBP alone, were identified by MALDI-ToF-MS. Identities are indicated as abbreviation and listed in Table 4.3.

Table 4.3. Identities of proteins suppressed in glucose.

Sr. No	Spot ID	Accession No.	Description	Score	Sequence coverage
1	Suc C	gil294011145	Succinyl - CoA synthetase subunit-ß of *Sphingobium japanicum* UT26S	86	44%
2	Mdh	gil 515750053	Malate dehydrogenase of *Sphingobium xenophagum*	88	42%
3	Asd	gil 334344389	Aspartate-semialdehyde dehydrogenase of *Sphingobium chlorophenolicum*	118	46%
4	Idh	gil 523702957	Isocitrate dehydrogenase of *Sphingobium*	132	49%
5	RPL 25	gil294012140	50S ribosomal protein L25 of *Pseudomonas entomophila* (strain L48)	52	39%
6	Act	gil 544820338	Acetyl-CoA acetyltransferase of *Sphingobium quisquiliarum*	67	41%
7	3-OACPase	gi | 523702901	3-oxoacyl- ACP synthase of *Sphingobium*	66	42%
8	Xf-Tr	gil545451216	Putative Xre family transcriptional regulator of *Novosphingobium tardaugens*	61	31%
9	GroEL	gil523701097	Molecular chaperone GroEL of *Sphingobium*	90	36%
10	Arg RS	gil294012801	Arginyl-tRNA synthetase of *Sphingobium japonicum* UT26S	88	27%

4.4. Isolation of TBP non-degrading mutants by Tn5 mutageneis

In our lab, previously, an alkaline phosphatase (*phoK*) was successfully cloned from *Sphingomonas* using Tn5 random mutagenesis approach [71]. Tn5 mutagenesis approach was also attempted for investigating the gene(s) responsible for TBP degradation. The method involved transfer of a suicide vector, *ANU 1041* (carrying Tn5 transposon with *nptII* (kan^r) gene) present in *E.coli* to RSMS strain by conjugation. Such conjugation yielded about $5X10^{-6}$ kan^r colonies (conjugation frequency). Several transconjugants (~8000, pooled from several conjugation events) were then screened for inability to degrade TBP using the zone of clearance approach on Petri plates supplemented with TBP. In three conjugation experiments, more than 50 TBP non degrading mutants were isolated by Tn5 mutagenesis method. Wild type colonies showed zone of degradation of TBP on solid LB agar medium supplemented with 10 mM^e TBP, whereas the mutants did not show a zone of clearance.

All the putative mutants were tested for their ability to utilize TBP both as carbon and phosphorous source in liquid MMM supplemented with 10 mM^e TBP (Fig. 4.7a). Wild type could grow when TBP was supplied as the sole source of carbon and phosphorous where as none of the mutants could degrade TBP or grow using TBP as sole source of carbon and phosphorous (Fig. 4.7a). About 50 TBP non-degrading kan^r mutants were grown in MMM supplemented with 1% glucose and 5 mM KH_2PO_4 and stored as glycerol stocks at -70°C. One of the mutants (Mutant SS22) was taken for further analysis (Fig. 4.7b). The 16s *rRNA* gene sequence of the mutant SS22 was found to be completely identical to the RSMS (Fig. 4.8). Besides, results of 41 biochemical tests performed on the mutant were also found to be identical to the wild type (Table 4.4) indicating that the mutant SS22 indeed derived from the wild type RSMS strain. Growth pattern of the Mutant SS22 found to be similar to RSMS in LB or TGY or MMM supplemented with 1% glucose and 5 mM KH_2PO_4.

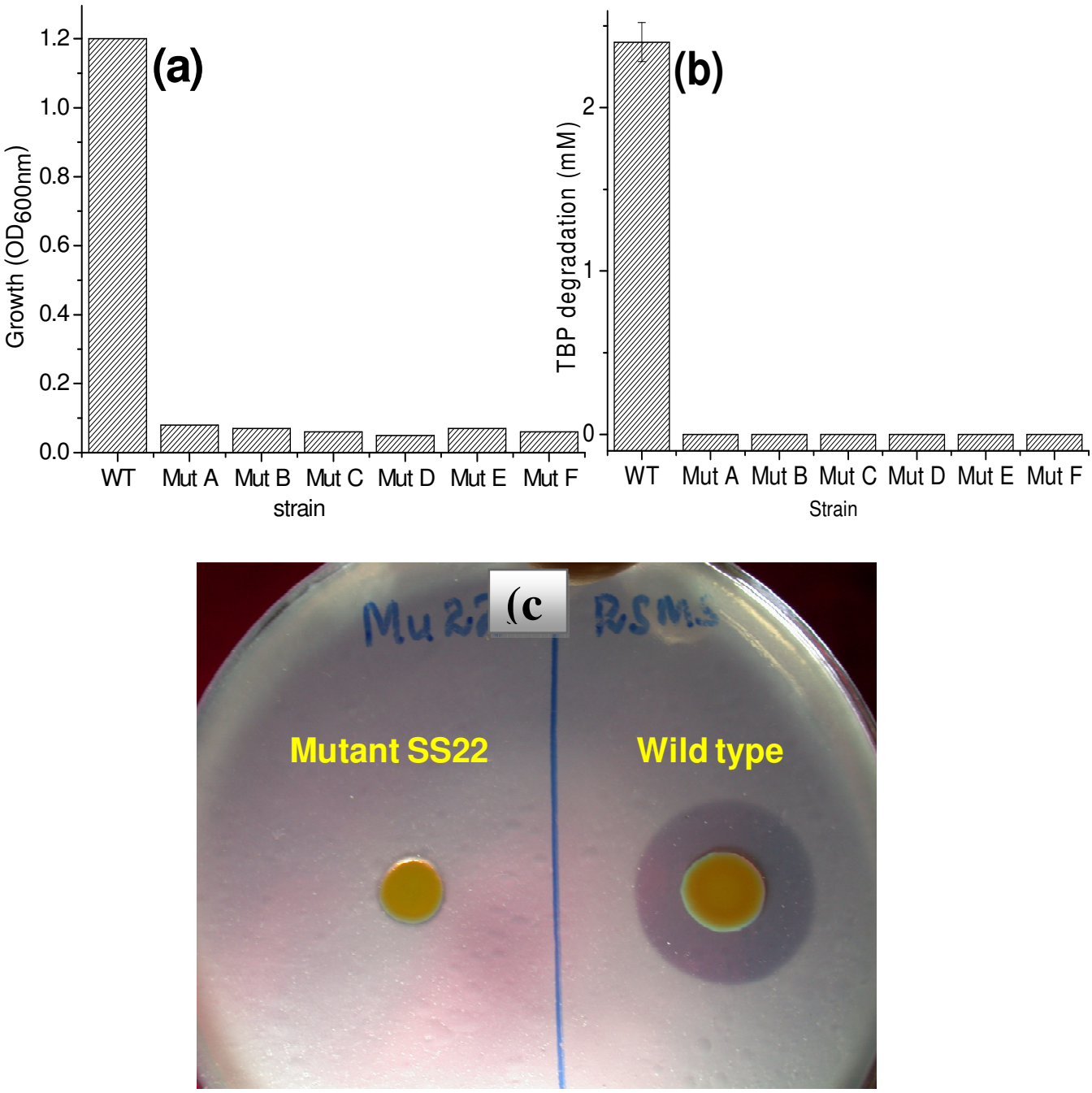

Fig. 4.7. Inability of the TBP non-degrading mutants to degrade and utilize TBP. Six selected putative TBP non-degrading mutants were assessed for their ability to (a) utilize, and (b) degrade TBP when supplied as the sole source of carbon and phosphorous. (c) Inability of one of the mutants (mutant SS22) to form zone of clearance was assessed by spotting ~20µl of 3 OD_{600nm} cell/ml suspensions on to Luria Bertani agar (LBA) medium supplemented with 10 mM^e TBP. The zone of clearance was noted after ~ 1 week of incubation at 30^oC. The wild type strain was used as positive control for comparison.

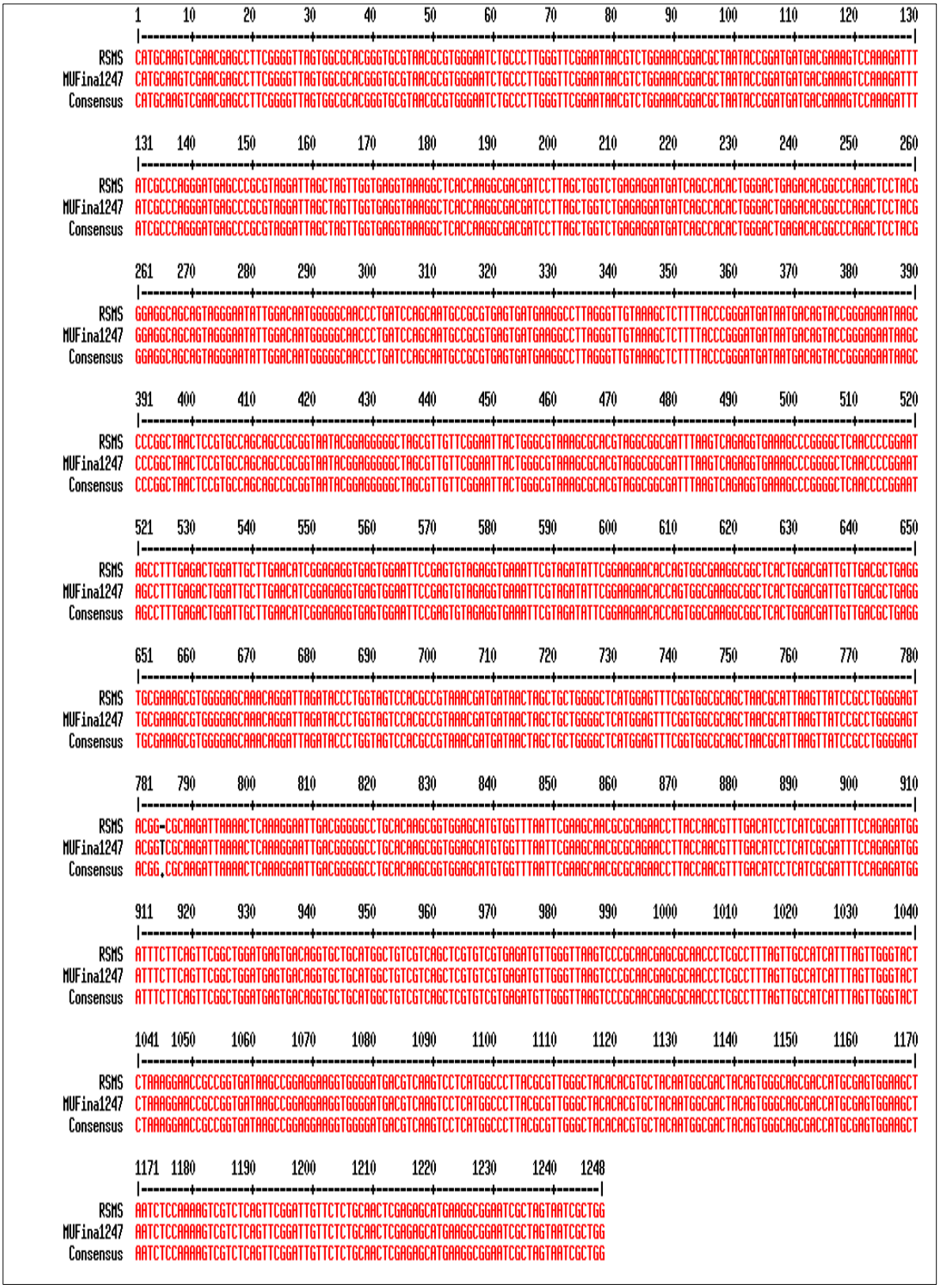

Fig. 4.8. Sequence alignment for 16s *rRNA* gene of RSMS and mutant SS22

Table 4.4. Comparison of biochemical tests of RSMS and mutant SS22 strains.

	Test	Result				Result	
		RSMS	Mutant		Test	RSMS	Mutant
1	Lactose	-	-	18	Inositol	-	-
2	Xylose	+	+	19	Sorbitol	-	-
3	Maltose	+	+	20	Mannitol	-	-
4	Fructose	-	-	21	Adonitol	-	-
5	Dextrose	+	+	22	Alpha Methyl-D-Glucoside	-	-
6	Galactose	+	+	23	Ribose	±	±
7	Raffinose	-	-	24	Rhamnose	±	±
8	Trehalose	±	±	25	Cellobiose	±	±
9	Melibiose	-	-	26	Melezitose	-	-
10	Sucrose	-	-	27	Xylitol	-	-
11	L-Arabinose	+	+	28	ONPG	-	-
12	Mannose	-	-	29	Esculin	±	±
13	Inulin	-	-	30	D-Arabinose	+	+
14	Sodium Gluconate	-	-	31	Citrate	±	±
15	Glycerol	-	-	32	Malonate	-	-
16	Salicin	-	-	33	Sorbose	-	-
17	Glucosamine	+	+				
Other biochemical tests							
1	Lysine decarboxylase	+	+	5	Nitrate reduction	-	-
2	Ornithine decarboxylase	+	+	6	H2S production	-	-
3	Urease	-	-	7	Oxidase	+	+
4	Deamination	-	-	8	Catalase	+	+

+, - and ± indicates positive, negative and weakly positive reactions respectively

4.5. Inability of mutant SS22 to use TBP/DBP as the sole source of carbon or phosphorous.

Mutant SS22 could not degrade 10 mM^e TBP or 20 mM DBP supplied in MMM, as assessed by phosphate released by their degradation (Table 4.5). Mutant could not grow in MMM supplemented with TBP or DBP, or with TBP/DBP + 5 mM KH_2PO_4, or TBP/DBP along with 1% glucose, while the wild type cells degraded both TBP and DBP and utilized them as sole source of carbon and phosphorous. Both wild type and mutant SS22 showed growth in the medium supplemented with 20 mM butanol and inorganic phosphate (5 mM KH_2PO_4) (Table 4.5), the expected or likely product of TBP or DBP degradation. Results indicate that the mutant could neither degrade nor utilize TBP/DBP as carbon or phosphorous source.

Table 4.5. Inability of Mutant SS22 to utilize TBP or DBP as carbon or phosphorous source

Added in MMM	**Growth after 24 h (OD_{600nm})**		**TBP/DBP degradation after 24 h (mM)**	
	Wild type	**Mutant**	**Wild type**	**Mutant**
10 mM^e TBP	1.2 (± 0.15)	0.08 (± 0.02)	2.5 (± 0.2)	nil
20 mM DBP	1.1 (± 0.10)	0.08 (± 0.02)	1.8 (± 0.2)	nil
10 mM^e TBP + 5 mM KH_2PO_4	1.5 (± 0.12)	0.07 (± 0.03)	NA	NA
20 mM DBP + 5 mM KH_2PO_4	1.3 (± 0.15)	0.08 (± 0.02)	NA	NA
10 mM^e TBP + 1% glucose	2.5 (± 0.15)	0.13 (± 0.03)	2.8 (± 0.2)	nil
20 mM DBP + 1% gluccose	2.2 (± 0.10)	0.12 (± 0.03)	2.5 (± 0.15)	nil
20 mM butanol + 5 mM KH_2PO_4	1.8 (± 0.18)	1.7 (± 0.15)	-	-

Ability of the mutant SS22 to use TBP or DBP as the sole source of carbon and phosphorous was assessed by inoculating mutant SS22 in MMM (at 0.1 OD_{600nm}) supplemented either with 10 mM^e TBP or 20 mM DBP. The ability of the mutant SS22 to use TBP or DBP as sole source of carbon or phosphorous was assessed, by supplementing TBP/DBP in MMM along with 5 mM KH_2PO_4 or 1% glucose, respectively. Growth was monitored as increase in OD_{600nm}, and degradation of TBP or DBP was measured spectrophotometrically as inorganic phosphate released after 24 h of incubation. RSMS strain (wild type) was used as a positive control.

4.6. Probing of Tn5 transposon in TBP non degrading mutantSS22.

Chromosomal walking in the genome of mutant SS22, around the Tn5 transposon can lead to the identification of the gene(s) responsible for the degradation of TBP in RSMS strain. The method involved the probing of Tn5 transposon in the genome of mutant SS22. In our lab, using similar approach an alkaline phosphatase gene (*phoK*) was successfully cloned from a strain of *Sphingomonas*. sp strain BSAR-1 (BSAR-1) by chromosomal walking approach around the Tn5 transposon [71]. In similar way, attempts were made to probe the Tn5 transposon in the mutant SS22.

In spite of several efforts the Tn5 transposon could not be detected by DNA-DNA hybridization in the mutant (Fig. 4.9). Attempts were made by changing several factors (such as usage of different restriction enzymes for digestion, amount of time for restriction digestion, temperature of hybridization, amount of time for hybridisation, and stringency for hybridization, etc..) to detect the *nptII*, but none of them worked out for detection of Tn5 in the mutant. In all the experiments the positive controls (restriction digestion fragments of Kn20 and PCR amplified fragment of *nptII* gene) were always detected (Fig. 4.9) indicating that methodology used was correct. Therefore this approach had to be abandoned for characterization of the gene(s) responsible for TBP degradation. Instead, the protein profile differences between mutant SS22 and RSMS were exploited for further molecular investigation of TBP degradation in RSMS, as described in section 4.8 and 4.9 below.

4.7. Isolation of spontaneous mutants of TBP non degradation.

In spite of several efforts the Tn5 transposon in mutant SS22 could not be probed. These observations suggested that such mutants may have arisen through spontaneous mutagenesis. Attempts were therefore made to see if spontaneous TBP non-degrading mutants could arise in

RSMS strain during repeated subculturing over a period of time. Spontaneous TBP non-degrading mutants of RSMS strain were obtained by repeated subculturing of RSMS strain in MMM supplemented with 1% glucose and 5 mM KH_2PO_4 and plating on TBP supplemented plates. Lack of a zone of TBP degradation around the colony was used as a screen for isolating spontaneous mutants. Colonies which did not show any zone of clearance around them were considered as putative TBP non-degrading spontaneous mutants (Fig. 4.10).

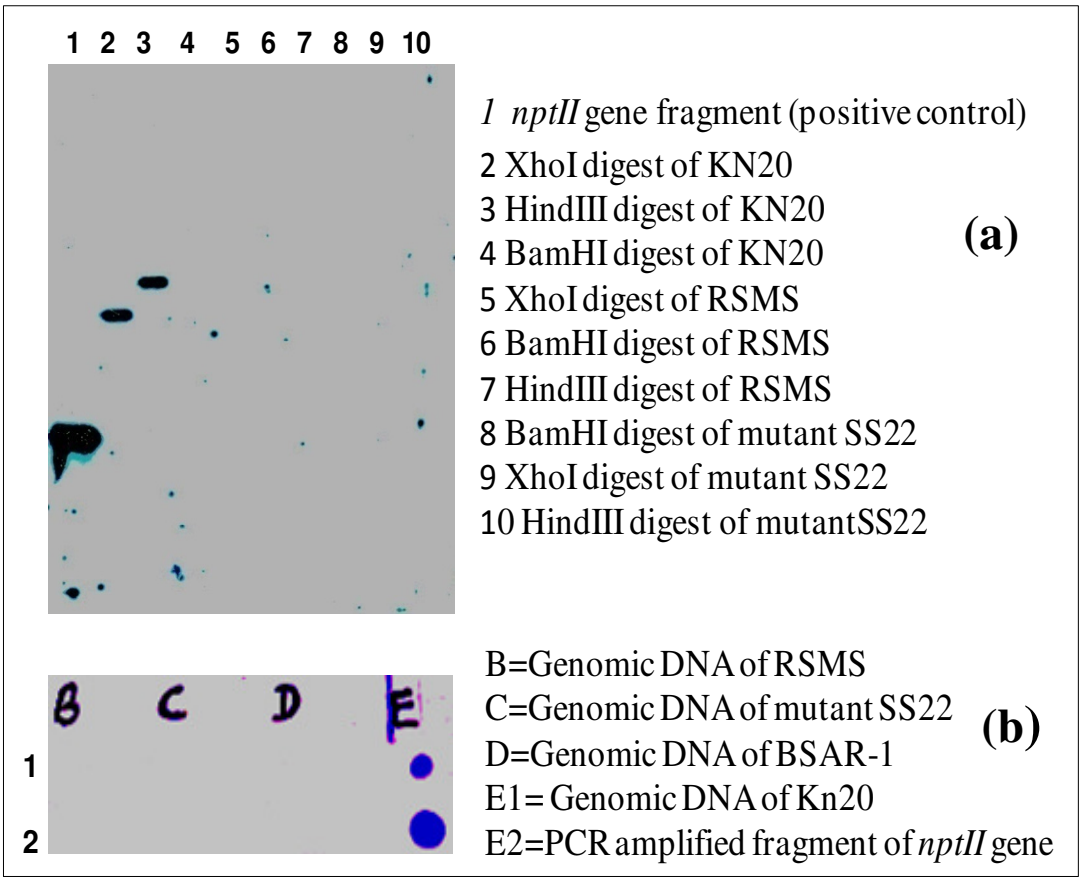

Fig. 4.9. Probing for Tn5 transposon in the mutant SS22. (a) Genomic DNA of the mutant SS22 was digested with specified restriction enzymes and resolved on 0.85% agarose gel. The restriction fragments generated were transferred to a nylon membrane and hybridized with digoxigenin (DIG) labeled *nptII* gene. The detection was carried out using an anti DIG antibody and CDP star substrate. The chemiluminiscence resulting due to the reaction was recorded on a X ray film. (b) Similar probing was also carried out using dot blot and hybridization was detected using NBT-BCIP as substrate.

The putative TBP non-degrading spontaneous mutants were further tested for their ability to degrade and use TBP/DBP as the sole source of carbon and phosphorous. None of the putative TBP non-degrading spontaneous mutants could degrade or use TBP/DBP for their growth (data not shown). About 10 such spontaneous mutants were stored as glycerol stocks at -70^{o}C.

The data presented in section 4.7 suggested that the TBP non-degrading Tn5 mutants may actually be spontaneous mutants which have lost the sensitivity to kanamycin and also lost the ability to degrade TBP. This would also explain why Tn5 could not be detected in these mutants. The mutant SS22 was considered as a spontaneous mutant and taken for further analysis since it carried a selectable kanamycin (kan^{r}) marker, and was found to be otherwise identical to RSMS strain (Fig. 4.8 and Table 4.4). It was, therefore, preferred over any spontaneous mutant.

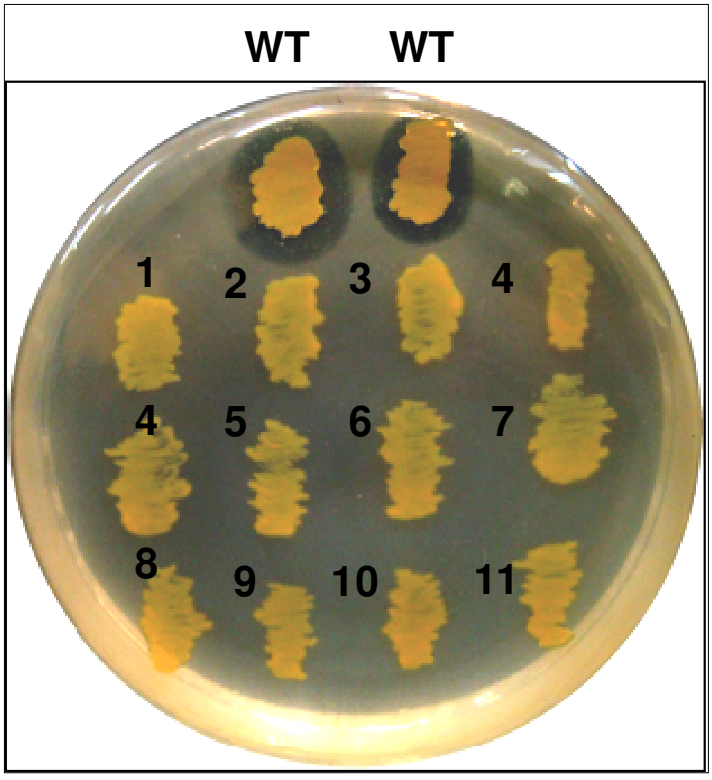

Fig. 4.10. Isolation of TBP non-degrading spontaneous mutants. Absence of the zone of clearance around the colonies of spontaneous TBP non-degrading mutants (1-11). The LB agar was supplemented with 10 mM TBP. As a positive control RSMS (WT) cells were used.

4.8. Protein profile differences between TBP non-degrading mutant SS22 and RSMS strain.

TBP non degrading mutant SS22 and wild type were compared in terms of their protein profiles during growth on 1% glucose + 5 mM $KH2PO_4$ (since the mutant could not grow on TBP). The protein(s) which were absent in the mutant and present in the wild type may be involved in the biodegradation of the TBP, and so attempts were made to investigate them. One dimensional SDS-PAGE resolution, revealed the absence of only one protein (~45 kDa) in the mutant SS22, in comparison to wild type (Fig.4.11). Further resolution by two dimensional gel electrophoresis (2-D electrophoresis) revealed that the protein profiles of wild type and mutant SS22 were nearly identical, except for three proteins which were lacking or absent in the mutant (Fig. 4.12).

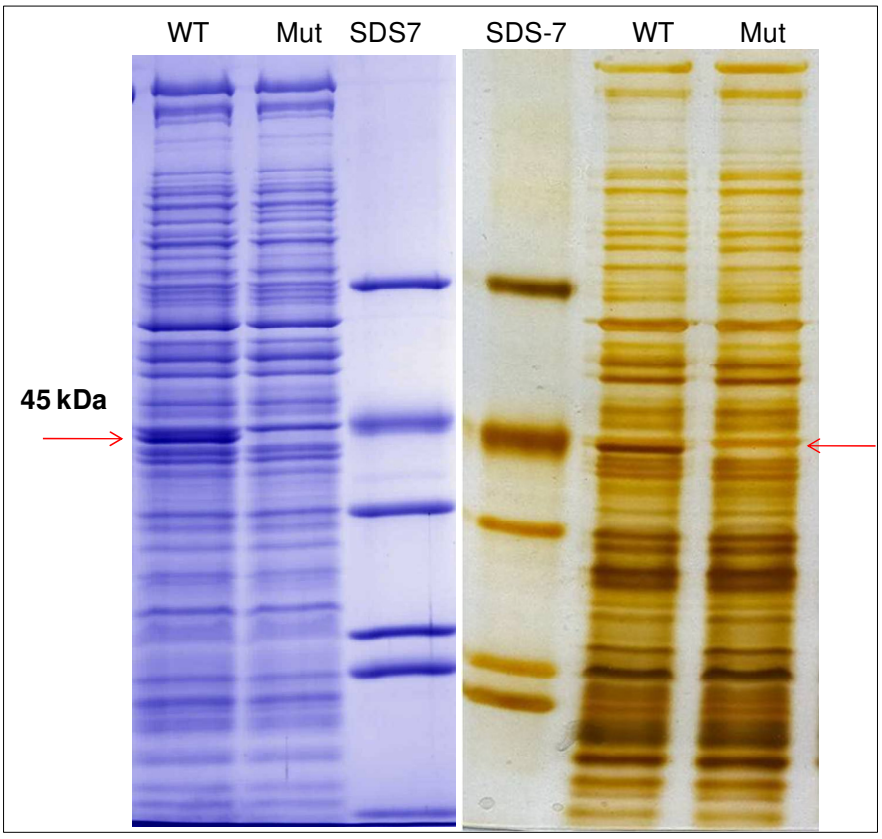

Fig. 4.11. Protein profile of RSMS and mutant SS22. Cell free protein extracts of the RSMS and Mutant SS22 were resolved by 10% SDS-PAGE. The resolved proteins were visualized, either using CBB or silver staining methods. The sole difference in protein profiles of wild type and mutant is marked with an arrow.

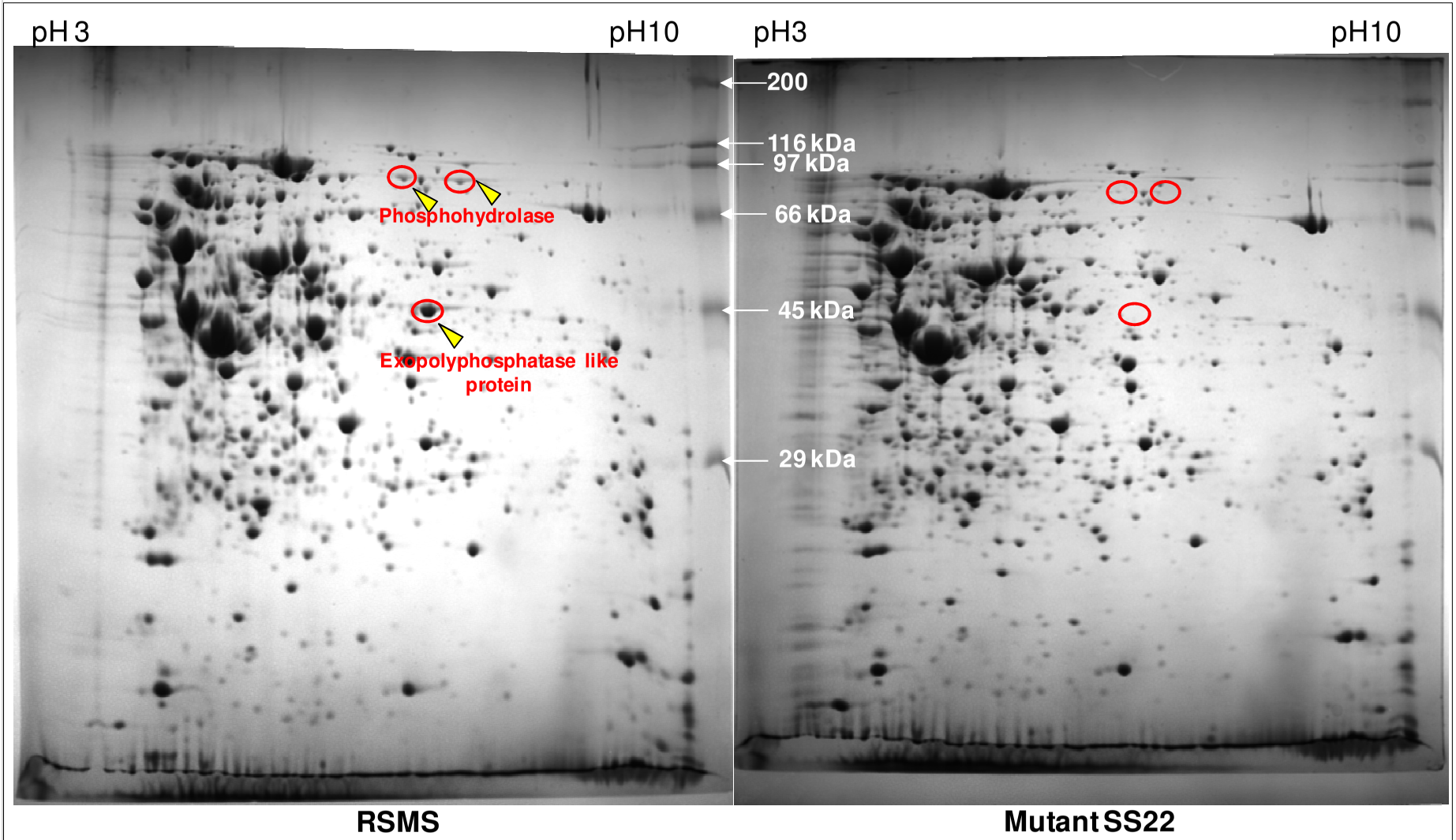

Fig. 4.12. Two dimensional electrophoretic resolution of proteins from RSMS and mutant SS22 strains, grown with 1% glucose and 5 mM KH_2PO_4. Cell free proteins extracted from wild type and mutant SS22 (grown in MMM containing 1% glucose and 5 mM KH_2PO_4) cultures were iso-electrophoretically separated on 17 cm IPG strips in 3 to 10 pH range. Proteins resolved on IPG strips were further separated by 14% SDS-PAGE in second dimension. The proteins that were absent in the mutant SS22 are marked and their identity is given. The results were reproducible among 3 biological replicates and a representative gel is shown.

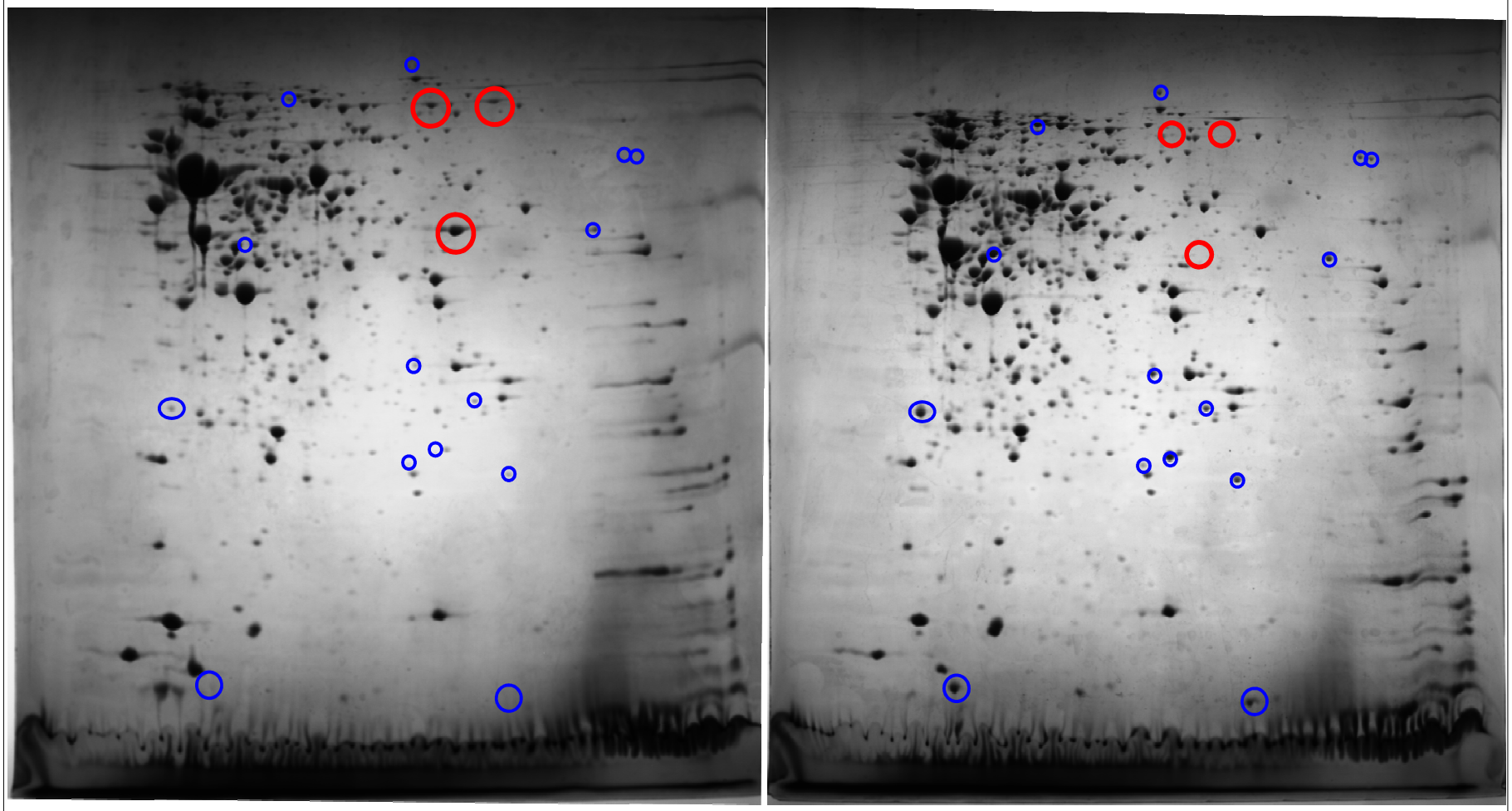

Fig. 4.13. Two dimensional electrophoretic resolution of proteins from RSMS and SS22 strains, grown in TBP. Cell free proteins extracted from wild type and mutant SS22 cultures incubated in 10 mM TBP were iso-electrophoretically separated on 17 cm IPG strips in 3 to 10 pH range. Proteins resolved on IPG strips were further separated by 12% SDS-PAGE in second dimension. The proteins absent in mutant SS22 are marked in red circles and proteins that are absent or present in low amounts in wild type are marked in blue coloured cirlces . The results were reproducible among 3 biological replicates and a representative gel is shown.

Irrespective of the medium (either MMM supplemented with TBP alone or with 1% glucose and 5 mM KH_2PO_4), these three proteins were absent in the mutant as compared to the wild type (Fig. 4.12 and 4.13). Interestingly, mutant SS22 showed several proteins, which were induced in TBP in comparison to the wild type (Fig. 4.13), but these were not identified as they were not considered relevant since mutant did not degrade TBP.

4.9. Identification of proteins absent in the TBP non-degrading SS22 mutant.

The apparent molecular mass of the three proteins absent in mutant were ~ 45 kDa and ~100 kDa (2 proteins). These were identified using MALDI-ToF-MS.

4.9.1. Identification of ~100 kDa proteins.

The enzymatic digestion of the two ~100 kDa proteins (absent in mutant) with trypsin resulted in several peptides. Among these, 21 peptides showed similarity with the phosphohydrolase of *Alivibrio fischeri* with a MASCOT score of 97 (Fig. 4.14). Both the ~100kDa proteins showed similar m/z spectrum, and similarity to phosphohydrolase. The 21 peptides matched with Phosphohydrolase with ~30% protein coverage (Fig. 4.14 b and 4.15). Phosphohydrolase is a Phosphodiesterase, known to degrade several phosphodiester compounds such as c-AMP, dimethyl phosphate (DMP), diethyl phosphate (DEP), Bis-pNPP, dementon [64,94-98]. Most of the phosphohydrolases also show non-specific activity for phosphomonoesters [94,95]. A phopsphohydrolase from *Enterobacter aeogenes* is known to act on trimethylene phosphate (TMP), which is a phoshotrietser [94]. DBP is similar to and a higher homologue to the DMP and DEP, while TBP is a phosphotriester and a higher homologue to TMP (Fig. 4.16). Further analysis of phosphohydrolase of *Alivibrio fischeri* (using BLAST program) showed similarity with phosphohydrolases belonging to *Alivibrio* and *Vibrio* species. Few of the hits also showed bacteria other than *Vibrio*, and one of them also belonged to *Pseudomonas.*

The identities have ranged from 100% to 35%, suggesting that these proteins have high diversity, but have conserved the function of phosphodiesterase (Fig. 4.16).

(a) Identity of 100 kDa protein

1. gi | 491564748 **Mass:** 107007 **Score:** 97 **Expect:** 0.0048 **Matches:** 21
phosphohydrolase [Alivibrio fischeri]

(b) Overlap between tryptic digest of 100 kDa RSMS protein with Phosphohydrolase from *Alivibrio fischeri*

Start	End	Observed	Mr(expt)	M	Peptide
1	15	1751.7701	1750.7628	0	-.MNDILLEANSTIFDR.I
16	25	1240.5458	1239.5385	1	R.IANETRFSMR.D + Oxidation (M)
22	40	2289.1018	2288.0945	1	R.FSMRDEYRPAFNAVEELSK.K
42	52	1345.7242	1344.7169	1	K.EIIKTNTLEER.K
46	52	862.4366	861.4293	0	K.TNTLEER.K
208	226	2192.9619	2191.9546	0	R.VLYDEENIVYASNYSANTK.L
229	247	2090.9947	2089.9874	1	K.NQAVMLKDIFNQSAIAALK.Q + Oxidation (M)
248	257	1279.7660	1278.7587	0	K.QLHLDQEIQR.Y
341	352	1394.6762	1393.6689	1	K.FDFSPLKQSPTK.I
480	493	1699.7306	1698.7233	0	K.DEDTWSLTFPLFTK.G
560	588	3227.3789	3226.3716	1	K.SPYTGNHCQKVPVLTQWLAEVADQSSSPK.F
614	626	1464.7494	1463.7421	1	K.ITTPEYVVDKATK.L
686	707	2582.1558	2581.1485	1	K.MNVGDEFLSEQDATQIQEISRR.T+ Oxidation (M)
708	726	2249.9798	2248.9726	1	R.TWVQTLDSSLGLAWEEKMR.L
725	734	1162.6393	1161.6320	1	K.MRLNDSGNQK.V
725	734	1178.5602	1177.5529	1	K.MRLNDSGNQK.V + Oxidation (M)
816	827	1275.5885	1274.5813	1	K.DIAKIAGGHHEK.V
848	866	2225.1158	2224.1085	0	K.MMAIADIFEALTSHDRPYK.K + Oxidation (M)
870	883	1677.7666	1676.7593	1	K.TLTESLKIMHFMVK.D
903	921	2330.1296	2329.1223	1	K.KYADTYLKPEQCDDVDIEK.F
904	921	2201.9832	2200.9759	0	K.YADTYLKPEQCDDVDIEK.F

Fig. 4.14. MASCOT similarity search results for ~100 kDa protein. (a) The peptides obtained trypsin digestion of the ~100 kDa protein of RSMS strain was analysed for similarity using MASCOT program. The protein phosphohydroalse from *Alivibrio fischeri* showed the highest similarity. (b) The tryptic digest peptides of the phosphohydrolase which matched with the tryptic digest peptides of the ~100 kDa protein.

1	MNDILLEANS	TIFDRIANET	RFSMRDEYRP	AFNAVEELSK	KEIIKTNTLE
51	ERKAYFSELL	MLLKLNNHVS	TYRITYPNGD	WFGVGIIENH	SLKKELNAPH
101	AARYYFINTL	LETKTTSTIY	FYDKTNKLID	SQVFEIPNND	PRNEDWFKKS
151	TINQTILSKP	RYFSAINQFG	LTIHQKADND	VVISADLLMS	KVDEVLKSTS
201	NHTSSVRVLY	DEENIVYASN	YSANTKLKNQ	AVMLKDIFNQ	SAIAALKQLH
251	LDQEIQRYQH	NNEDWYGKIF	SVPINQQRTL	NLLVAVKASE	LLSSASVIRN
301	QAILWSFLVL	FLSIPCVYFV	SQRISKPIKQ	ATEQARLISK	FDFSPLKQSP
351	TKILEIDNLN	KSVTSMKSAI	ENYFNLTNTI	LEEHEIDDLI	QIIGRNTANA
401	TQAAGAYLL	INDDETHIEP	HFAWRATQN	EDISELKRYS	LGDKEIAKNL
451	EYILVKKKPF	EDLSIQKLDI	NEQEKLGLKD	EDTWSLTFPL	FTKGKQTIGA
501	MVLVFDIKNS	EAIRNDKLDY	FESLINFTAI	ALHGKMLQS	QKDLLESFIQ
551	VMAGAIDTKS	PYTGNHCQKV	PVLTQWLAEV	ADQSSSPKFT	HFTLNNVQKE
601	ELRIASWLHD	CGKITTPEYV	VDKATKLETI	YNRIHEIRTR	FEVLKRDVEI
651	EQWKQAKQE	LPQKNKEILQ	NEWQKLDDEF	TFIAKMNVGD	EFLSEQDATQ
701	IQEISRRTWV	QTLDSSLGLA	WEEKMRLNDS	GNQKVPKVN	LLQDNLFHLI
751	PREIDLPYDE	RFTLQPTEHE	NNLGEIYNLL	IQRGTLNNEE	RFVINNHIIQ
801	TIQMLETLPF	PKTMKDIAKI	AGGHHEKVNG	TGYPMGLTSE	QMPLTAKMMA
851	IADIFEALTS	HDRPYKKAKT	LTESLKIMHF	MVKDQHDSD	LFDLFLTSGL
901	KKYADTYLK	PEQCDDVDIE	KFLS		

Fig. 4.15. Protein sequence coverage of the matched tryptic peptides from 100 kDa protein from RSMS strain. The red text indicates the peptides from phosphohydolase of the *Alivibrio fscheri* which showed good match with the peptides of the 100 kDa protein absent in mutant.

The phosphohydrolase of *Pseudomonas* showed only 35% similarity. There were several stretches of amino acids which were highly conserved in all the bacterial strains that were compared (Fig. 4.16 and 4.17).

Among conserved regions of phosphohydrolase, were found the HD and HAMP region of the protein, which are the signature domains of the Phosphoesterases. The HD domain is a conserved domain found in a superfamily of enzymes with a known phosphohydrolase (phosphodiesterase) activity. These enzymes appear to be involved in nucleic acid metabolism. The HAMP (present in Phosphatases) linker domain is an approximately 50-amino acid alpha-helical region. HAMP domain can be found in combination with HD domain. The mass spectrum of 100 kDa protein (absent in mutant) showed peptides of HD domain and HAMP domain (Fig. 4.17).

a) Sequence similarities of Phoshohydrolase of *Alivibrio fischeri* with other proteins

Protein	Percent identity
phosphohydrolase (Aliivibrio fischeri) gb|EHN69044.1	100
membrane protein (Aliivibrio logei)	68
phosphohydrolase (Tolumonas auensis) gi|237808279	36
phosphohydrolase (Plesiomonas shigelloides) gb|EON89267.1	37
phosphohydrolase (Pseudomonas sp. UW4) gb|AFY18654.1	35

b) Sequence alignment between Phoshohydrolase of *Alivibrio fischeri* with other proteins

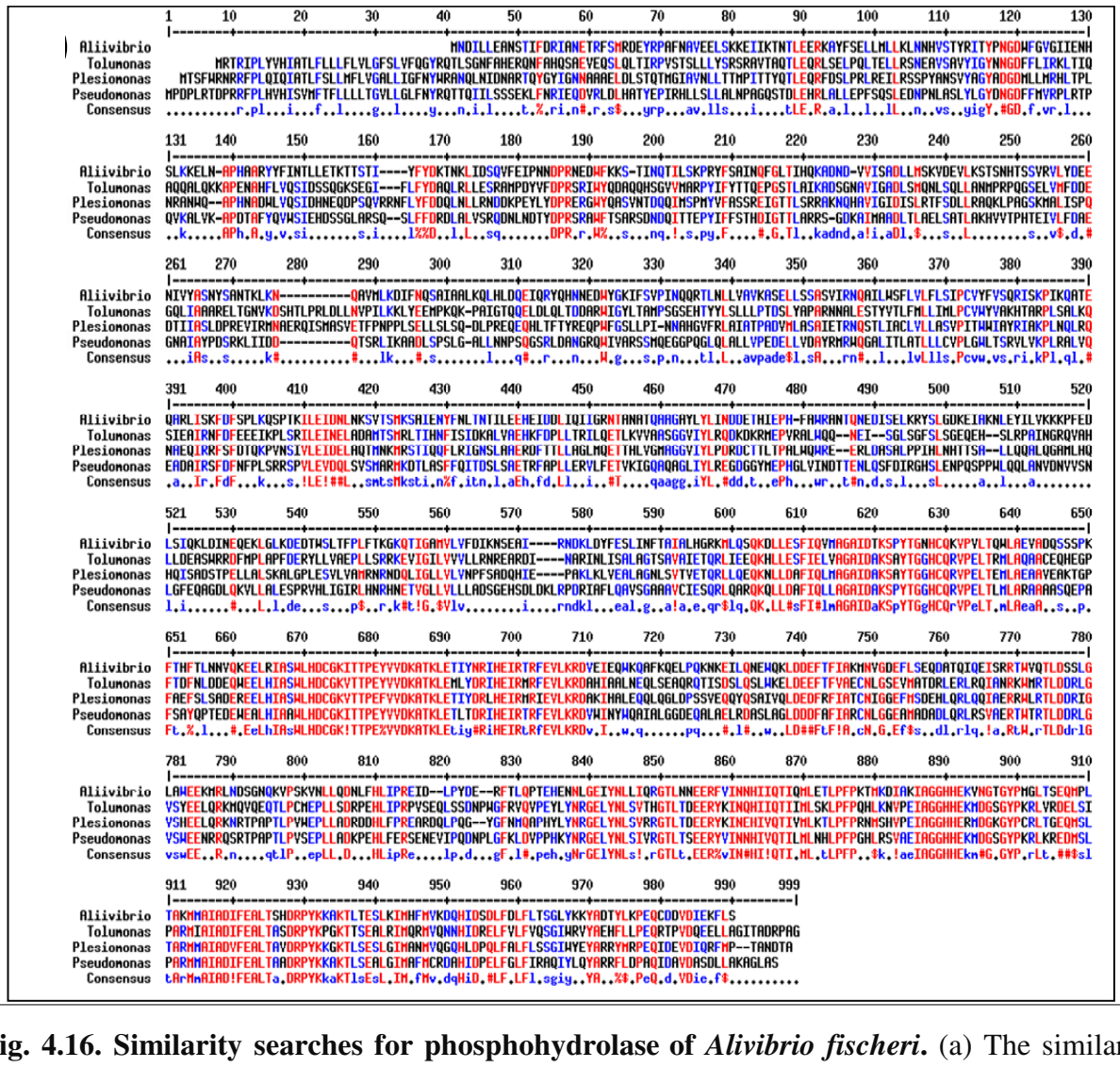

Fig. 4.16. Similarity searches for phosphohydrolase of *Alivibrio fischeri*. (a) The similarity search was carried out using BLAST program, and five most similar proteins are listed here. (b) Multiple sequence alignment for Phosphohydrolase of the *Alivibrio fischeri* with nearest phosphohydrolase relatives (obtained through BLAST search). The red text depict the most conserved amino acids.

The amino acid from 410 to 416, 615 to 619, 669 to 690, 860 to 870 and 916 to 924 were the most conserved regions of the protein among all the bacterial strains (Fig. 4.16b). The oligonucleotide probe derived from such highly conserved regions would be useful to probe the corresponding gene in the RSMS strain.

MNDILLEANSTIFDRIANETRFSMRDEYRPAFNAVEELSKKEIIKTNTLEERKAYFSELLMLLKLN
NHVSTYRITYPNGDWFGVGIIENHSLKKELNAPHAARYYFINTLLETKTTSTIYFYDKTNKLIDSQ
VFEIPNNDPRNEDWFKKSTINQTILSKPRYFSAINQFGLTIHQKADNDVVISADLLMSKVDEVLKS
TSNHTSSVRVLYDEENIVYASNYSANTKLKNQAVMLKDIFNQSAIAALKQLHLDQEIQRYQHNN
EDWYGKIFSVPINQQRTLNLLVAVKASELLSSASVIRNQAILWSFLVLFLSIPCVYFVSQRISKPIK
QATEQARLISKFDFSPLKQSPTKILEIDNLNKSVTSMKSAIENYFNLTTTNTILEEHEIDDLIQIIGRN
TANATQAAGAYLYLINDDETHIEPHFAWRANTQNEDISELKRYSLGDKEIAKNLEYILVKKKPFE
DLSIQKLDINEQEKLGLKDEDTWSLTFPLFTKGKQTIGAMVLVFDIKNSEAIRNDKLDYFESLINF
TAIALHGRKMLQSQKDLLESFIQVMAGAIDTKSPYTGNHCQKVPVLTQWLAEVADQSSSPKFTH
FTLNNVQKEELRIASWLHDCGKITTPEYVVDKATKLETIYNRIHEIRTRFEVLKRDVEIEQWKQAF
KQELPQKNKEILQNEWQKLDDEFTFIAKMNVGDEFLSEQDATQIQEISRRTWVQTLDSSLGLAW
EEKMRLNDSGNQKVPSKVNLLQDNLFHLIPREIDLPYDERFTLQPTEHENNLGEIYNLLIQRGTLN
NEERFVINNHIIQTIQMLETLPFPKTMKDIAKIAGGHHEKVNGTGYPMGLTSEQMPLTAKMMAIA
DIFEALTSHDRPYKKAKTLTESLKIMHFMVKDQHIDSDLFDLFLTSGLYKKYADTYLKPEQCDD
VDIEKFLS

Fig. 4.17. Detailed analysis of Phosphohydrolase of the *Alivibrio fischeri*. Red colour indicates the peptides of Phosphohydrolase of *Alivibrio fischeri*, matched with the peptides generated for ~100 kDa RSMS protein digested by trypsin. Yellow colour indicates the most conserved regions in the Phosphohydrolase as assessed by multiple sequence alignment (Fig. 4.16). Underlined are the peptides generated by trypsin digestion of 100 kDa RSMS protein, which are the most conserved regions of the protein. The peptides present in the boxes above and below respectively represent the HAMP and HD domain.

4.9.2. Identification of the ~45kDa protein

The enzymatic digestion of ~45 kDa protein that was absent in the mutant SS22, with trypsin also resulted in several peptides. Among these, 10 peptides showed similarity with hypothetical proteins of *Vibrio cholera* (Fig. 4.18). But, none of these hypothetical proteins showed similarity (through BLAST search) with any of the known protein from protein database. The ~45 kDa protein also showed similarity with Exopolyphosphatase-like protein of *Marinomonas mediterranea* MMB-1, with a MASCOT score of 68 (Fig. 4.18). The 10 peptides of 45 kDa RSMS protein matched with exopolyphosphatase-like protein with 36% protein coverage (Fig. 4.19a and b). The exopolyphosphatase of *Marinomonas mediterranea* MMB-1 protein showed maximum of 11% similarity with other proteins (Table 4.6). The protein also showed maximum similarity of 11% with a phosphoesterase of *Bacillus cereus*, and with few other proteins including several phosphoesterases and oligoribonucleases. The multiple sequence alignment of these sequences revealed very little similarity among the sequences (Fig. 4.20).

1.	gi | 487839028	**Mass:** 43399	**Score:** 80	**Expect:** 0.24	**Matches:** 10
	hypothetical protein [Vibrio cholerae]				
	gi | 487933263	**Mass:** 43400	**Score:** 80	**Expect:** 0.24	**Matches:** 10
	hypothetical protein [Vibrio cholerae]				
	gi | 487821780	**Mass:** 44075	**Score:** 79	**Expect:** 0.27	**Matches:** 10
	hypothetical protein [Vibrio cholerae]				
	gi | 487826572	**Mass:** 44058	**Score:** 79	**Expect:** 0.27	**Matches:** 10
	hypothetical protein [Vibrio cholerae]				
	gi | 487858920	**Mass:** 44015	**Score:** 79	**Expect:** 0.27	**Matches:** 10
	hypothetical protein [Vibrio cholerae]				
2.	gi | 326796303	**Mass:** 35568	**Score:** 68	**Expect:** 3.5	**Matches:** 10
	exopolyphosphatase-like protein [Marinomonas mediterranea MMB-1]				

Fig. 4.18. MASCOT similarity search results for ~45 kDa protein. The peptides obtained from trypsin digestion of ~45 kDa protein from RSMS strain (absent in the mutant) was analysed for similarity using MASCOT programme. Exopolyphosphatase-like protein from *Marinomonas mediterranea* showed the highest similarity. All other similarities belonged to hypothetical proteins.

a) Overlap between tryptic digest of 45 kDa RSMS protein with Exopolyphosphatase-like protein from *Marinomons mediterranea*

Start	End	Observed	Mr (expt)	M	Peptide
12	38	3138.3907	3137.3834	1	R.SDFDGLVCAVLLKDMDMIDDILFVHPK.D + 2 Oxidation (M)
95	105	1320.7557	1319.7484	1	R.VVYDYYGGKEK.F
120	146	3152.4471	3151.4398	1	K.GDSAQFNEDEVLNPKDWVLLNFIMDAR.T + Oxidation (M)
120	146	3178.6426	3177.6353	1	K.GDSAQFNEDEVLNPKDWVLLNFIMDAR.T + Acetyl (K)
120	146	3194.6663	3193.6591	1	K.GDSAQFNEDEVLNPKDWVLLNFIMDAR.T + Acetyl (K); Oxidation (M)
154	165	1601.8381	1600.8308	1	R.EFRISNYQLMMK.L + Acetyl (K)
157	165	1127.5760	1126.5687	0	R.ISNYQLMMK.L
172	187	1979.0347	1978.0275	1	K.DMSIEEIQELEDVKER.T + Oxidation (M)
188	199	1521.8387	1520.8314	1	R.TELYFDHDAKAK.E + 2 Acetyl (K)
299	305	828.5637	827.5564	0	K.LNDIIAK.I + Acetyl (K)

b) Sequence coverage of matched tryptic peptides from 45 kDa protein of RSMS strain

1	MTEKKFRLVT	RSDFDGLVCA	VLLKDMDMID	ILFVHPKDM	QDGKIEIGAN
51	DITTNLPYVA	GCNLAFDHHL	SETVRNDGNI	ENHIIDPEAP	SAARVVYDYY
101	GGKEKFPNIS	VDMMDAVDKG	DSAQFNEDEV	LNPKDWVLLN	FIMDARTGLG
151	RFREFRISNY	QLMMKLIDCC	KDMSIEEIQE	LEDVKERTEL	YFDHDAKAKE
201	QIQRCATVHN	NLVVLDLTNE	ETIYTTNRFV	IYALYPDCNI	SIHKMWGLKK
251	QNTVFAIGKS	ILNRSSNTNV	GELCLTYGGG	GHLNAGTCQV	ENDLADEKLN
301	DIIAKINQDG				

Fig. 4.19. Analysis of 45 kDa protein. (a) The tryptic peptides of the Exopolyphosphatase-like protein which matched with the tryptic peptides of the ~45 kDa protein. (b) Protein sequence coverage of the matched tryptic peptides. The red text depicts peptides from Exopolyphosphatase-like protein of the *Marinomonas mediterranea,* which showed a match with the peptides of the 45 kDa protein absent in mutant.

Table 4.6. Sequence similarity of the Exopolyphosphatase- like protein

Description (accession number)	Identity
Hypothetical protein (*Caldibacillus debilis*) (WP020156360.1)	11%
Phosphoesterase (*Bacillus cereus*) (WP016116598.1)	11%
Oligoribonuclease (*Bacillus* sp.) (WP010678408.1)	11%

Similarity searches for Exopolyphosphatase-like protein of *Marinomonas mediterranea* was carried out using BLAST program. Here the first three most similar sequences were presented.

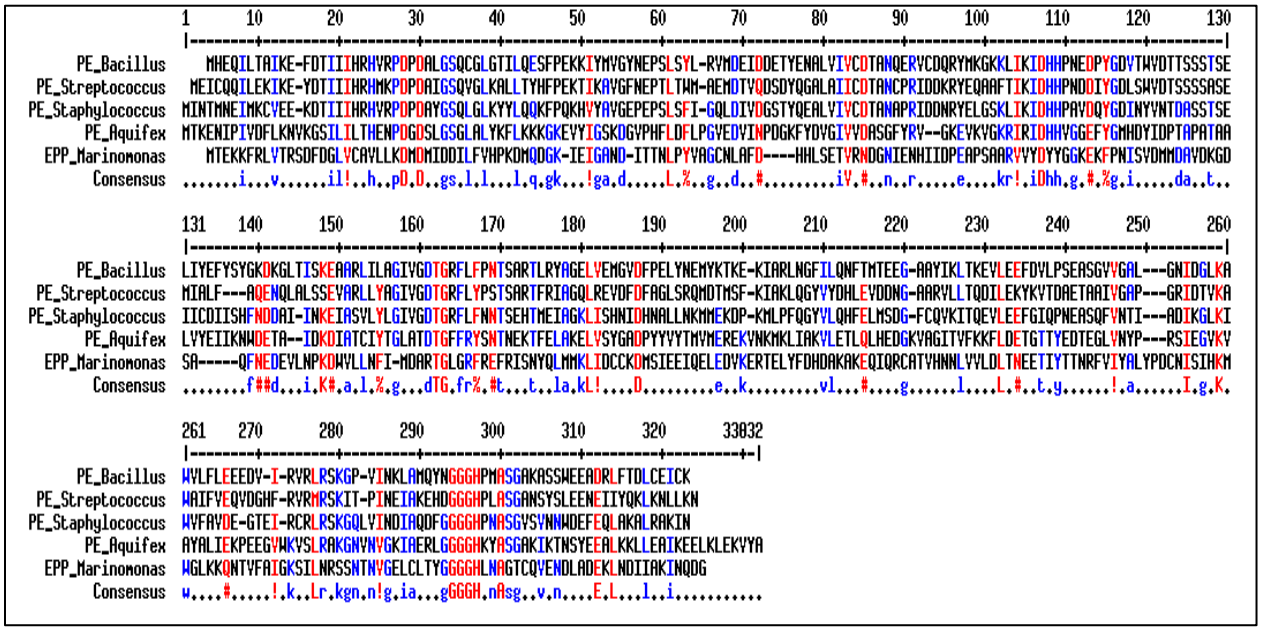

Fig. 4.20. Multiple sequence alignment for Exopolyphosphatase-like protein of the *Marinomonas mediterranea* with other proteins, obtained through BLAST search. The red coloured aminoacids are the most conserved amino acids. PE, EPP indicate phosphoesterase and Exopolyphosphatase-like protein, respectively.

Exopolyphosphatases are enzymes which play important role in the polyphosphate metabolism. The enzyme degrades polyphosphate with phosphomonoesterase activity. Further analysis showed that Exopolyphosphatase and Phosphohydrolase form part of the same operon in *E. coli* genome, and were located next to each other (Fig. 4.21). Although *E. coli* does not degrade TBP, the observed arrangement (Fig. 4.21) suggests that the corresponding genes in *Sphingobium* strain RSMS may be present in the same operon. This needs to be explored.

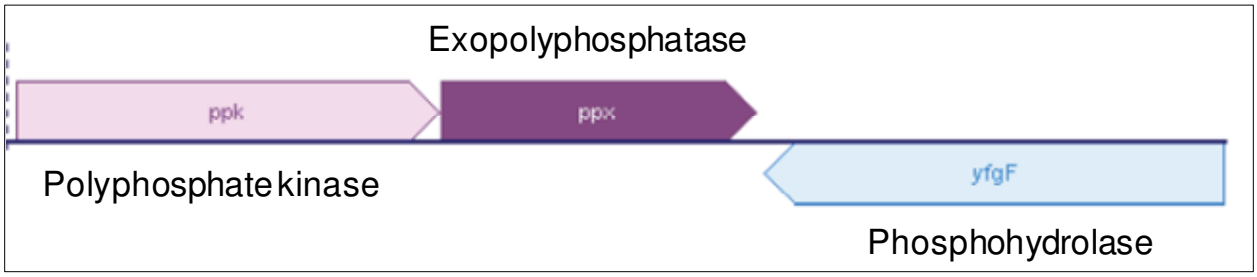

Fig. 4.21. Relative arrangement of *ppx* (Exopolyphosphatase) and *yfgF* (Phosphohydrolase) genes in the genome of *E. coli* strain K12. The operon has three genes which regulate the polyphosphate metabolism (http://ecocyc.org/ECOLI/NEW-IMAGE?Type=ENZYME &object =PPX-MONOMER)

TBP, DBP and mono butyl phosphate (MBP) are phosphoesters, which on degradation result in butanol and phosphoric acid, suggesting the involvement of phosphoestersaes. Hence, the genes encoding the 45 and 100 kDa proteins could be potential candidates for further studies towards understanding the molecular basis of TBP degradation.

Discussion

Till now more than 50 bacterial isolates have been reported to degrade TBP. There have been several attempts to identify the genes responsible for TBP degradation, for three decades, which did not succeed possibly for the following reasons: (1) Most of the strains showed very poor efficiency of degradation [24-26,29,34,81], and were difficult to work with, (2) None of the isolated strains showed any visible phenotype, which could have allowed the screening of TBP non-degrading mutant strains or which could have allowed isolation of mutants for further study, (3) several cultures which degraded TBP with somewhat better efficiency were not stable, even in the TBP containing medium and lost the trait during subculturing [36], (4) few strains were not really TBP degraders, but merely TBP sequesters [25,26], (5) Most of the strains showed poor tolerance even to low concentrations of TBP. There has been no clue, therefore, for the molecular basis of TBP degradation or its regulation.

TBP and DBP degradation in RSMS strain were strongly inhibited by glucose above 5%, indicating possible catabolite repression (Fig. 4.2). The mechanism of suppression in growth due to the suppression of TBP/DBP degradation is depicted in Fig. 4.22. The consequent unavailability of phosphorous (from TBP/DBP degradation) completely inhibited growth in TBP or DBP supplemented cultures containing 5% or 10% of glucose, indicating complete repression of TBP degradation. Such efficient regulation of TBP degradation by glucose has not been reported earlier. TBP degradation results in inorganic phosphate release which at high concentration can further inhibit TBP degradation and TBP dependent growth as reported earlier [36]. However, no adverse effect on TBP dependent growth was seen in the presence of 0–20 mM phosphate (supplied in the form of KH_2PO_4). Inorganic phosphate concentrations above 30 mM and 50 mM inhibited TBP (Fig. 4.1) degradation.

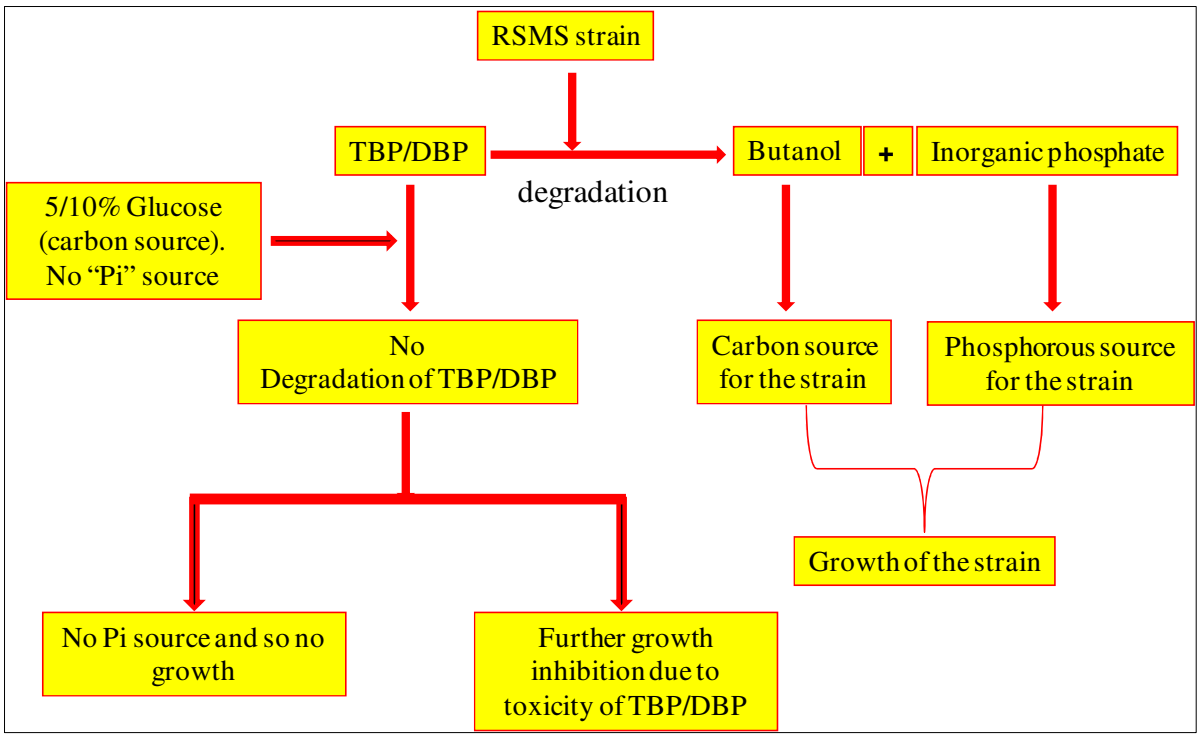

Fig. 4.22. Depiction of the mechanism of growth inhibition of RSMS strain due to suppression of the TBP/DBP degradation by 5 or 10% glucose.

The *Sphingobium* sp. RSMS strain exhibited a clear zone of TBP degradation around the colony. The zone was clearly visible at >5 mM^e of TBP concentrations. The strain being tolerant to high concentrations of TBP (30 mM^e TBP), such phenotype was exploited for the isolation of TBP non-degrading mutants. The colonies which did not show zone of TBP degradation were considered as putative TBP non-degrading mutants. A total of >50 such TBP non-degrading mutants were isolated by Tn5 mutagenesis in three attempts. This is the first study to report isolation of TBP non- degrading mutants.

In spite of several efforts the Tn5 transposon in mutant SS22 could not be probed (Fig. 4.9), which gave rise to speculation that these may be spontaneous mutants. Indeed, experiments involving repeated sub-culturing of RSMS strain in MMM supplemented with 1% glucose and 5 mM KH_2PO_4 resulted in the isolation of several TBP non-degrading spontaneous mutants

(Fig. 4.10). The spontaneous mutants lacked a zone of clearance around them, and did not use TBP/DBP as the sole source of carbon and phosphorous. These observations suggest that mutants generated through Tn5 mutagenesis were probably spontaneous double mutants, wherein the mutants lost the sensitivity to kanamycin as well as the ability to degrade TBP. The Tn5 generated (but spontaneous mutants) mutant SS22 was taken for further analysis since it derived from RSMS strain and it carried a selectable kanamycin (kan^r) marker.

When TBP or DBP were supplemented in MMM, wild type strain utilized each of them as sole source of carbon and phosphorous (Table 4.5). The butanol released in each step of the degradation could serve as the carbon source, whereas the inorganic phosphate released after complete mineralization (released only after last step of degradation) could serve as the phosphorous source. Mutant SS22 could not utilize TBP or DBP as sole source of carbon or phosphorous, thus suggesting that the entire pathway of TBP degradation was blocked in the mutant SS22. If, only the first step of the TBP degradation pathway was affected due to mutation, the mutant should have showed growth when DBP was supplemented in the MMM or, if, only the second step was affected due to mutation, the mutant should have shown growth when KH_2PO_4 (phosphorous source) was supplemented along with TBP, since first step of TBP degradation can release butanol which could serve as a carbon source. Both, wild type and mutant could utilize butanol and phosphorous for growth, which are the expected products of TBP or DBP degradation. These observations confirmed that the whole TBP degradation pathway was affected by the mutation, and also indicated the likely steps of TBP degradation.

Protein profile studies along with peptide mass finger printing identified three proteins absent in the mutant. These were identified as phosphoesterases. Among these two (~100 kDa) proteins showed identity to phosphohydrolase which is a known a phosphodiesterase, while the ~45 kDa

protein showed similarity with Exopolyphosphatase like protein, which is a known phosphomonoesterase. When analysed, these two proteins were found to be the part of the same operon in *E coli* genome, and located next to each other. Although *E. coli* does not degrade TBP, the observed arrangement (Fig. 4.21) suggests that the corresponding genes in *Sphingobium* strain RSMS may also be present in a similar operon. This needs to be explored.

Table. 4.7. Promiscuous activities of various enzymes

Enzyme	Primary activity	Promiscuous activity	Reference
Phosphohydroalse	phosphodiesterase	PTE	[94]
Arylsulfatase A	Sulfatase	PDE	[99]
Amino peptidase P	Peptide hydrolysis	PTE	[100]
Carboxyl esterase	carboxyl esterase	PTE	[101]
A-Esterase	Esterase	PTE	[99]
Carbonic anhydrase II	CO_2 Hydration	PTE	[99]
Carbonic anhydrase III	CO_2 Hydration	PME	[99]
Chymotrysin	Amidase	PTE	[99]
PTE (Bacterial)	Phosphotriesterase	PDE	[99]
Alkaline phosphatase	Phosphoryl transfer	PDE	[99]

PTE, PDE and PME indicate Phosphotriesterase, Phosphodiesterase and Phosphomonoesterase activities, respectively

TBP and DBP are both phosphoesters and the enzymes which degrade these compounds must be phosphoesterases in nature. There are several known phosphoesterases which show promiscuous activity towards other higher or lower order of phosphoesters (Table 4.7). For example, the primary activity of phosphohydrolase is phosphodiesterase activity, but it can also show the phosphotriesterase activity. Present study has identified two phosphoesterases which were absent in TBP non-degradation mutant. These proteins need to be investigated further to elucidate the genetic basis of TBP degradation.

Chapter V

Elucidation of biochemical pathway of TBP degradation

Isolation of the intermediates and products of the TBP degradation and their characterization is a major objective of this study**.** TBP degradation was proposed to be followed through the sequential release of each of the butanol from TBP, resulting in the formation of butanol and inorganic phosphate as the final products [36]. Though, the concept seemed logical, no proof of any intermediates or products could be obtained till now. A gas chromatographic and spectrophotometric approach was taken in this study to identify the intermediates and products, and to propose a biochemical pathway for TBP degradation. New extraction protocols and gas chromatography methods were developed for the analysis of TBP, dibutylphosphate (DBP) and butanol.

5.1. Standardisation of gas chromatographic methods.

Two types of solvents were used for the extraction of TBP and DBP from MMM. Dodecane was used for the extraction and analysis of TBP and 10% benzyl alcohol (in benzene, v/v) was used for TBP and DBP. Each of the solvent served a different purpose in the extraction and analysis of oranophosphates (TBP and DBP).

5.1.1. TBP and DBP extraction using dodecane.

Attempts were made to extract 20 mM DBP and 30 mM[e] TBP from MMM with equivolumes of dodecane. TBP was resolved well by gas chromatography, and showed a distinct peak with a retention time of 6.029 min (Fig. 5.1). Comparison between the peak areas of the pure TBP and of TBP from TBP-dodecane extractant suggested more than 97% extraction of the TBP from MMM, by dodecane. The method showed linearity of detection up to 30 nmoles of TBP (Fig. 5.4). However, dodecane failed to extract DBP from MMM. Hence, different solvents were used for the extraction of DBP from the MMM.

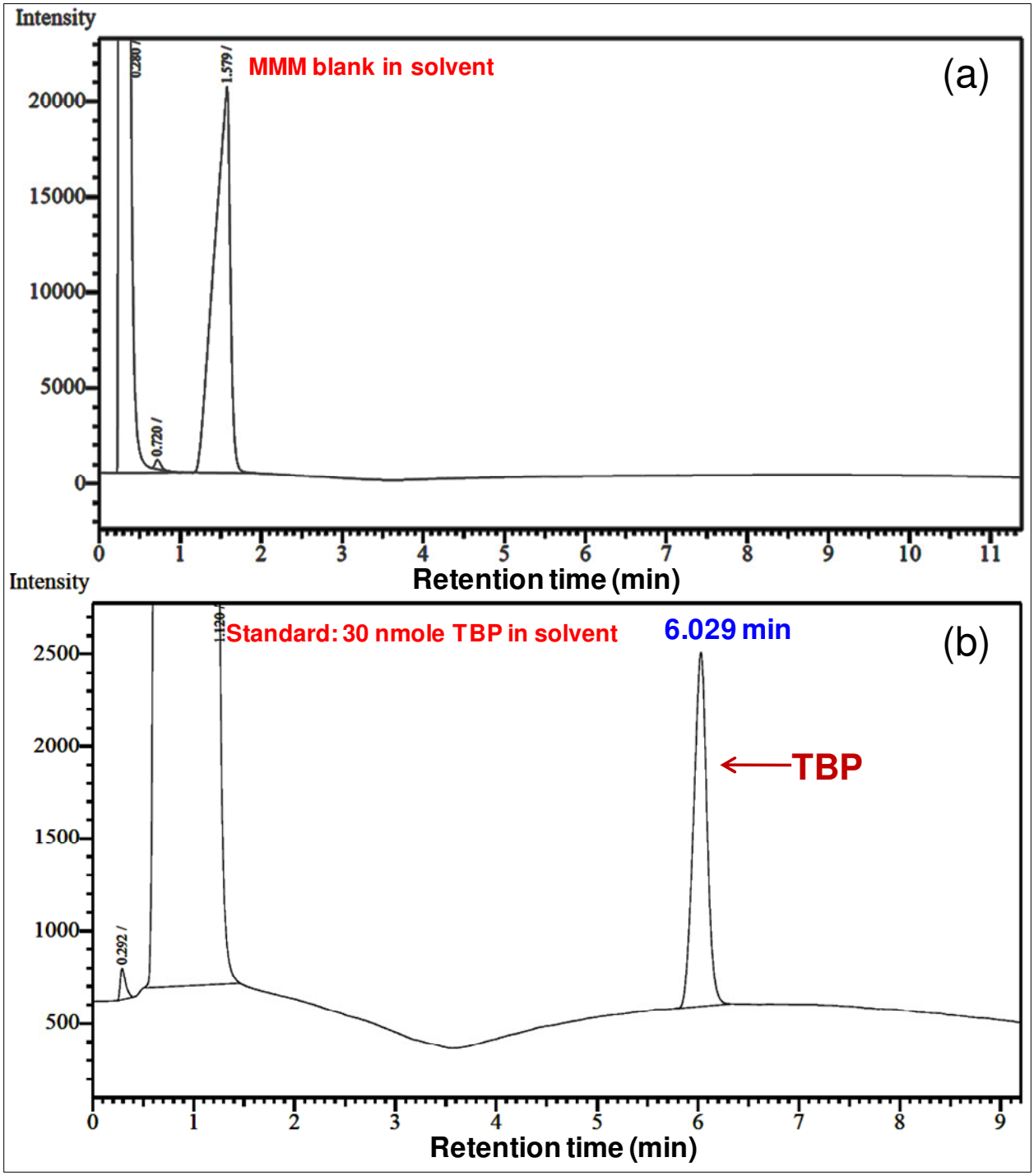

Fig. 5.1. Standardization of gas chromatographic analysis of TBP. Chromatogram obtained by injection of (a) 1µl MMM (blank) extracted in to equivolume of dodecane, (b) 1 µl of 30 mM[e] TBP (30 nmole) extracted in to equivolume of dodecane.

5.1.2. Extraction of TBP and DBP in benzyl alcohol

Predetermined amounts of TBP and DBP (as a mixture) were extracted with 10% benzyl alcohol in benzene (v/v) (henceforth referred to as BBA). Whole volume of MMM from the flask (containing TBP and DBP) was subjected to extraction, to avoid the problems of inhomogeneity which occurs if sample aliquots were taken from the bulk. The procedure extracted TBP and DBP, and resolved them as distinct peaks (Fig. 5.2). Retention time of the TBP and DBP showed dependence on the column temperature. At column temperature of 170^{o}C, the DBP and TBP showed, a retention time of ~1.185 and ~2.087 min, respectively (Fig. 5.2), and at 120^{o}C DBP and TBP were resolved at ~4.15 and 6.0 min respectively. The sensitivity of detection was 0.1 and 0.5 nmoles for TBP and DBP, respectively. The efficiency of BBA to extract the DBP and TBP was calculated by comparing the peak areas of the known amount of pure TBP and DBP with the peak areas of TBP and DBP from the BBA extractant, and were found to be more than 97% for the TBP and DBP extraction from MMM. The amount of DBP and TBP was calculated as nmoles from the slope of the peak areas. The slopes for the TBP and DBP estimations were 532 and 486, respectively (Fig. 5.4).

5.1.3. Analysis of butanol by gas chromatography

Attempts were made to extract butanol in to various solvents (dodecane, BBA, octane), but did not succeed. Hence, the aqueous samples were injected directly in to gas chromatograph. The method yielded well resolved butanol peaks from aqueous medium (MMM) (Fig. 5.3). For standard curve, known amounts of butanol dissolved in to MMM was injected in to gas chromatograph, and the peak areas obtained was plotted against the amount of butanol. All the negative control samples (including MMM blank) showed a small and insignificant peak at 0.6 min, where the butanol peak also appeared (Fig. 5.3).

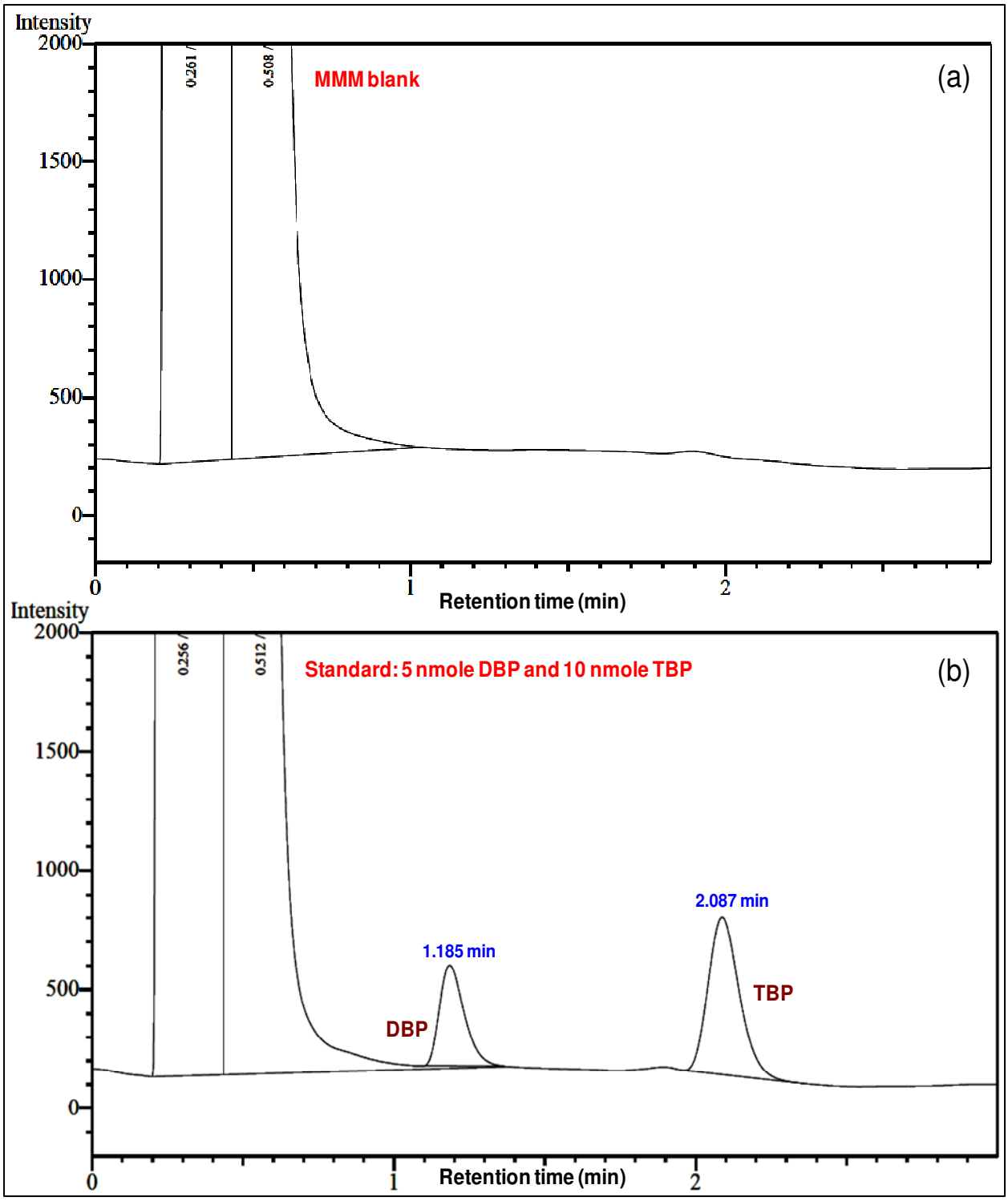

Fig. 5.2. Standardization of gas chromatographic analysis of TBP and DBP. Gas chromatogram obtained by injection of (a) a 1µl of MMM extracted in to equivolume of BBA and, (b) a 1µl of a standard mixture of 5 nmoles DBP and 10 nmoles TBP, extracted in to equivolume of BBA.

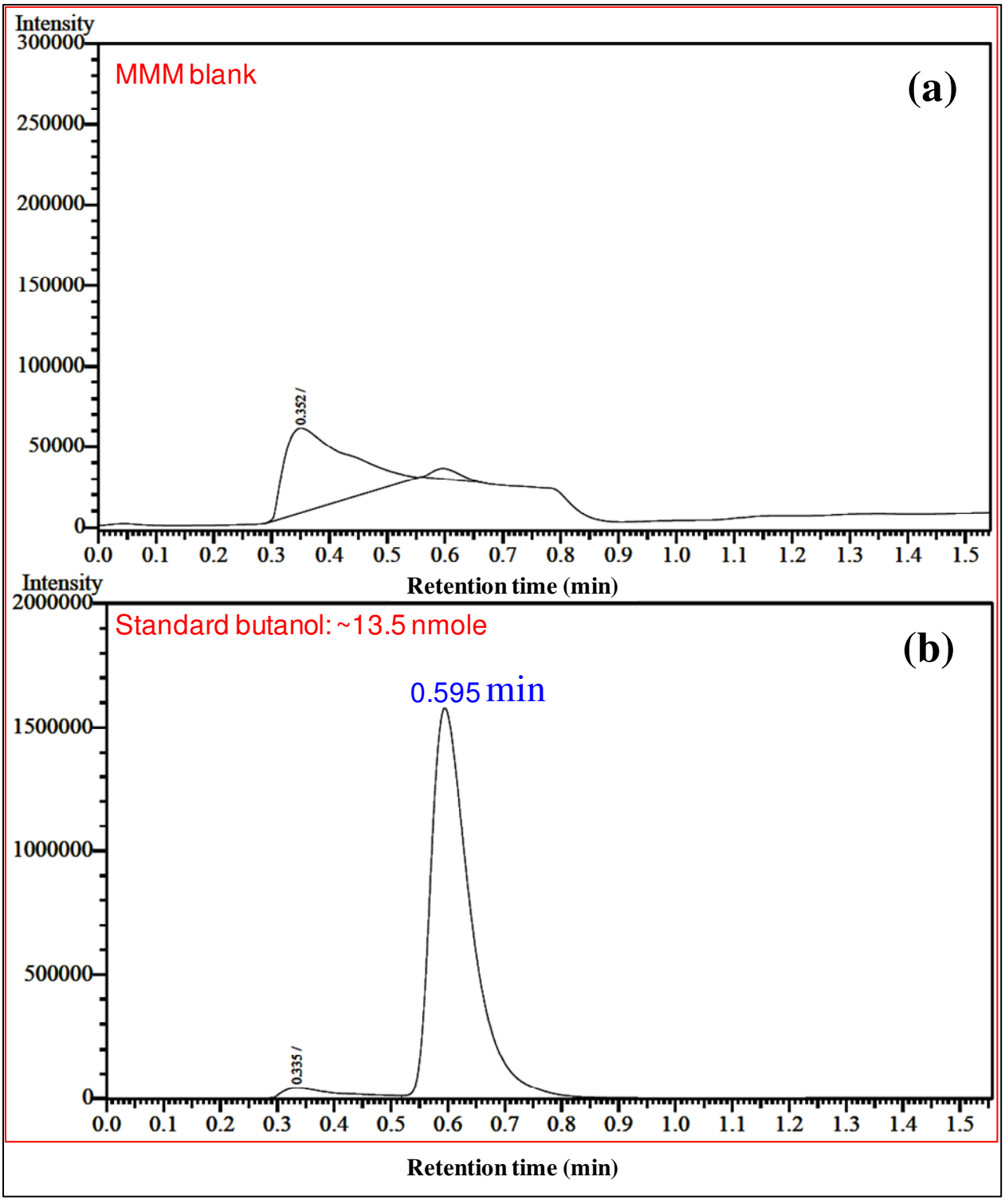

Fig. 5.3. **Standardization of gas chromatographic analysis of butanol.** Gas chromatogram obtained by direct injection of (a) 1µl of MMM, (b) 1 µl of 1000 ppm butanol in MMM, which is equivalent to 13.5 nmoles of butanol.

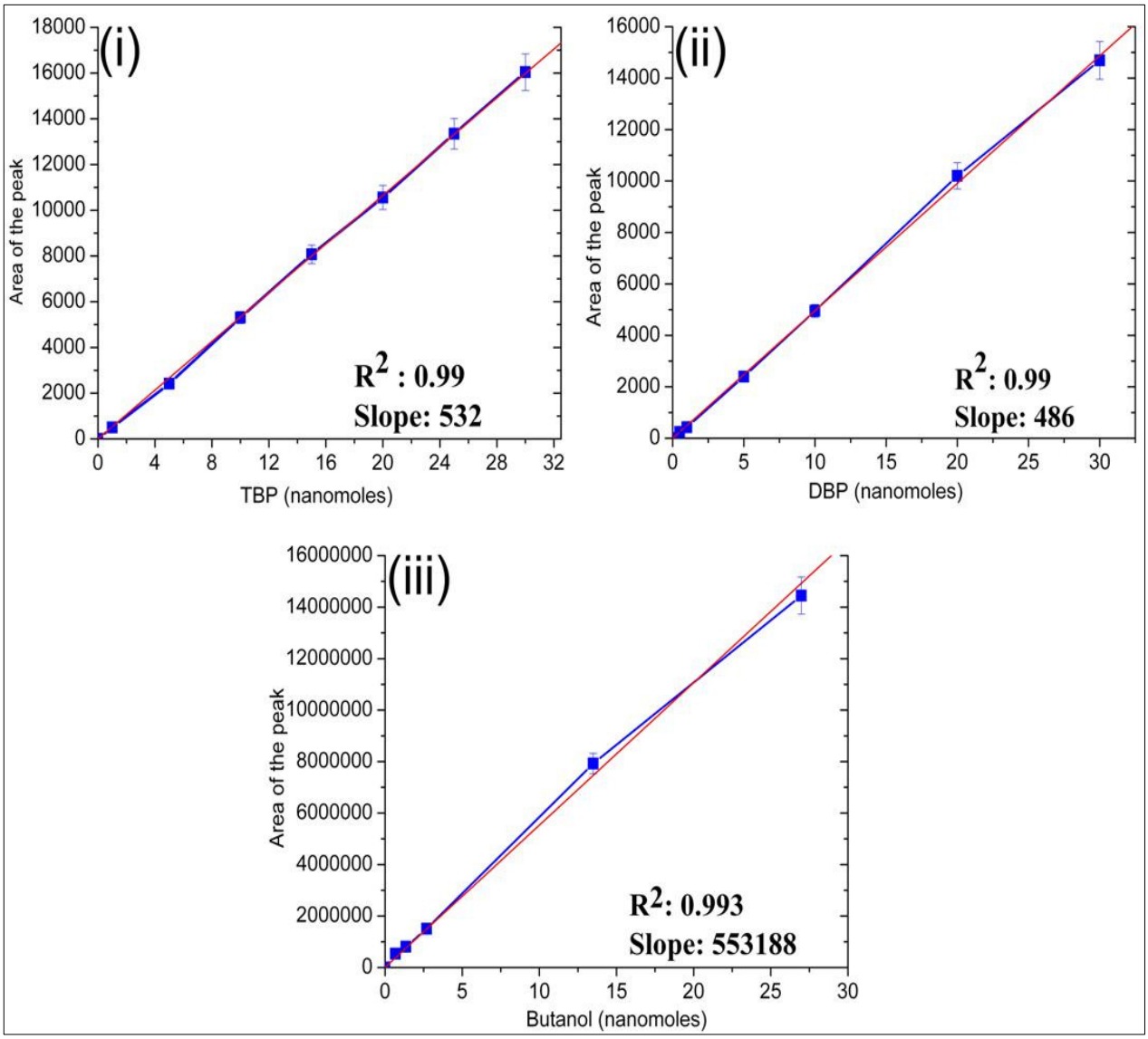

Fig. 5.4. Standard curves obtained for (i) TBP, or (ii) DBP, or (iii) butanol. The peak areas obtained from gas chromatograph was plotted against the known amounts of the analyte injected. The TBP was extracted either in dodecane/BBA, and DBP was extracted in BBA.

If, it were a butanol peak, the area corresponds to only ~0.5 pmoles of butanol, which was at least 1000 times less than the amount (nmoles) estimated in all further experiments.

The extraction method described in section 5.1.2 and 5.1.3 for TBP, DBP and butanol showed linearity up to 30 nmole for TBP, DBP and butanol with slopes of 532, 486 and 553188, respectively (Fig. 5.4)

5.2. TBP degradation analysis using RSMS strain.

The experiment was started by adding 30 mM^e TBP as a feed to the MMM containing 3 OD_{600nm}/ml cells of RSMS. But, only about 24-25 mM^e TBP was extracted in to BBA solvent, at 0 h (Fig. 5.6). This may be attributed to the binding to/uptake of rest of the TBP by the cells. Therefore, the TBP degradation was calculated with respect to the concentration of the TBP degradation products, such as, inorganic phosphate and DBP in the medium.

The RSMS strain completely degraded 30 mM^e TBP in 3 d (Fig. 5.5 and 5.8), with DBP and butanol detected as products of TBP degradation by gas chromatography (Fig. 5.6 and 5.7). Twenty four hours after inoculation, ~55% of 30 mM^e TBP was degraded, of which 35% underwent complete degradation to phosphate (10 mM) and butanol, while 20 % was converted to DBP (6 mM) (Fig. 5.8). The concentration of butanol remained at peak levels (~14 mM) in the first two days, whereas the DBP reached peak levels (~11 mM) at 48 h (Fig. 5.8).

The observed kinetics suggested that each day ~ 15 mM^e TBP was degraded to DBP, of which 10 mM DBP was completely degraded to phosphate and the rest remained in the medium (Table 5.1). Each day about 35 mM butanol was formed from TBP degradation, of which ~15 mM was detected in the medium and rest utilized for the growth (Table 5.1). Independent spectrophotometric measurements showed that after 3 days of inoculation, 30 mM^e TBP was completely degraded resulting in 28 mM of inorganic phosphate (Fig. 5.8) with rest used up for

growth. After 3 d, TBP or DBP or butanol were not detected in the culture medium, suggesting complete utilization of TBP and its degradation products as a carbon source for the growth (Fig. 5.8). Growth of the strain increased from a cell density of 3 OD_{600nm}, at inoculation to ~18 OD_{600nm} after 3 days (Fig. 5.8), which is equivalent to ~2.5 doublings.

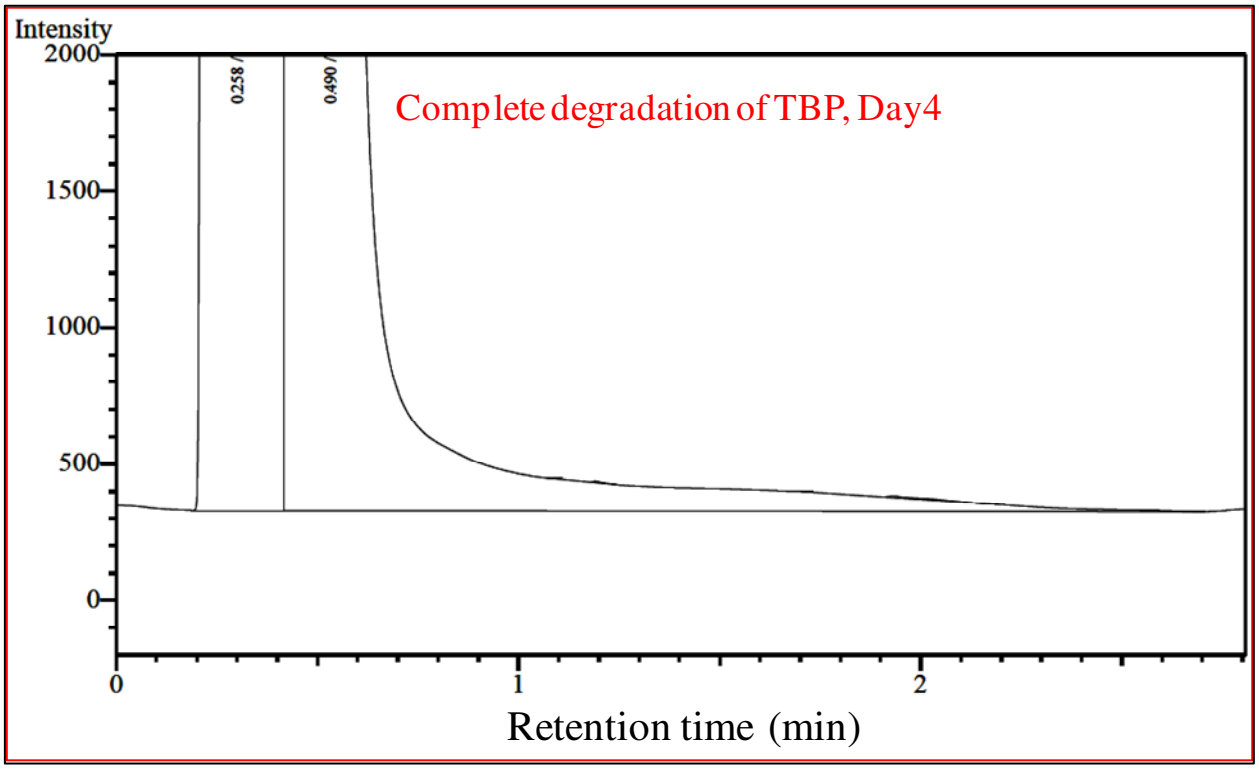

Fig. 5.5. Disappearance of 30 mM[e] TBP, 4 days after inoculation. Gas chromatogram obtained by injection of a sample four days after inoculation of RSMS strain in to MMM containing 30 mM[e] TBP. Absence of TBP and DBP peaks can be noted from the chromatogram.

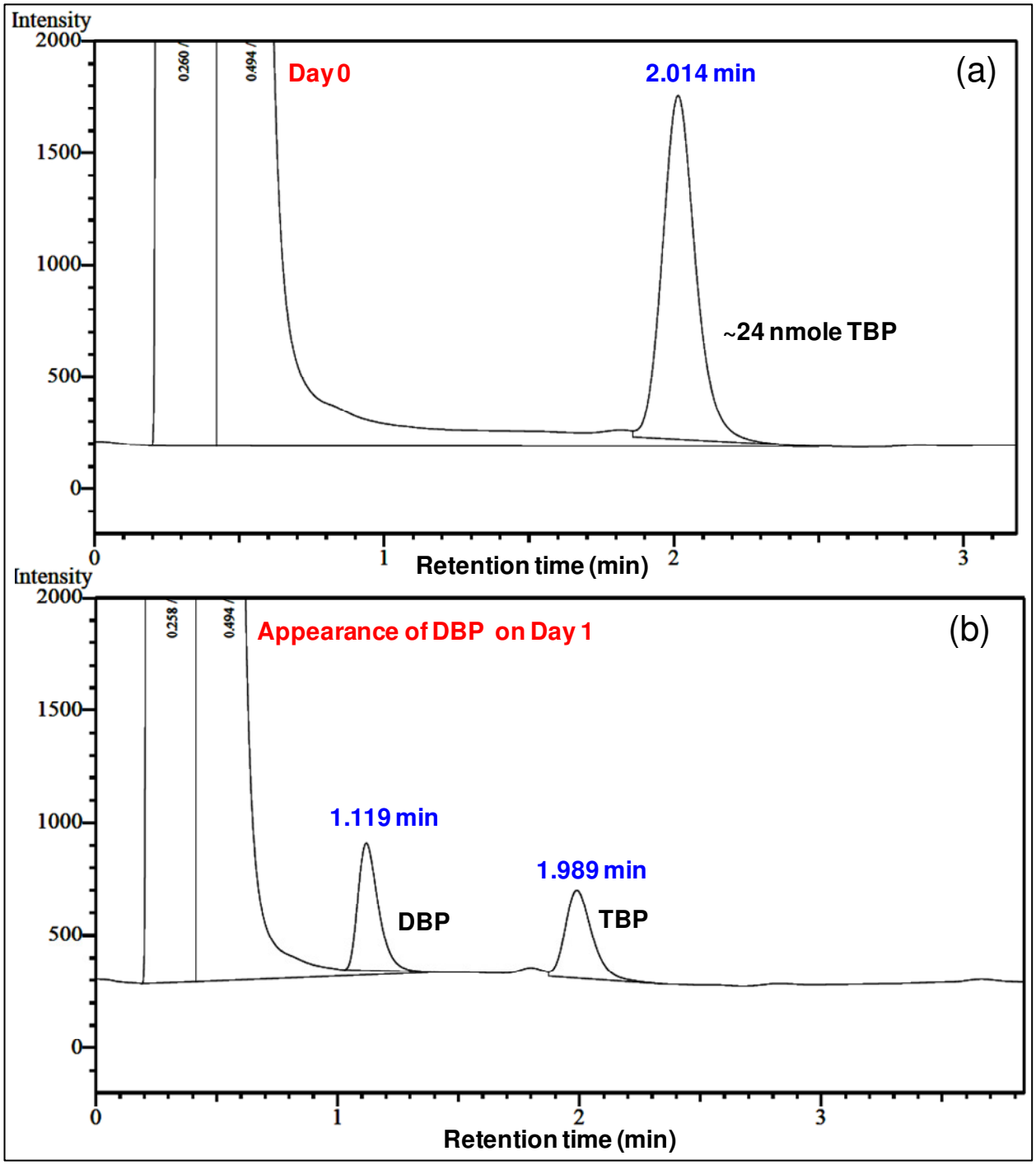

Fig. 5.6. Appearance of DBP as an intermediate of TBP degradation. Gas chromatogram obtained by injection of a sample at (a) 0 h (just after the inoculation), or (b) one day after inoculation of RSMS strain in MMM containing 30 mM[e] TBP. The amounts of TBP and DBP were calculated to be 6.7 nmole and 6 nmole, respectively.

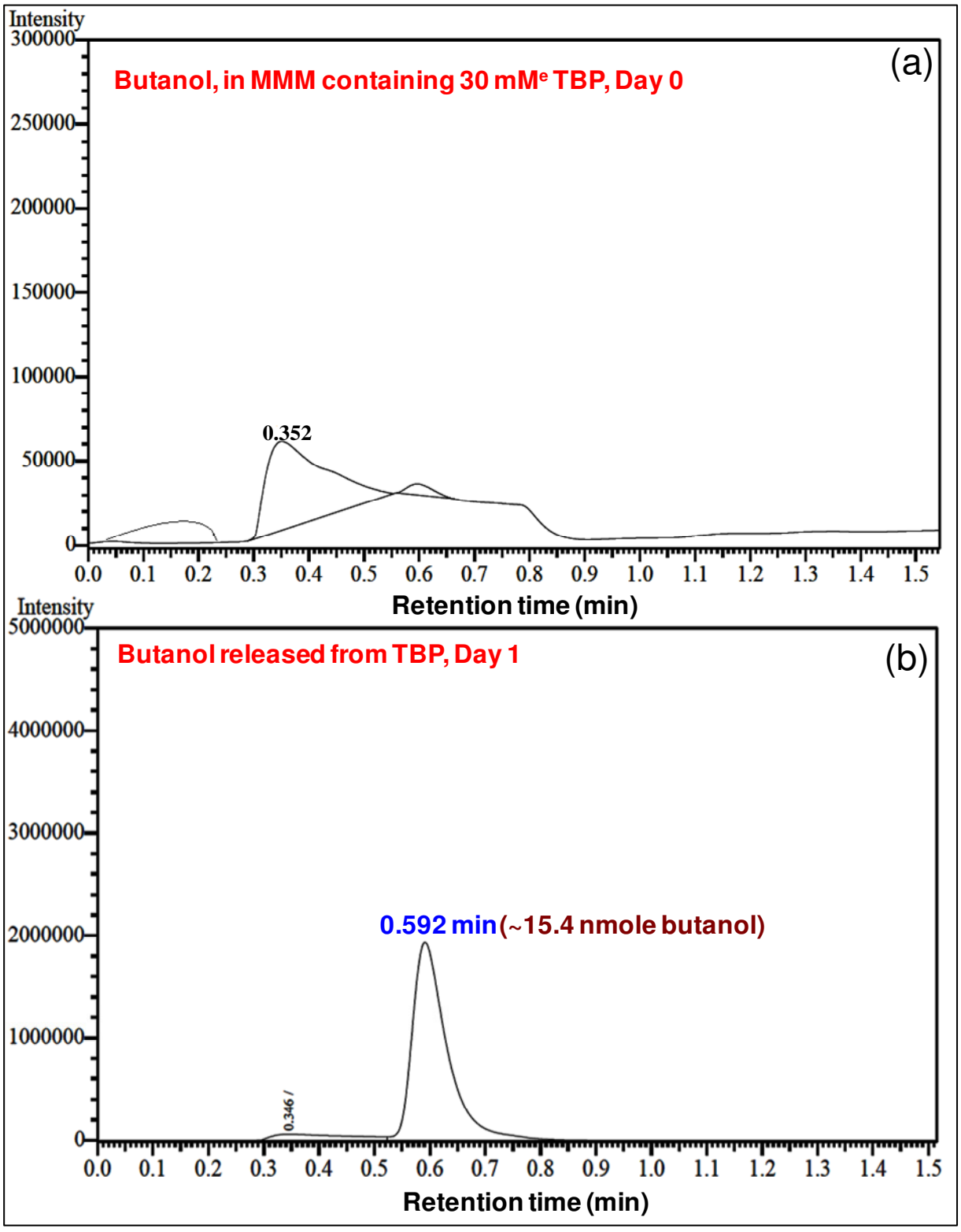

Fig. 5.7. Detection of butanol as a product of TBP degradation. Chromatogram obtained by injection of a sample at (a) 0 hours, or (b) one day after inoculation of RSMS strain in minimal medium supplemented with 30 mM^{e} TBP.

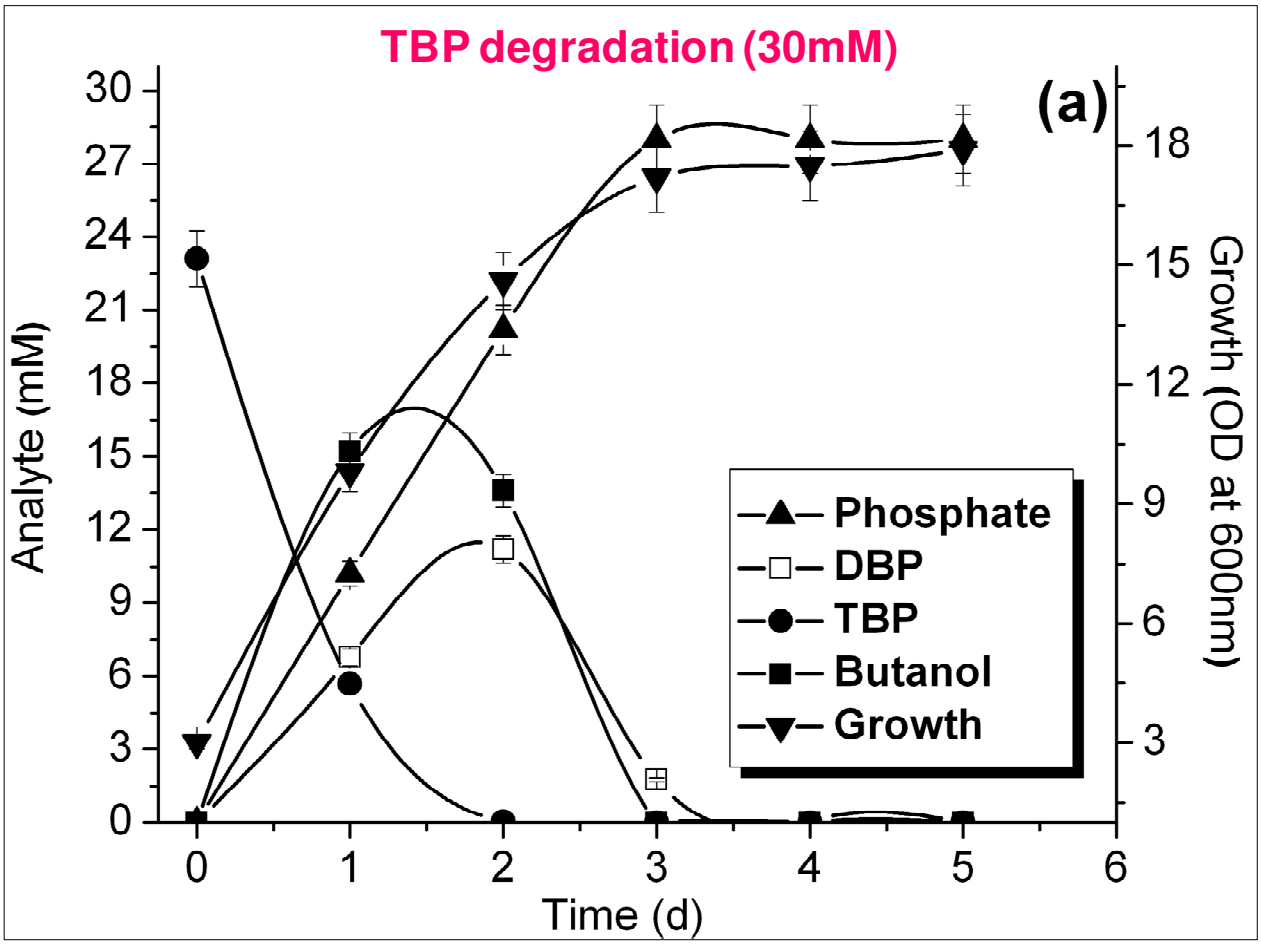

Fig. 5.8. Appearance of degradation products of TBP in MMM, assessed by gas chromatography in combination with spectrophotometric methods. Various symbols indicate levels of TBP (●), DBP (□), butanol (■), or inorganic phosphate (▲). Growth (▼) of the strain using the products of TBP degradation as the sole source of carbon and phosphorous.

Table 5.1. Kinetics of TBP degradation inferred from gas chromatography and sphectrophotometric methods.

Day	Detected TBP (mM^e) (A)	DBP (mM) (B)	iP (mM) (C)	Total TBP (mM^e) degradation, On a given day (D)	Butanol released on a particular day (E)	Butanol detected in the medium (F)	Butanol utilized for growth (mM), on a given day (G)
0	24	0	0	0	0	0	0
1	6	6.77	10.2	C1+ B1 = 16.97	(C1X3) +B1 = 37.37	15	(M1-N1) = 22.37
2	Nil	11.2	20.2	(C2-C1) + (B2-B1) = 14.43	(C2-C1)X3 + (B2-B1) = 34.43	14	(N1+M2)-N2 = 35.43
3	Nil	Nil	28	Almost whole DBP is degraded	B2 X 2 = 22.4	Nil	(N2+M3)-N3 = 36.4
4	Nil	Nil	28	Nil	Nil	Nil	Nil

RSMS strain was inoculated at 3 OD_{600nm} ($1.5x10^9$ CFUs/ml) in to minimal medium supplemented with 30 mM^e TBP, and each day the samples were analyzed for TBP, DBP and butanol content. The table gives estimated amount of TBP degraded, and intermediates (DBP) and products (inorganic phosphate and butanol) formed on each day. The amount of butanol utilized on each day for the growth was also calculated. Values represented in column A, B, C and F were observed values, while D, E and G are calculated values.

5.3. Dibutyl phosphate degradation by RSMS strain.

RSMS strain completely degraded 20 mM DBP in 3 d, when inoculated in to MMM containing 20 mM DBP as the sole source of carbon and phosphorous (Fig. 5.9). Gas chromatography analysis also detected butanol as the product of DBP degradation (Fig 5.10). The strain degraded 50% DBP in the first 24 h after inoculation (Fig. 5.11). Spectrophotometric analysis revealed inorganic phosphate as the end product. The strain released ~10 mM inorganic phosphate each day (Fig. 5.11). The butanol stayed at peak levels for first two days (Fig. 5.11) and was utilized for the growth of the strain. The growth of the strain was saturated on day 3 after inoculation. The strain's growth was less in 20 mM DBP in comparison to 30 mM[e] TBP. While butanol was completely used up as carbon source by day 3, phosphate released was in excess of growth requirement and accumulated in the medium. Appearance of butanol (an alcohol) and inorganic phosphate among the TBP and DBP degradation products suggests sequential cleavage of all ester bonds, resulting in the conversion of TBP to DBP and then to butanol and phosphate.

5.4. Biochemical pathway of TBP degradation.

Based on the combination of gas chromatographic and spectrophotometric analyses, a biochemical pathway for TBP degradation was proposed (Fig. 5.12). The pathway involves the release of butanol and DBP in the first step of TBP degradation. DBP could be subsequently degraded in two possible ways, (a) it may be degraded in a single step to release two molecules of butanol along with one molecule of inorganic phosphate as the final products, or (b) the DBP could be degraded in two steps, first step resulting in the release of monobutyl phosphate (MBP) and butanol from DBP, followed by the degradation of MBP to butanol and inorganic phosphate in the second step. The involvement of MBP as an intermediate could not be established or ruled out, because of the unavailability of MBP in pure form.

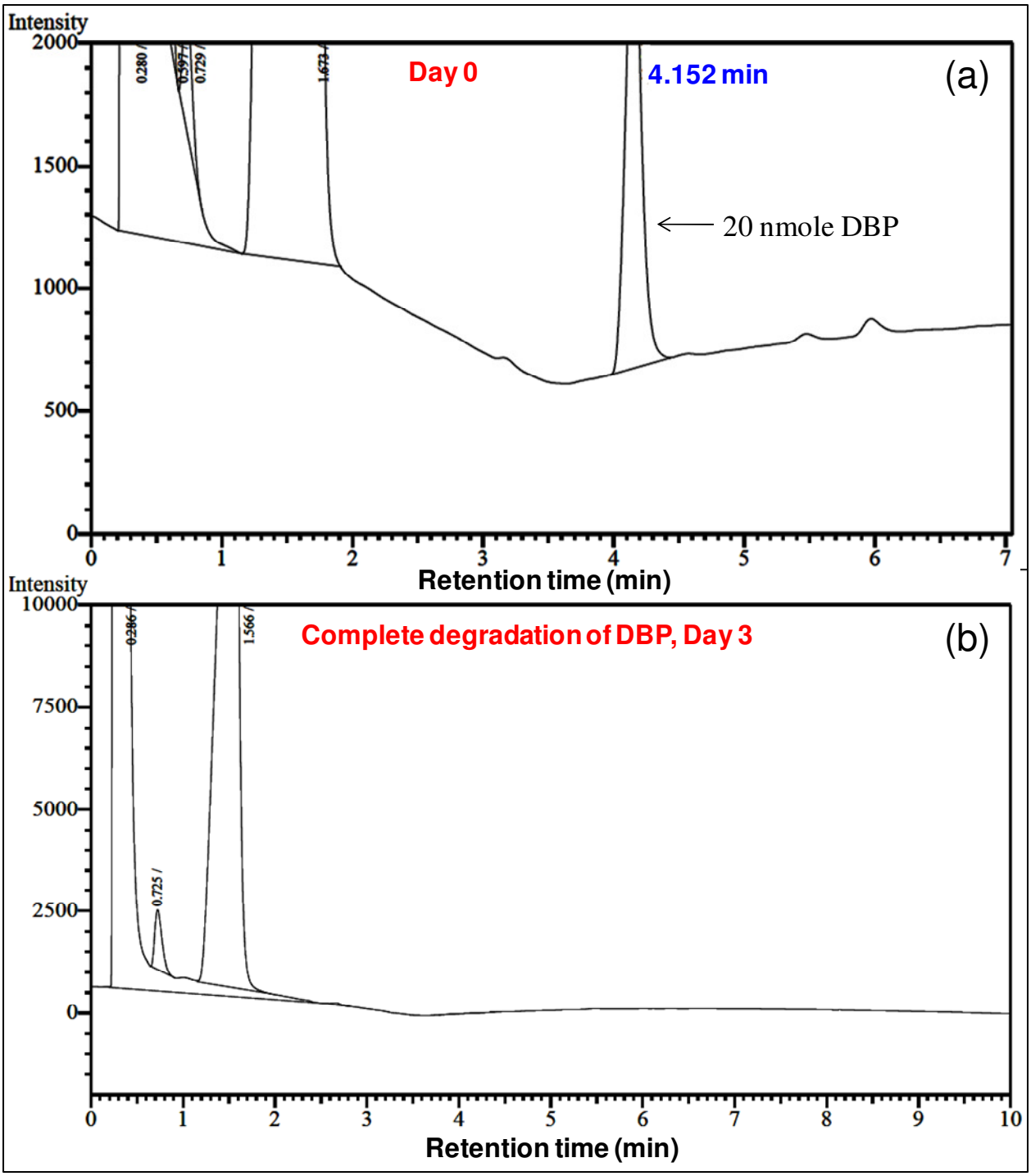

Fig. 5.9. Gas chromatographic analysis of DBP degradation. Chromatogram obtained by injection of a sample at (a) 0 h (just after the inoculation), or (b) 3 days after inoculation of RSMS strain in MMM containing 20 mM DBP.

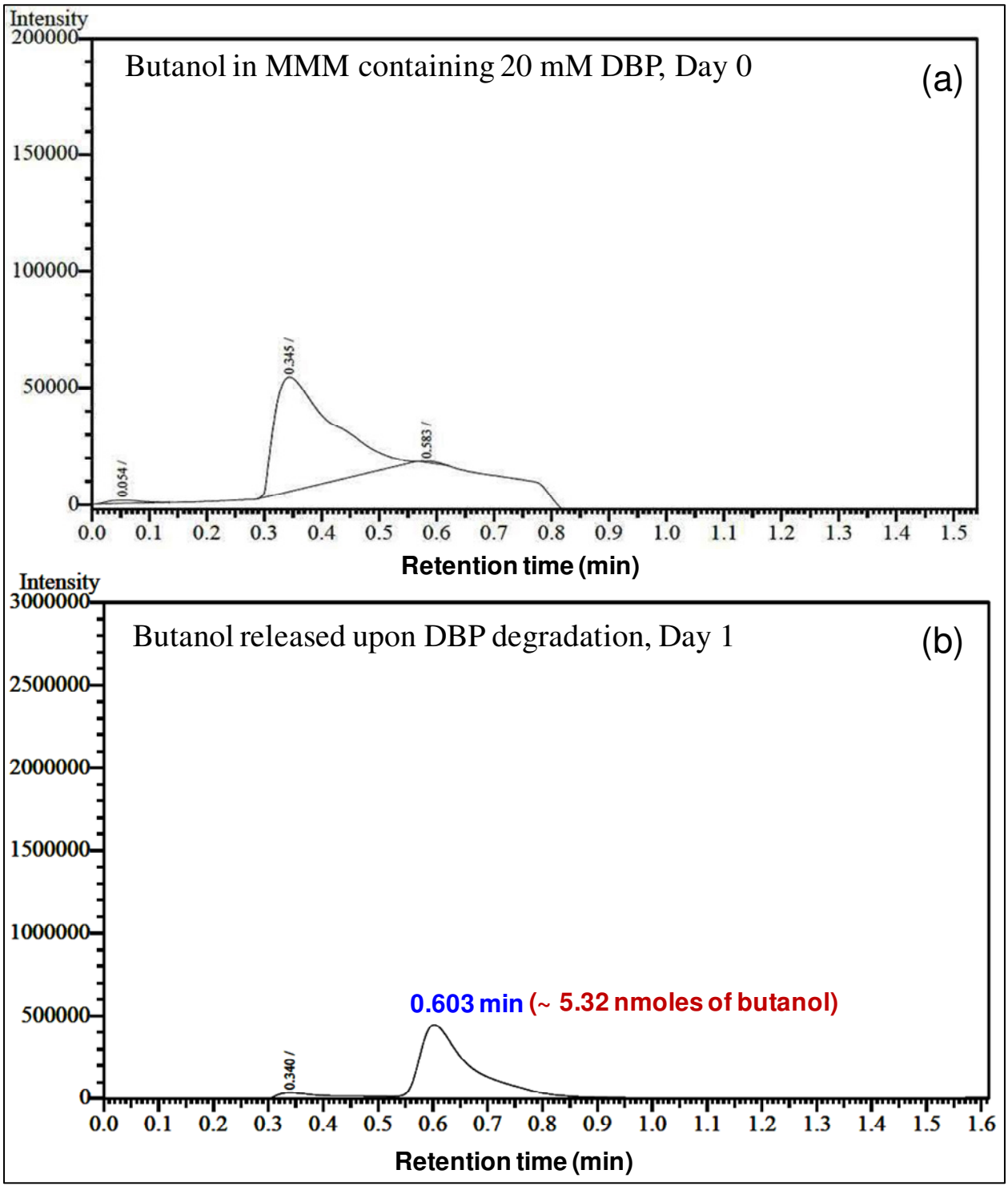

Fig. 5.10. Detection of butanol as a product of DBP degradation. Chromatogram obtained by injection of sample at (a) 0 hours, (b) one day after inoculation of RSMS strain in minimal medium supplemented with 20 mM DBP.

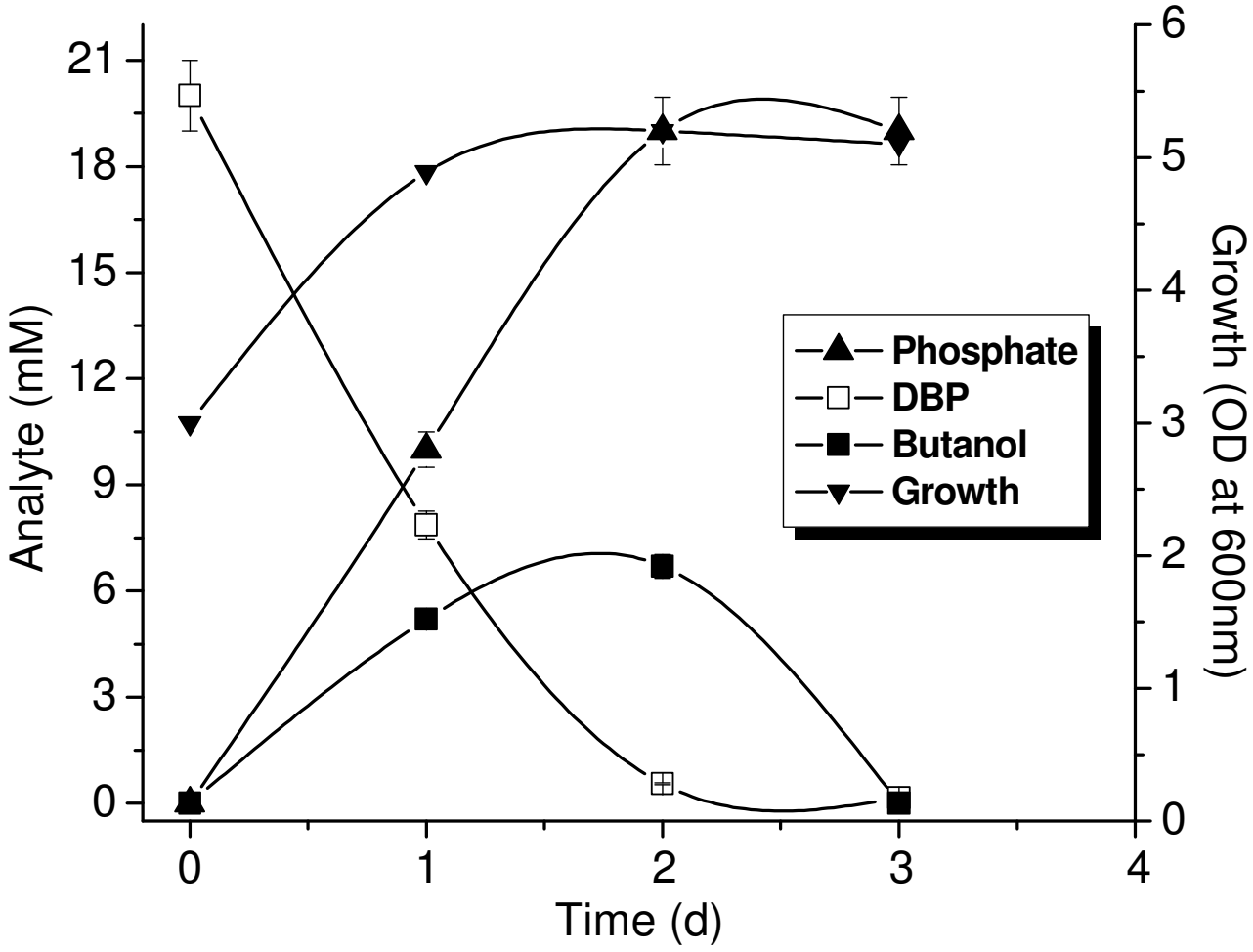

Fig. 5.11. Analysis of DBP degradation by RSMS strain. Appearance of degradation products of DBP in MMM, assessed by gas chromatography in combination with spectrophotometric methods. Various symbols indicate levels of DBP (□), butanol (■), or inorganic phosphate (▲). Growth (▼) of the strain using the products of DBP degradation as the sole source of carbon and phosphorous.

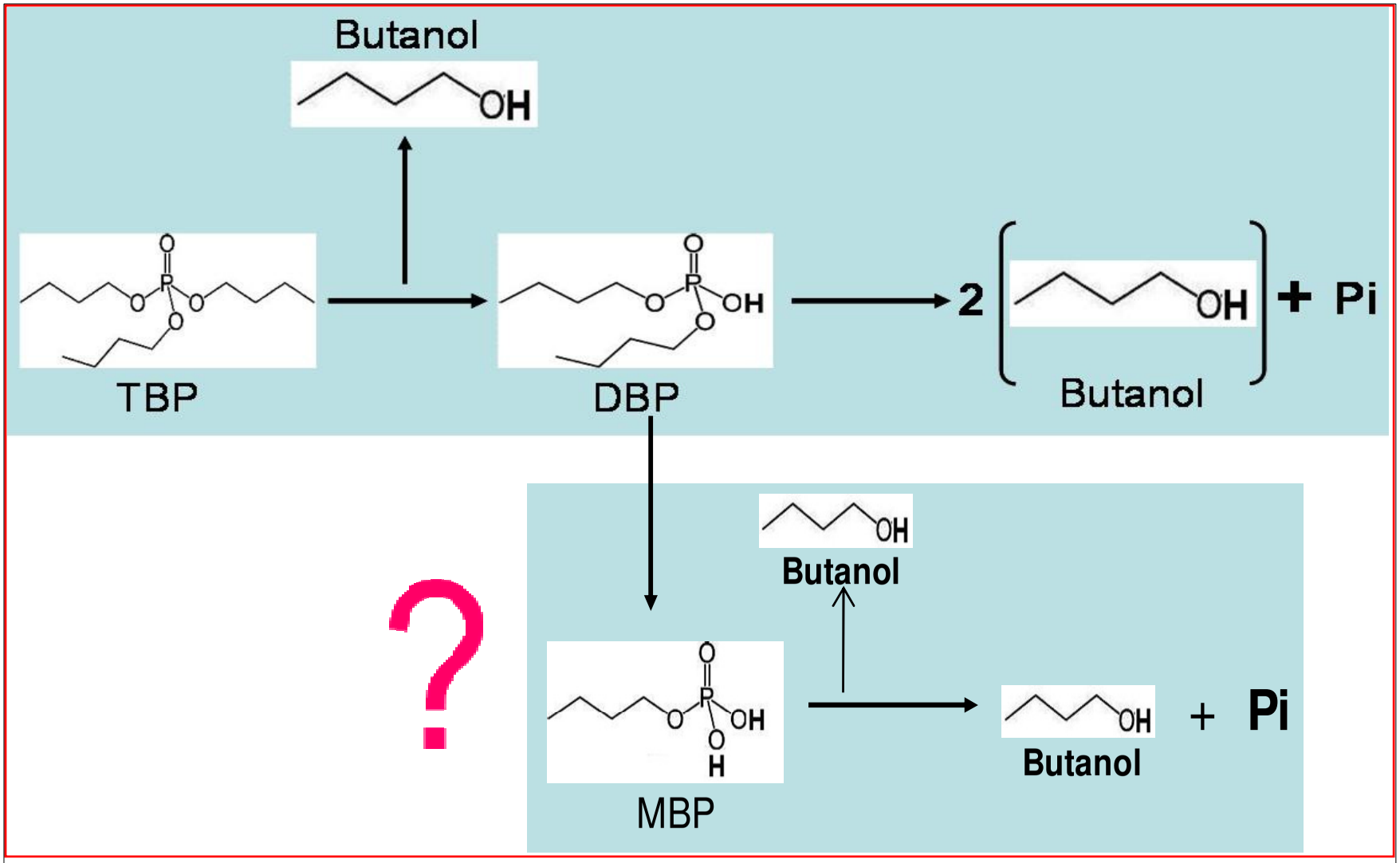

Fig . 5.12. Proposed biochemical pathway for TBP degradation by RSMS strain.

5.5. TBP and DBP degradation analysis in the mutant SS22.

No reduction in TBP or DBP concentration was observed following 3 days of incubation of the mutant cells in the test solutions supplemented either with 30 mM[e] TBP or 20 mM DBP (Fig. 5.13). Also, no butanol (Fig. 5.14) or phosphate was detected in the medium, suggesting no TBP or DBP degradation occurred in the mutant cells.

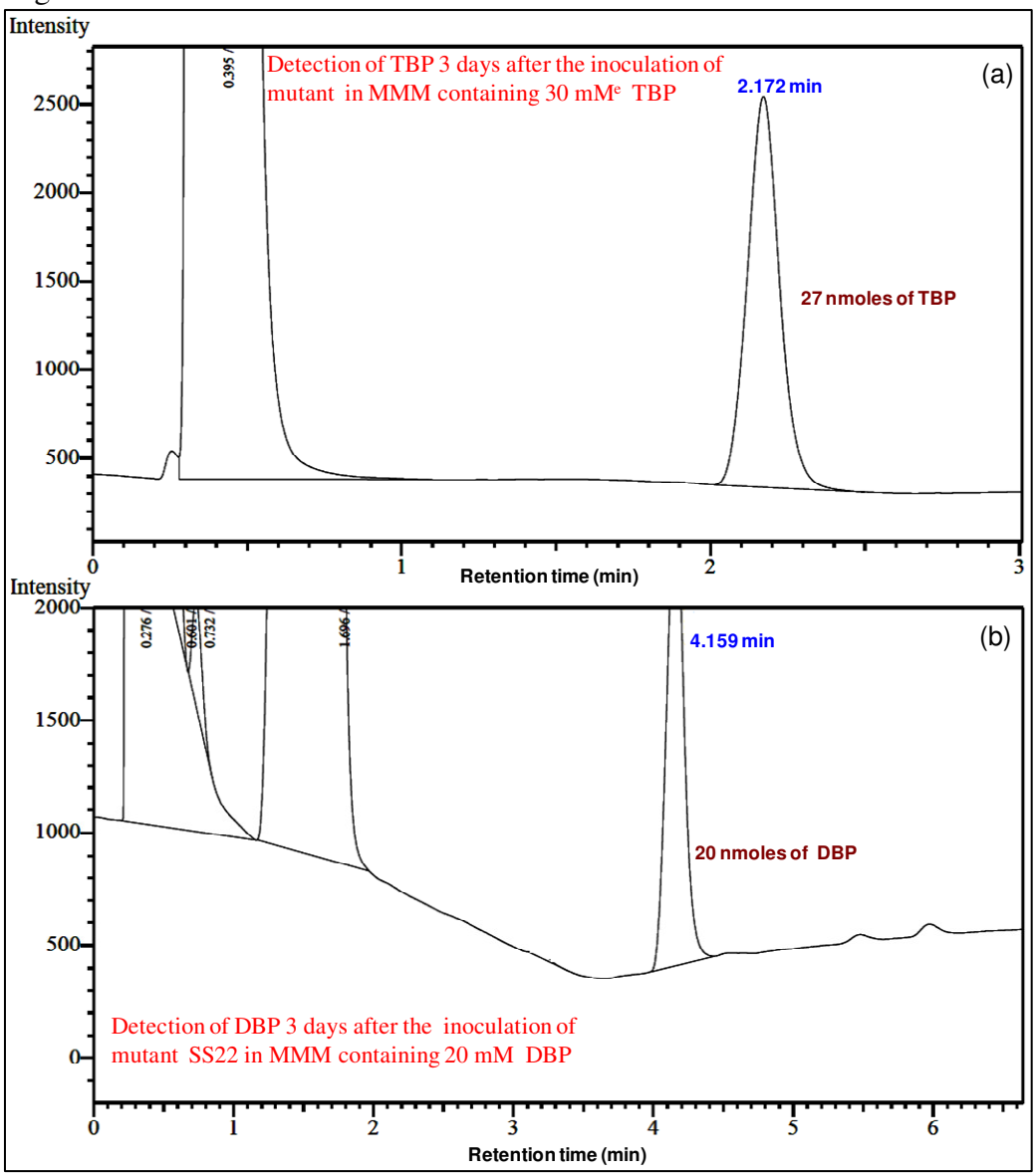

Fig. 5.13. Gas chromatographic analysis of TBP and DBP degradation by mutant SS22. Gas chromatogram obtained by injection of (a) 1 µl sample, obtained after 3 days of mutant SS22 inoculation in to MMM containing 30 mM[e] TBP, or (b) 1 µl sample, obtained after 3 days of mutant SS22 inoculation in to MMM containing 20 mM DBP.

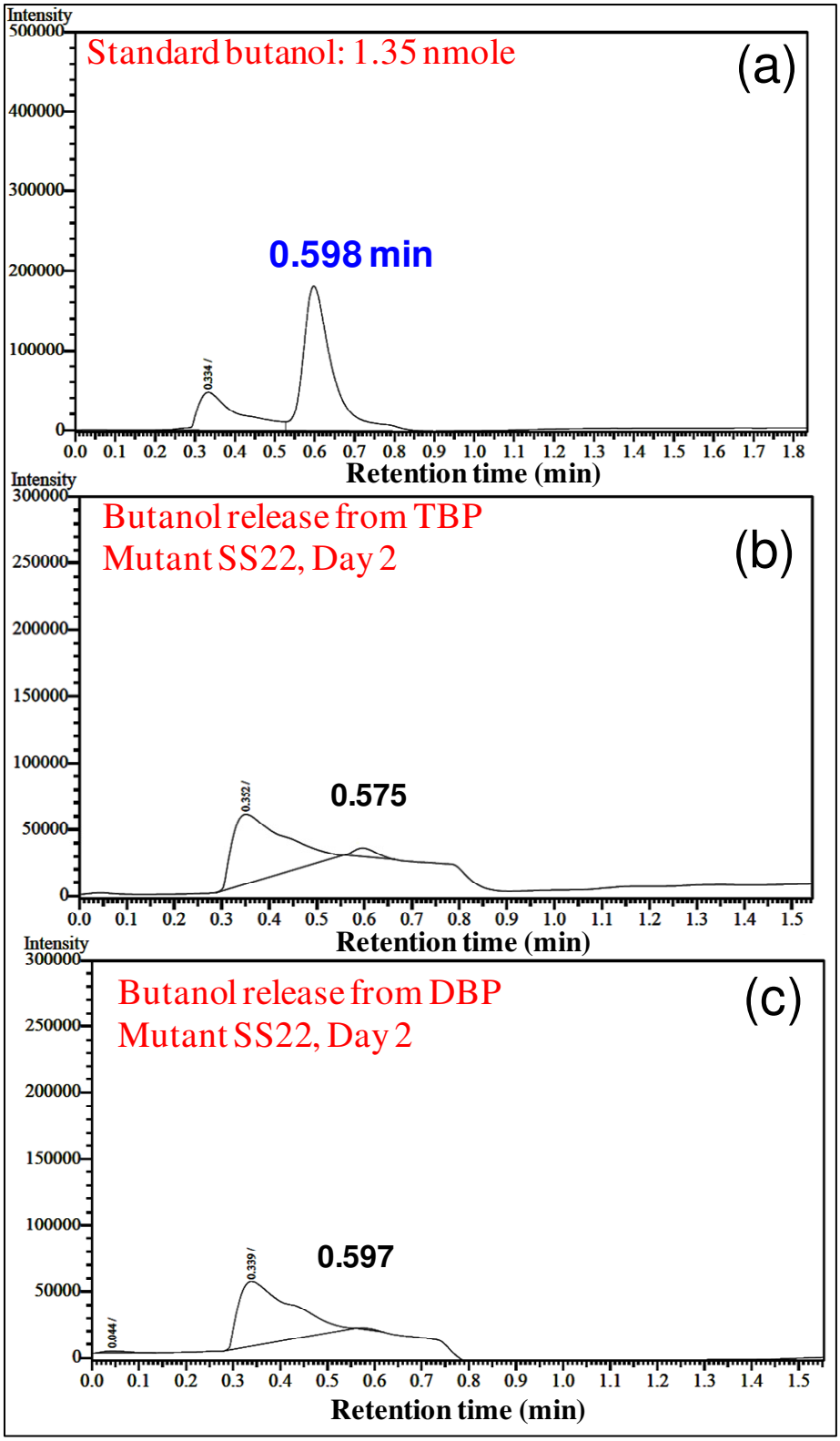

Fig. 5.14. Gas chromatographic analysis of butanol release from TBP and DBP by mutant SS22. Chromatogram obtained by injection of (a) standard 1 µl of 1.35 mM (1.35 nmole) butanol, or (b) 1 µl sample, obtained after 2 days of mutant SS22 inoculation in to MMM containing 30mM[e] TBP, (c) 1 µl sample, obtained after 2 days of mutant SS22 inoculation in to MMM containing 20 mM DBP. The standard butanol (a) is presented here for the comparison of chromatograms.

5.6. Characterisation of Phosphoesterases

Gas chromatography analysis revealed that the degradation of phosphotriester (TBP) showed an alcohol (butanol) and phosphodiester (DBP) as intermediates, while DBP degradation showed butanol and phosphoric acid as the end products. The observed intermediates and products released could be attributed to phosphoesterase activity or activities. The mutant SS22 could neither degrade TBP nor DBP, suggesting that it lacks the relevant phosphoesterases. Besides, protein profile studies (Chapter 4, 4.12 and 4.13) also indicated the absence of certain phospho-mono and di-esterases in the TBP non degrading mutant SS22. Therefore, phosphoesterases from the RSMS strain were partially characterized.

5.6.1. Phosphodiesterase and phosphomonoesterase activities in RSMS and mutant SS22.

Phosphodiesterase and phosphomonoesterase analyses were carried out with the mutant SS22 and wild type RSMS strain. Phosphomonoesterase (PME) and phosphodiesterase (PDE) assays were carried out with standard substrates. Para-nitrophenolphosphate (pNPP) and Bis-para-nitro phenol phosphate (Bis-pNPP) were used as substrates for the PME and PDE assays, respectively. Para-nitrophenol (pNP) released in PDE and PME assays was measured spectrophotometrically.

Cell free protein extracts of wild type cells released about 13, 7, and 2 μmoles of pNP released/h/mg of protein, from Bis-pNPP (phosphodiester) at pH 7, 8, and 9, respectively. In comparison, the activity was almost nil for the mutant SS22 at any of these pH values (Fig 5.15a). However, prolonged incubation of cell free extracts of mutant SS22 showed very negligible activity for Bis-pNPP, at pH 7 (0.5 μmole pNP released/mg protein/h). The wild type strain also showed about 28, 18 and 11 μmoles of pNP released/h/mg of protein, from pNPP (a phosphomonoester). The activity in the mutant was ~10 times less at pH 7 or 3.5 times less at pH 8, in comparison to wild type activity (Fig. 5.15b). Further analysis showed that PME activity in mutant SS22 was nearly comparable to wild type at pH 9 or 10 (Fig. 5.15).

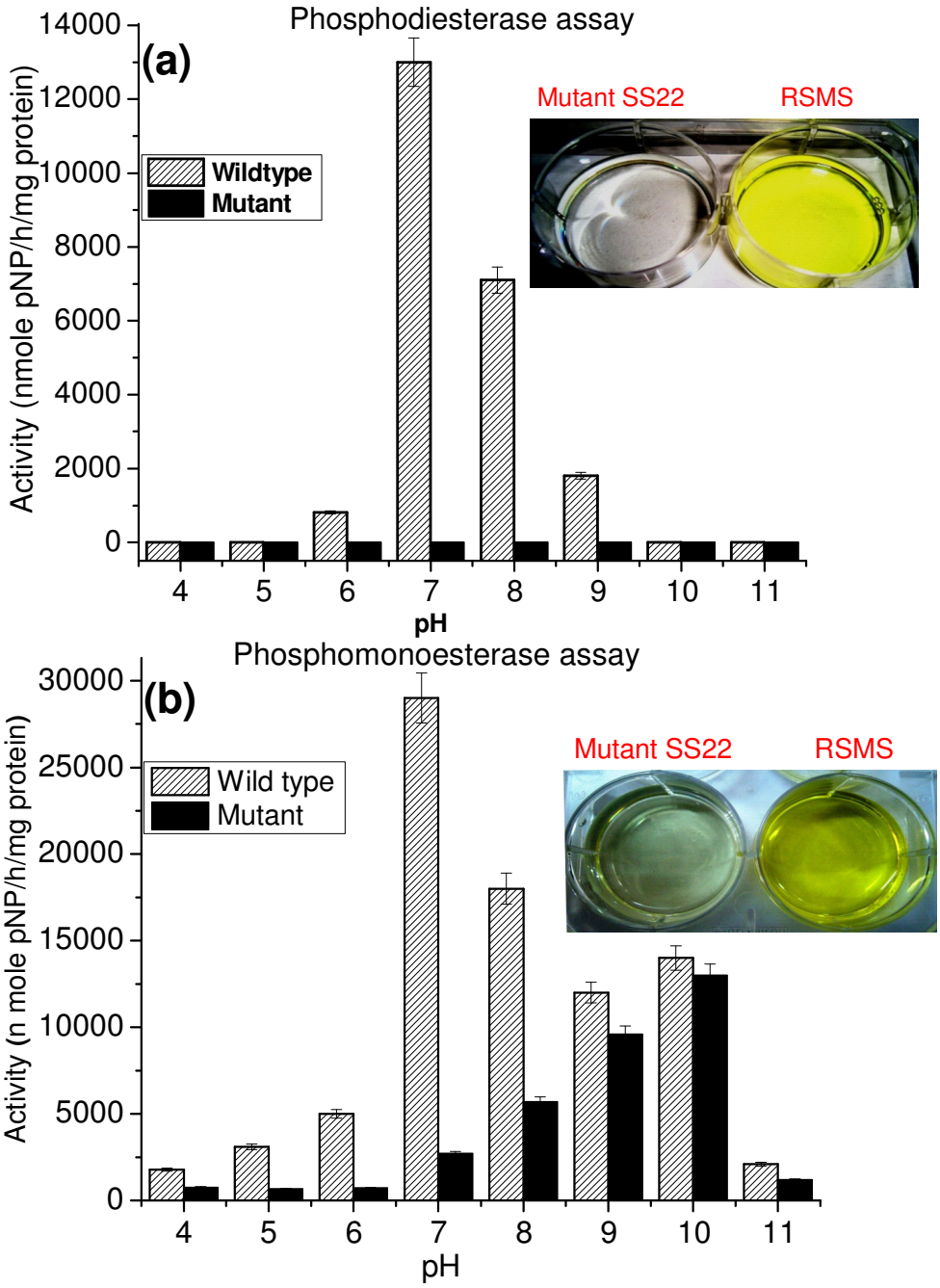

Fig. 5.15. Di- and mono-phosphoesterase activities of RSMS and mutant SS22 strains. Cell free extracts of wild type or mutant SS22 cells were incubated with (a) Bis-pNPP, for phosphodiesterase activity, or (b) pNPP for phosphomonoesterase activity. Released product, p-nitrophenol was measured spectrophotometrically. The differences in the para-nitrophenol released, in the reaction can be visualized as differences in the intensity of yellow colour, as shown in the (a) and (b).

The differences in PDE activity and PME activity between wild type and mutant could also be visualized as difference in intensities of yellow coloured product (pNP) released during Bis-pNPP and pNPP degradation (Fig. 5.15).

5.6.2. In gel PDE and PME assays

In gel PDE and PME assays were carried out with cell free extracts of wild type and mutant SS22. Since the product of the PDE and PME activities (paranitrophenol) is highly diffusible, large amount of native protein (~1.5 mg) was loaded in 4 lanes of a mini native PAGE gel. High loading allowed high PDE and PME activity, which avoided problems in visualization of yellow colour in short time since para-nitrophenol diffuse quickly.

Wild type extracts showed two pNP bands for both PDE and PME activity (Fig. 5.16). In comparison, mutant extracts showed only single para-nitrophenol band both for PDE and PME activities. Since the gel was electrophoresed in the native conditions the molecular mass of the protein related to the band could not be identified. However, the position of the band was roughly equivalent to the ~100 kDa protein missing in the mutant (Fig. 5.16). The experiment clearly suggested that the proteins showed non specific activity for both phosphodiester (Bis-pNPP) and phosphomonoesters (pNPP). The observations also suggested that one of the phosphoesterase is absent in the mutant, and is capable of acting both on phosphodiesters and phosphomonoesters.

5.6.3. DBP degradation assay

PDE activity was also assayed with DBP (a phosphodiester, and intermediate of TBP degradation), and released Pi was measured after 24 h of incubation. Cell free extracts of RSMS cells incubated with DBP at pH 7 for 24 h showed 15 µmole of DBP degradation/d/mg protein, which was ~5 times more activity than that of the mutant SS22 cells (Fig. 5.17). The DBP

degradation was also observed at basic pH values. It is to be noted that the release of inorganic phosphate from DBP requires both PDE and PME activities.

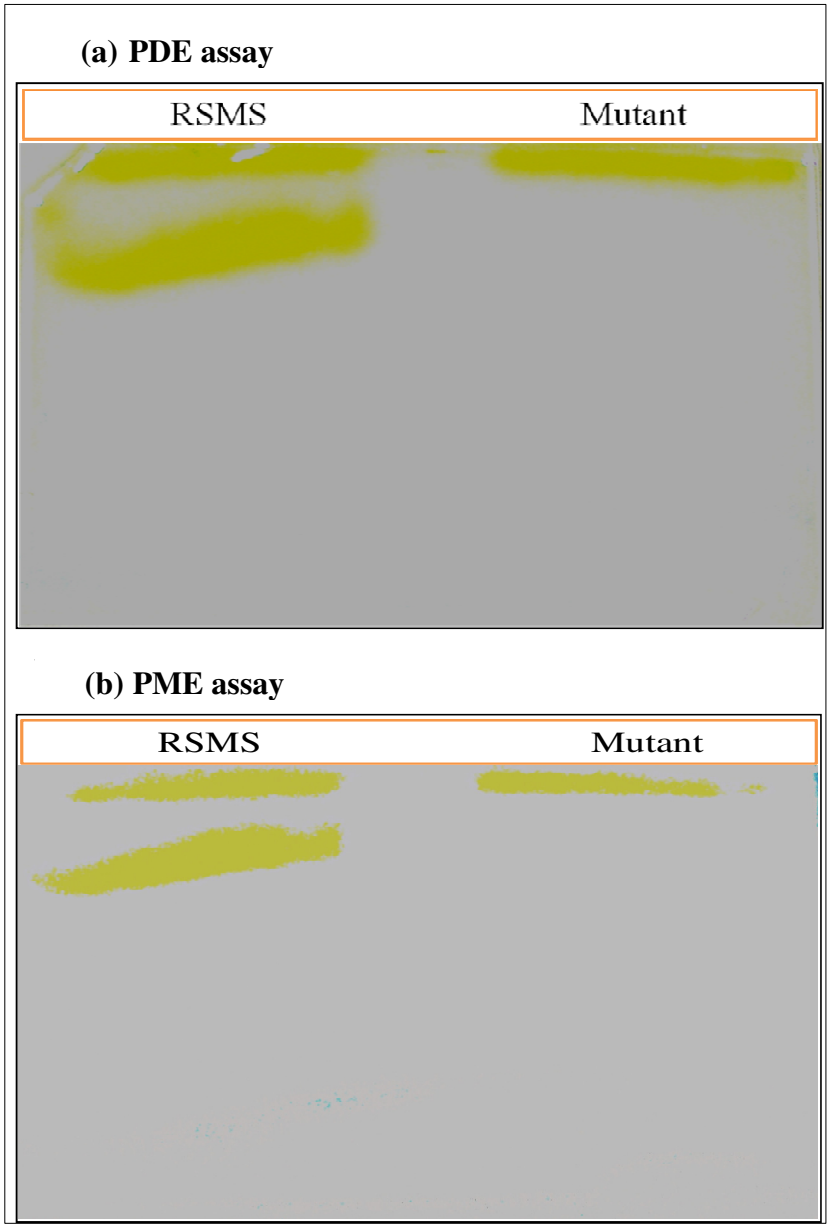

Fig. 5.16. In gel PDE and PME assay. Cell free protein extracts (Total of 1.5 mg in 4 lanes) from wild type and mutant cells were resolved by native-PAGE on 12% gel. The gel was washed with MMM, and incubated with MMM containing (a) 10 mM Bis-pNPP, or (b) 10 mM pNP. The released yellow colored product (pNP) due to PDE and PME activity was noted after 0.5 h. The 4 lanes were merged to get prominent PDE and PME activity. The para-nitrophenol in soluble and so diffusible in the MMM and so could not yield sharp bands.

The DBP degradation was also observed at 60°C, and was similar to that at 30°C (Table 5.2) indicating that the activity was thermostable. When 1 mM caffeine (a known phosphodiesterase inhibitor) was added to the same reaction mixture, DBP degradation was completely inhibited (Table 5.3).

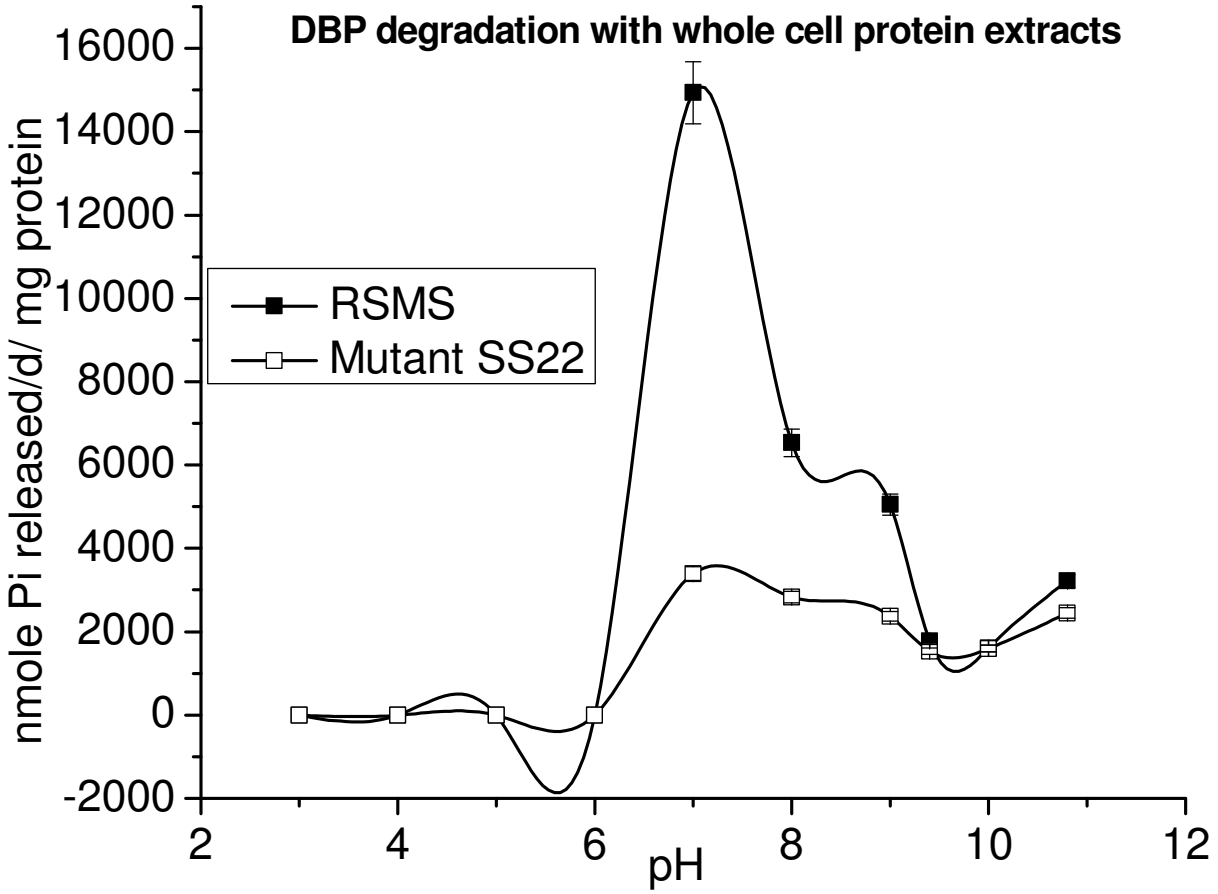

Fig. 5.17. DBP degradation with cell free protein extracts of RSMS and mutant SS22. Whole cell protein extracts (100 µg) from wild type or SS22 mutant cells were incubated with 10 mM DBP, in buffers of various pH values for 24 hours. The inorganic phosphate (Pi) released due to DBP degradation was measured spectrophotometrically.

Table 5.2. Phosphodiesterase activity at high temperatures

Substrate	Incubation temperature (°C)	Activity*
10 mM DBP	30	15,100 (± 700)
10 mM DBP	60	14,800 (± 750)
Bis -pNPP	30	13,800 (± 950)
Bis- pNPP	60	13,500 (± 870)

***** n moles of phosphate released/d/mg protein, or n mole para-nitrophenol released/h/ mg protein, respectively for DBP and Bis-pNPP.

The phosphodiesterase assays were carried out in MMM (pH 7) containing 100 µg of whole cell protein extracts of RSMS and 10 mM DBP or 10 mM Bis-pNPP. The mixture was incubated at 30°C or 60°C, and released inorganic phosphate or para-nitrophenol, respectively from DBP or Bis-pNPP was spectrophotometrically estimated.

5.6.4. Monobutyl phosphate (MBP) degradation activity

The MBP is available only as a mixture of MBP and DBP. The inhibition of DBP degradation or phosphodiesterase activity by caffeine was exploited, to assess the ability of the strain to degrade MBP (considered as an intermediate by several previous studies [36]). Whole cell protein extracts of wild type cells treated with caffein showed 9,200 nmoles of inorganic phosphate release/d/mg of protein using MBP (mixture of MBP and DBP), which was >2 fold higher than that of mutant. Decreased PME activity in mutant and the aforesaid decrease in MBP degradation activity suggest plausible involvement of MBP as an intermediate of TBP degradation.

Table 5.3. Degradation assay of dibutyl phosphate (DBP) and monobutyl phosphate (MBP)

Additions to the MMM	Activity (n moles of phosphate released/ mg protein/d)	
	Wild type	**Mutant**
5 mM DBP	15,100 (± 700)	2,900 (± 300)
5 mM DBP + 1mM caffeine	Nil	Nil
5 mM MBP (contains ~ 5.50 mM DBP)	25,500 (± 1100)	6, 490 (± 395)
5 mM MBP (contains ~ 5.50 mM DBP) + 1 mM caffeine	9,200 (± 490)	4, 200 (± 325)

The phosphodiesterase (with DBP) / monoesterase (with MBP + caffeine) assay was carried out in MMM (pH 7) containing 100 μg of cell free extracts of wild type or mutant, and 5 mM DBP or 5 mM MBP (as MBP and DBP mixture). The mixture was incubated at 30°C for 24 h and released inorganic phosphate was spectrophotometrically estimated.

Discussion

Several methods are available for the analysis of TBP. All these methods estimate only the soluble TBP. Such analysis is not useful for the present study, where high amounts of insoluble TBP were present in the medium. In the present study, a new method for the extraction and analysis of TBP, DBP and butanol was developed. The method showed >97% extraction of analytes, and well resolved detection with a good sensitivity and linear range by gas chromatography (Fig. 5.4).

Over the last three decades, mineralization of TBP has been proposed to be mediated by phosphoesterases, wherein the tri-, di- and monoesterases would sequentially act on TBP to release DBP and MBP as intermediates, and butanol and phosphate as final products. But no

such intermediates or products were ever detected or reported earlier. The present study clearly showed DBP as the most immediate intermediate in TBP degradation, and butanol and phosphate as the end products of TBP degradation by the RSMS strain (Fig. 5.6 and 5.7). Butanol and phosphate were also found to be the products of DBP degradation (Fig. 5.10 and 5.11). The mutant SS22 did not produce any of the intermediates or products of TBP/DBP degradation (Fig. 5.13 and 5.14).

The scheme of TBP degradation revealed by the present investigation is delineated in (Fig. 5.12). The first step of TBP degradation involves the release of butanol and DBP. DBP could be subsequently degraded in two possible ways: (a) it may be degraded in a single step to release two molecules of butanol along with one molecule of inorganic phosphate as the final products, or (b) the DBP could be degraded in two steps, first step resulting in the release of monobutyl phosphate (MBP) and butanol from DBP, followed by the degradation of MBP to butanol and inorganic phosphate in the second step. It is possible that DBP degradation to butanol and phosphate proceeds through monobutyl phosphate (MBP), but unavailability of purified MBP precluded the gas chromatography experiments which could establish/rule out this possibility. However, reduced PME activity (Fig. 5.15) and 2 fold less MBP degradation (Table 5.3) activity in whole cell protein extracts of mutant SS22 strongly suggest the likelihood of involvement of MBP as an intermediate in TBP degradation.

Gas chromatography analysis also revealed complete absence of TBP and DBP degradation activities in the mutant (Fig. 5.13). Release of butanol and phosphoric acid from TBP and DBP suggests the involvement of phosphoesterases in the TBP degradation. Besides, protein profile studies suggested absence of PDE (phosphohydrolase) and PME (Exopolyphosphatase like protein) like activities in the mutant (Fig. 4.12 from 4th chapter). With these clues, relative

activities of PDE and PME for the mutant SS22 and wild type were compared. PDE activity towards Bis-pNPP was found to be much less or absent in mutant (Fig. 5.15). The PDE was found to be active even at alkaline conditions such as pH 8. Similarly, 10-fold difference for PME activity was observed between wild type and mutant SS22, at pH 7 (Fig. 5.15). Alkaline phosphatases/ alkaline PMEs were found to be unaffected in mutant SS22. The yellow colored product (pNP) of the Bis-pNPP and pNPP could be exploited for the purification of the proteins involved in the TBP degradation. In gel PDE and PME assays revealed that there were at least two proteins which showed activity both on phosphomonoesters and phosphodiesters. One of the proteins was found to be absent in the mutant (Fig. 5.16).

Cells of RSMS strain could degrade TBP and DBP (and release butanol and phosphate as the final products) (Fig. 5.8 and 5.11) whereas cell free extracts of RSMS could degrade only DBP (Fig. 5.17) but not TBP (data not shown), suggesting that the first step of TBP degradation to DBP and butanol probably requires intact cells. As a result, *in vitro* TBP degradation experiments could not be carried out with cell free extracts. Cell free extracts of RSMS showed 5 times more DBP degradation activity in comparison to the mutant (Fig. 5.17). Cell free extracts of wild type showed DBP degradation even at 60°C (Table 5.2). The DBP degradation by cell free extracts was completely inhibited by caffeine, which is a well known phosphodieterase inhibitor (Table 5.3). Involvement of MBP as an intermediate in TBP degradation could not be established, because of its commercial unavailability in pure form. The MBP is available only as a mixture of MBP and DBP. The inhibition of DBP degradation by caffeine was exploited, to assess the ability of the strain to degrade MBP. Cell free extracts of wild type showed 9,200 nmoles of inorganic phosphate release/mg protein/d, which was >2 fold higher than that of mutant SS22 (Table 5.3). Since, caffeine was found to be toxic to the RSMS strain, similar kind

of experiments could not be conducted to assess the ability of RSMS to use MBP as sole source of carbon and phosphorous.

The unraveling of the biochemical pathway of TBP degradation and PDE, PME assays provide very clear clues that the genes involved in the TBP degradation belong to phosphoesterase family. Proteomic data further suggest involvement of phoshohydrolase and exopolyphosphate like protein in TBP/DBP degradation.

Annexure

TBP degradation coupled to uranium precipitation

Alkaline or acid phosphatases release inorganic phosphate from several organophosphates, which can be coupled to precipitate uranium from acidic or alkaline aqueous solutions in the form of uranyl phosphate [71,102]. Since TBP biodegradation releases considerable excess phosphate, studies were carried out to couple the inorganic phosphate released in the TBP degradation to the precipitation of uranium from aqueous solutions. At circumneutral pH, uranium predominantly occurs as hydroxide and gets precipitated easily. Uranyl carbonate is the highly soluble uranium species at pH 7.5. Even at high concentrations of uranyl carbonate (>10 mM) in MMM, no precipitation is seen in the presence of 200 mM MOPS concentration, which is optimal buffer concentration for TBP degradation. Hence, uranium precipitation studies were carried out in 200 mM MOPS buffer using uranyl carbonate at pH 7.5.

6.1. Tolerance of the RSMS strain to high concentrations of uranium

First, the tolerance of RSMS strain to uranium, TBP and to combined presence of uranium and TBP was evaluated. No significant growth suppression of RSMS strain was observed up to 1.2 mM of uranyl carbonate, but at 1.5 mM uranyl carbonate, the growth was reduced 2.5 times, in comparison to control medium containing no uranyl carbonate, and no growth was observed at and above 1.7 mM of uranyl carbonate (Fig. 6.1a). On solid LB medium supplemented with uranyl carbonate, the strain showed no reduction in CFUs up to 1.5 mM uranyl carbonate, but the size of the colonies was small in comparison to that at lower concentrations (Fig. 6.1b). No growth was, however, observed in the medium supplemented with 1.7 mM of uranyl carbonate. Thus, MIC of uranium for RSMS strain was estimated as 1.7 mM (Fig. 6.1a and b).

The tolerance of the strain to uranium in the presence of different concentrations of TBP was also assessed in MMM (Table 6.1).

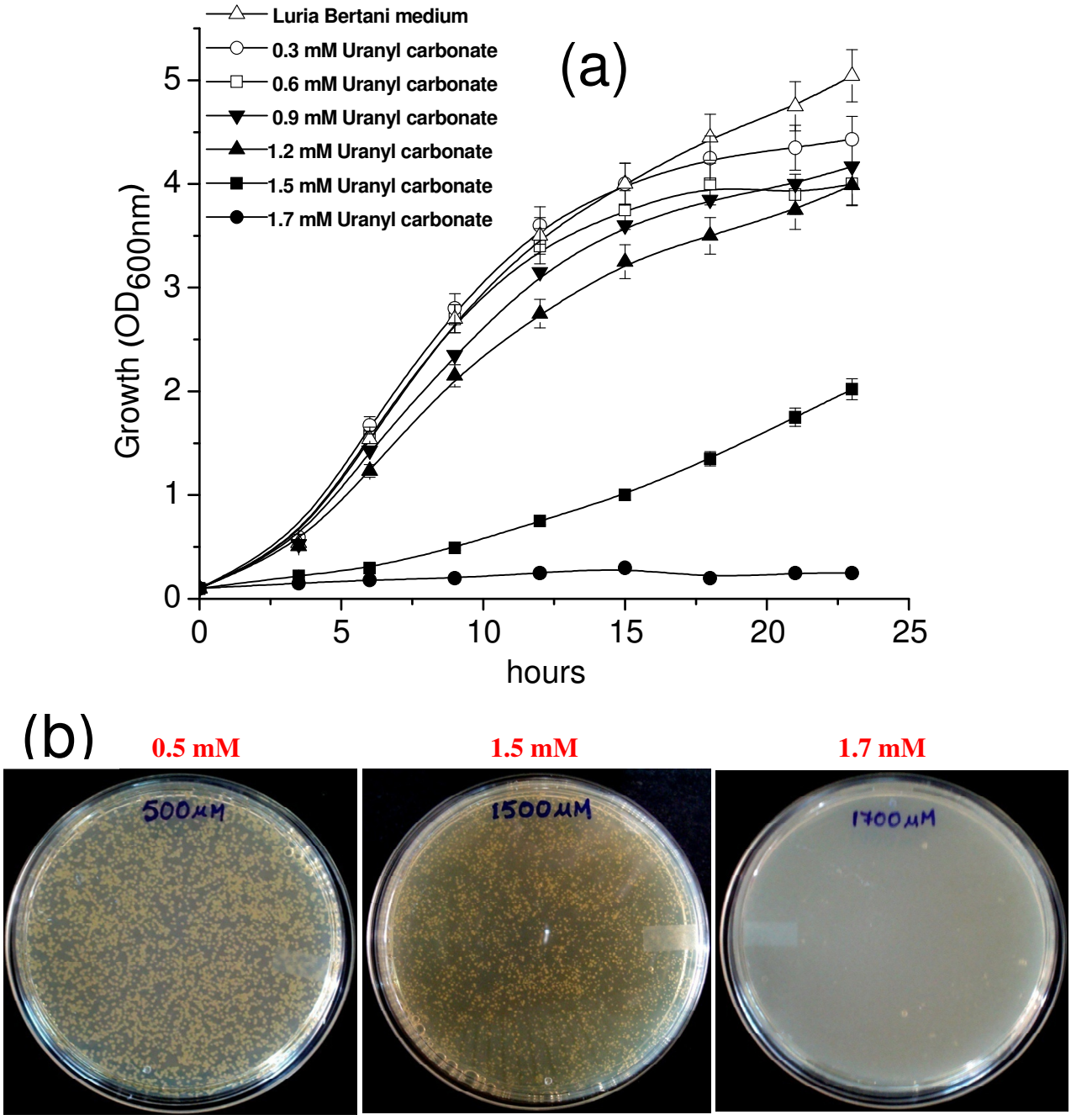

Fig. 6.1. Growth of RSMS strain in (a) liquid LB medium, or (2) on LB agar medium, supplemented with various concentrations of uranyl carbonate. The liquid cultures were grown at 30°C and 180 rpm. Growth was measured as increase in turbidity at 600 nm. Solid LB agar plates were incubated for 2 days at 30°C under static condition. MIC was calculated as the concentration of uranyl carbonate at which no growth was observed.

Table 6.1. Tolerance of the RSMS strain to combined presence of uranium and TBP.

Medium	Growth of the strain after 24 h (OD_{600nm})
LB (control)	4.5 (± 0.3)
LB + 1 mM uranyl carbonate	4.4 (± 0.25)
LB + 1mM uranyl carbonate + 2 mM^e TBP	4.4 (± 0.22)
LB + 1 mM uranyl carbonate + 5 mM^e TBP	4.3 (± 0.23)
LB + 1 mM uranyl carbonate + 10 mM^e TBP	0.085 (± 0.03)
LB + 1 mM uranyl carbonate + 20 mM^e TBP	0.05 (± 0.02)

The RSMS strain was inoculated at a cell density of 0.1 OD_{600nm}/ml, in to LB medium supplemented with 1 mM uranyl carbonate and various concentrations of TBP. The culture was incubated at 30°C and 180 rpm, and growth was measured 24 h after inoculation, as increase in turbidity at 600nm.

The strain could tolerate 1 mM uranyl carbonate in the presence of 5 mM^e TBP, but could not tolerate TBP concentrations above 5 mM^e at 1 mM uranyl carbonate (Table 6.1). Uranium precipitation experiments with RSMS cells were therefore carried out with 5 mM^e TBP and 1 mM uranyl carbonate.

6.2. Precipitation of uranium at different cell densities of RSMS strain

About 0.1 mM of the uranyl carbonate was spontaneously precipitated in MMM inoculated with RSMS strain at 0.1 to 1 OD_{600nm} cells (Table 6.2). The strain could precipitate >90% of the added uranyl carbonate in the form of uranyl phosphate in 13-17 h, when inoculated at 1 OD_{600nm} in to MMM containing 1 mM uranyl carbonate (Table 6.2). The rate of precipitation increased with increasing cell density up to 1 OD_{600nm}.

Table 6.2. Precipitation of uranium using various cell densities of RSMS strain

Cell density (OD600nm)	Uranium precipitation (mM) with time (h)					
	0 h	**2 h**	**5 h**	**8 h**	**13 h**	**17 h**
Blank (no cells)	0.1±0.005	0.1±0.003	0.1±0.004	0.1±0.005	0.1±0.005	0.1±0.005
0.1	0.1±0.004	0.1±0.005	0.21±0.004	0.38±0.004	0.78±0.005	0.95±0.004
0.5	0.1±0.005	0.1±0.005	0.32±0.01	0.49±0.015	0.92±0.012	0.92±0.014
1	0.1±0.002	0.1±0.003	0.30±0.01	0.62±0.02	0.95±0.015	0.98±0.05

Uranium precipitation was assayed by inoculating RSMS strain at different cell densities in to MMM containing 5 mM^e TBP and 1 mM uranyl carbonate at pH 7.5 and compared with controls kept without cells. At specified time intervals samples were subjected to centrifugation and the uranium present in the supernatant was estimated by Arsenazo III method and corrected for blank value. The values represent average of three independent experiments. MMM containing 5 mM^e TBP and 1 mM uranyl carbonate (without cells) served as a blank or negative control.

6.3. Immobilization of RSMS strain for the precipitation and recovery of uranium

Though precipitation of uranium could be achieved using free cells of RSMS strain, recovery of such precipitated uranium was rather difficult. Further, it was anticipated that if the direct contact of the cells and its components to uranyl carbonate is avoided through immobilisation, the tolerance of the strain could be increased for combined presence of uranium and TBP. This would allow usage of high concentrations of TBP which could increase the TBP degradation, phosphate availability and uranium precipitation. Indeed, when immobilized in calcium alginate beads, RSMS strain tolerated 1.5 mM of uranyl carbonate and up to 30 mM^e TBP, and precipitated uranyl carbonate (Table 6.3a). The calcium alginate beads containing RSMS cells at

various densities showed precipitation of uranium in the form of uranyl phosphate (Table 6.3a). The precipitated uranium present in the beads was extracted using concentrated HCl and estimated by Arsenazo III method.

Table 6.3. Precipitation of 1.5 mM uranyl carbonate using immobilized RSMS cells

a) Concentration of TBP (mM^e) added to MMM	**Uranium (mM) precipitated using different density of cells**			
	Control (mutant SS22)	Beads obtained from 5 ml of 0.5 OD_{600nm}/ml solution	Beads obtained from 5 ml of 1 OD_{600nm}/ml solution	Beads obtained from 5 ml of 3 OD_{600nm}/ml solution
10	0.2	0.22	0.61	0.9
30	0.2	0.78	1.1	1.22
b)	**Amount of free inorganic phosphate (mM) in the medium**			
10	Nil	1.7	1.2	1.8
30	Nil	1.2	1.75	3.5

About 40 RSMS immobilised beads (formed from 5 ml of sodium alginate solution containing 0.5 OD_{600nm}/1 OD_{600nm}/3 OD_{600nm} cells per ml solution) were incubated in 100 ml MMM (pH 7.5) supplemented with 1.5 mM uranyl carbonate and 10/30 mM^e TBP. (a) Uranium present in the beads was estimated after 24 h using Arsenazo III method. (b) The amount of free inorganic phosphate released in the solution was also estimated. Immobilised mutant SS22 beads (~40 beads, formed from 5 ml of sodium alginate solution containing 3 OD_{600nm}/ml of mutant SS22 cells) inoculated in to MMM supplemented with 1.5 mM uranyl carbonate and 30 mM TBP, served as a negative control.

The immobilized mutant cell beads showed about 20 µmole of uranium, which was equivalent to the disappearance of 0.2 mM of uranyl carbonate from the solution. The immobilized RSMS cells incubated with 30 mM TBP exhibited ≥ 95% of 1.5 mM uranium in the beads in 24 h

(Table 6.3a). The uranium precipitation with 30 mM TBP at cell density 1 OD_{600nm} was found to be 1.1 mM, which is equivalent to 73% of precipitated uranium (Fig. 6.3a). Though free inorganic phosphate was observed in the medium, complete uranium precipitation was not achieved with beads containing 0.5 or 1 OD_{600nm} cells (Table 6.3b).

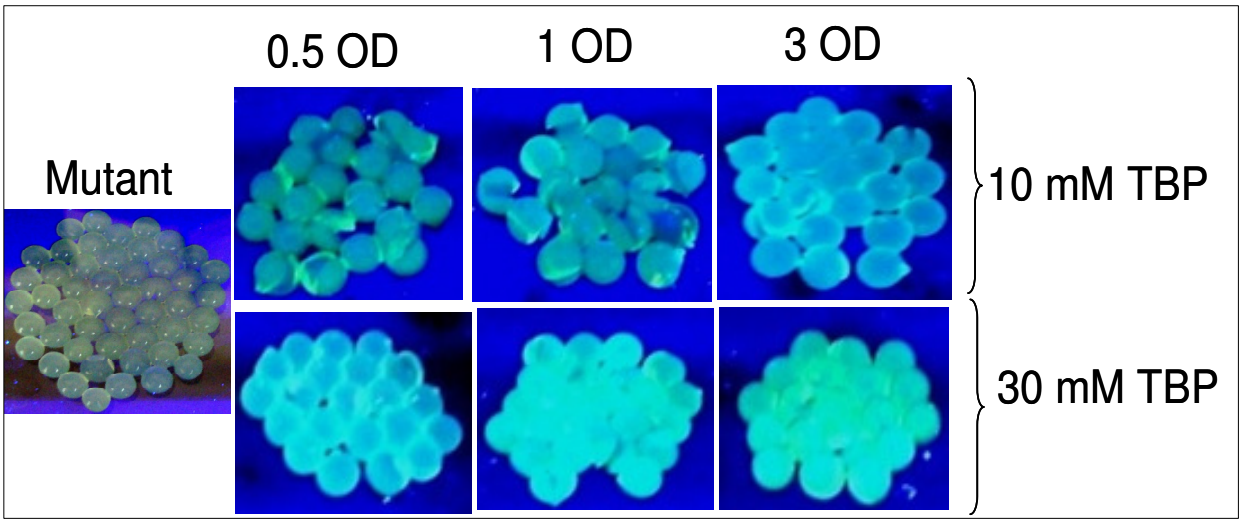

Fig. 6.2. Visualisation of uranium precipitation in the immobilized beads of RSMS cells. About ~40 beads of RSMS strain (obtained from 5 ml of sodium alginate solution containing 0.5 OD_{600nm}/1 OD_{600nm}/3 OD_{600nm} cells per ml solution) were incubated in MMM + 1.5 mM uranyl carbonate and 10/30 mM^{e} TBP at 30°C for 24 h. TBP non degrading mutant SS22 derived from RSMS was used as a control at a cell density of $3OD_{600nm.}$ The precipitated uranium in beads was visualized as green fluorescence under UV illumination.

Uranyl phosphate formed due to uranium precipitation exhibits green fluorescence (Fig. 6.2). The intensity of the fluorescence was directly proportional to the concentration of precipitated uranyl phosphate. The beads carrying TBP non degrading mutant cells (negative control) did not show any fluorescence, suggesting the absence of uranium precipitation (Fig. 6.2). The beads containing RSMS cells at various densities showed precipitation of uranium, and intensity of

fluorescence increased with increasing cell densities and TBP concentration. The intensity was higher in beads incubated in MMM containing 3 OD_{600nm} cells and with 30 mM^e TBP (Fig. 6.2).

Discussion

TBP degradation was successfully linked to the precipitation of the uranium. The tolerance of the RSMS strain for the uranyl carbonate alone was found to be 1.7 mM. But, the tolerance of the RSMS strain for both uranyl carbonate and TBP was low. The strain could tolerate only 1 mM uranyl carbonate in the presence of 5 mM^e TBP. The strain could precipitate >90% of 1 mM uranium in 17 hours when inoculated at 1 OD_{600nm} in MMM containing 5 mM^e TBP.

Though, the TBP degradation could be coupled to uranium precipitation, the recovery of the precipitated uranium from the solution was rather difficult. Hence, the strain was immobilized in calcium alginate beads. Immobilized cells easily tolerated combined effect of 30 mM^e TBP + 1.5 mM uranium. Beads containing RSMS cells could precipitate ≥ 95% of 1.5 mM uranium in 24 h. Thus, it is possible to achieve simultaneous degradation of TBP and removal of uranium by precipitation from the waste. The possibility needs to be explored further and optimized.

Summary and conclusions

- A TBP degrading bacterial strain isolated from the RSMS site in BARC, was initially identified to be a *Sphingomonas* strain. Using an array of biochemical tests, 16s *rRNA* gene sequencing and fatty acid methyl ester (FAME) analysis, the strain was identified as a *Sphingobium.*
- The *Sphingobium* sp. strain RSMS was found to completely degrade up to 30 mM^e TBP from immiscible suspensions and 50 mM DBP from solutions in 3 and 4 days, respectively.
- The RSMS strain also degraded Tri-iso amyl alcohol phosphate (TAP), which is a higher homologue of TBP. The strain degraded 20 mM^e TAP in 4 days and utilized it as sole source of carbon and phosphorous. This is the first study to report the TAP biodegradation.
- The RSMS strain also individually utilized TBP, DBP, TAP and butanol as the sole source of carbon and phosphorous for its growth.
- The ability of the RSMS strain to degrade TBP was also demonstrated on solid MMM/LB agar media in the form of a zone of TBP degradation around the RSMS colony. Such phenotype has not been reported for any of the earlier strains reported to degrade TBP.
- The RSMS strain tolerated ~100 mM^e TBP, 50 mM DBP and 50 mM butanol, which is truly remarkable. Best known strain reported earlier tolerated a maximum of 5 mM^e TBP.
- High concentrations of inorganic phosphate (final product of TBP degradation) and glucose were found to negatively regulate TBP and DBP degradation and TBP/DBP dependent growth of the RSMS strain. Inorganic phosphate has been reported earlier to negatively regulate TBP degradation. However, catabolite repression of TBP degradation by glucose has not been reported earlier.

- Gas chromatography and spectrophotometric analyses revealed the involvement of DBP as an intermediate, and butanol and inorganic phosphate as the end products of TBP degradation. Based on these results, a biochemical pathway for TBP degradation was proposed. The involvement of monobutyl phosphate (MBP) as an intermediate downstream of DBP was strongly suggested by several results, but could not be ascertained due to unavailability of pure MBP.
- Ability of the RSMS colony to exhibit a zone of TBP degradation around the colony was used as a screen for the isolation of TBP non degrading mutants. The mutant SS22 failed to grow on TBP or DBP and did not generate any of the intermediates or products of TBP/DBP degradation, suggesting that the entire pathway of TBP degradation was blocked in the mutant.
- Mutant SS22 showed loss of two proteins from its proteome in comparison to the wild type strain. By MALDI-ToF-MS based peptide mass finger printing, these proteins were found to be similar to Phosphohydrolase and Exopolyphosphatase like proteins from other bacteria, which are known to exhibit phosphodiesterase and phosphomonoesterase activities.
- The cell free protein extracts of mutant SS22 showed significantly reduced activity towards phosphodiesters and phosphomonoesters, in comparison to the RSMS strain. They also showed ~5 times lesser DBP (phosphodiester) degradation in comparison to the RSMS strain.
- The gas chromatographic and proteomic analyses of wild type and mutant, reduced phosphodiesterase and phosphomonoesterase activities and reduced DBP degradation activity in mutant SS22, strongly suggest the involvement of phosphoesterase like proteins in TBP degradation by RSMS strain.

❖ The excess inorganic phosphate released in TBP degradation could be coupled to uranium precipitation. The RSMS strain, immobilized in alginate beads, could precipitate ≥ 95% of 1.5 mM uranium in 24 h, using phosphate released from degradation of 30 mM[e] TBP.

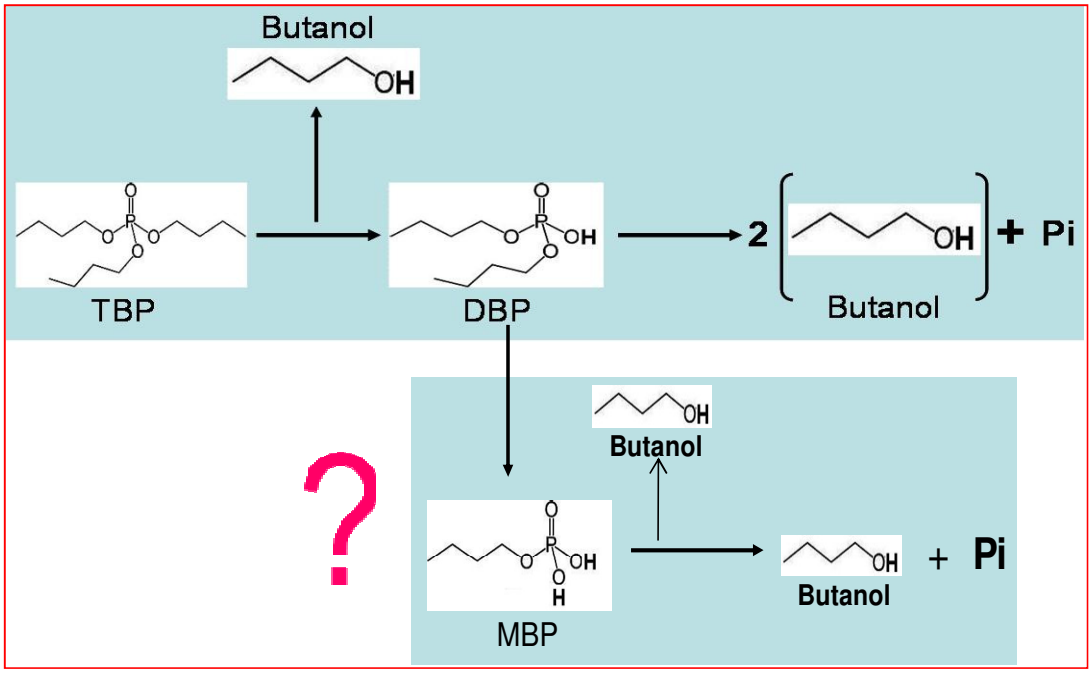

Fig. 7.1. Proposed biochemical pathway for TBP degradation by RSMS strain

In conclusion, the study has resulted in following major findings:

(1) The novel TBP degrading S*phingobium* strain RSMS was found to be far superior to any of the previously reported TBP degrading strains in terms of TBP tolerance, stability and TBP degradation/utilization efficiency.

(2) The following biochemical pathway of TBP degradation by RSMS strain was revealed for the first time (Fig. 7.1).

(3) The *Sphingobium* strain RSMS completely degraded and utilized TBP, DBP and also TAP, leaving no toxic products or residues. Such efficient utilization is desirable in

bioremediation, since it can prevent accumulation of toxic byproducts such as butanol formed due to TBP degradation in the medium.

(4) The study also revealed that the TBP degradation can be coupled to uranium precipitation, thus achieving simultaneous degradation of TBP and removal of uranium from waste.

The work has suggested possible involvement of phosphoesterases in the TBP/DBP degradation. The two proteins absent in the mutant SS22 appear to be potential candidates for further investigation on the molecular basis of TBP/DBP degradation.

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ENVIRONMENTAL BIOTECHNOLOGY

Tributyl phosphate biodegradation to butanol and phosphate and utilization by a novel bacterial isolate, *Sphingobium* sp. strain RSMS

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Abstract A *Sphingobium* sp. strain isolated from radioactive solid waste management site (RSMS) completely degraded 7.98 g/L of tributyl phosphate (TBP) from TBP containing suspensions in 3 days. It also completely degraded 20 mM dibutyl phosphate (DBP) within 2 days. The strain tolerated high levels of TBP and showed excellent stability with respect to TBP degradation over several repeated subcultures. On solid minimal media or Luria Bertani media supplemented with TBP, the RSMS strain showed a clear zone of TBP degradation around the colony. Gas chromatography and spectrophotometry analyses identified DBP as the intermediate and butanol and phosphate as the products of TBP biodegradation. The RSMS strain utilized both TBP and DBP as the sole source of carbon and phosphorous for its growth. The butanol released was completely utilized by the strain as a carbon source thereby leaving no toxic residue in the medium. Degradation of TBP or DBP was found to be suppressed by high concentration of glucose which also inhibited TBP or DBP dependent growth. The results highlight the potential of *Sphingobium* sp. strain RSMS for bioremediation of TBP and for further molecular investigation.

Keywords Tributyl phosphate · Biodegradation · *Sphingobium* · Biochemical pathway

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Introduction

Tributyl phosphate (TBP) is a phosphotriester used in large volumes (~3,000–5,000 tons/annum) in nuclear industry for extraction of uranium and plutonium (Hernandez 2001). TBP is also used as a plasticizer in the plastic industry, as a component of aircraft hydraulic fluid, and as an antifoaming agent (Nakamura 1991). Chemical destruction or incineration is generally used for the treatment of TBP waste (Anon 1992). Alkali treatment of TBP at high temperatures results in the formation of dibutyl phosphate and butanol (Healy 1976; Schulz and Navratil 1984), while incineration leads to formation of P_2O_5, a corrosive product (Manohar et al. 1999). Management of the byproducts formed by both these methods is rather difficult.

Bacterial strains have been reported to degrade TBP, either as mixed cultures or as single isolates (Ahire et al. 2011; Berne et al. 2005, 2004; Jeong et al. 1994; Owen et al. 1992; Rosenberg and Alexander 1979; Thomas and Macaskie 1998, 1996 Thomas et al. 1997). For example, *Serratia odorifera* or *Rhodopseudomonas palustris*, respectively, degraded 0.6 mM TBP within 8 h or 1.6 mM of TBP in 3 weeks. However, these strains could not mineralize or utilize TBP as carbon or phosphorous source for growth (Berne et al. 2005, 2004). In contrast, mixed cultures containing *Pseudomonas* have been shown to degrade 2 mM TBP in ~3 days and utilize TBP both as carbon and phosphorous source (Thomas et al. 1997). But, the corresponding *Pseudomonas* strain irreversibly lost the ability to degrade or utilize TBP after eight serial subcultures (Thomas et al. 1997). Recently, 15 bacterial strains were reported for their ability to degrade and utilize TBP as the sole

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source of carbon and phosphorous (Ahire et al. 2011) but showed rather poor rates of TBP degradation (1–3.05 mM TBP degradation in 4 days). No sphingomonad has been reported to degrade TBP so far. The intermediates or products of TBP degradation formed by any of the strains reported so far have not been identified.

This paper reports isolation of a *Sphingobium* sp. strain RSMS (hereafter referred to as RSMS strain), which efficiently mineralized high level of TBP in media to butanol and phosphate and utilized them as carbon and phosphorous source for its growth. The strain exhibited excellent stability in terms of degradation and utilization of TBP over repeated subcultures. The biochemical pathway of TBP degradation by RSMS strain has been elucidated for the first time with dibutyl phosphate (DBP) as an intermediate of the process, which results in the formation of butanol, which is fully utilized, and excess inorganic phosphate as the end products.

Materials and methods

Isolation of the strain A TBP degrading bacterial strain was isolated from the TBP waste storage tank located at the RSMS in Bhabha Atomic Research Centre, Mumbai, and denoted as RSMS strain. Swabs were taken from the side walls of the TBP waste storage tank with sterile cotton and immersed in 10 ml of sterile normal saline (0.85 % NaCl), shaken well and added to 250 ml conical flask containing 50 ml of Lurea Bertani medium supplemented with 2 mM TBP. The flasks were incubated under shaking (150 rpm) at 30 °C for 3 days. The cells were harvested, washed with saline, and directly plated on to mineral medium (hereafter referred as MM) agar (Thomas et al. 1997) plates supplemented with 2 mM TBP, as the sole source of carbon and phosphorous. The plates were incubated at 30 °C for 3 days. The colonies which grew and showed clearance of TBP around them were considered as putative TBP degraders. Such colonies were isolated and purified. The purified colonies were inoculated in to MM supplemented with 2 mM TBP, as the sole source of carbon and phosphorous and grown under shaking (180 rpm) at 30 °C. Cells from isolated colonies were washed with sterile saline and stored in 15 % glycerol at −70 °C. Luria Bertani agar (LBA) or MM agar plates supplemented with TBP were prepared by suspending 2.66 g/L TBP (equivalent to 10 mM TBP when dissolved) of medium and shaking them well. Due to insolubility of TBP, such plates were opaque in nature (Fig. 1). The TBP degradation by the strain was visualized by spotting aliquots of 20 μl of 20 OD_{600nm} cells on such plates and incubating them at 30 °C for 7 days or until a clearly visible zone of clearance of TBP appeared.

Identification of the TBP degrading strain Identification of the strain was carried out by 16S *rRNA* gene sequencing. The 16S *rRNA* gene amplification was carried out by using 8F (5'-AGAGTTTGATCCTGGCTCAG-3') (Eden et al. 1991) and 1387R (5'-GGGCGGA/TGTGTACAAGGC-3') (Marchesi et al. 1998) primers, and the purified DNA product was sequenced. Similarity searches for the obtained 16S *rRNA* gene sequence were carried out using the BLAST algorithm available at http://www.ncbi.nlm.nlh.gov (Altschul et al. 1990). The nucleotide sequence (1,248 nt) was submitted to Genbank (accession no. EU629211.2). Nitrate reductase test was performed as described earlier (Skerman 1967). The strain was deposited with Microbial Type Culture Collection and Gen Bank, Chandigarh, India (http://mtcc.imtech.res.in) under the accession number 11630.

Media and growth conditions A mineral medium described earlier (Thomas et al. 1997) was used as basal medium for the growth of the strain. The mineral medium was buffered with 100 mM 3-(N-morpholino) propane sulfonic acid at pH 7.0 (hereafter referred as modified mineral medium or MMM). Glucose and 5 mM KH_2PO_4 or 20 mM DBP or the required amounts of TBP were added to MMM as the sole source of carbon and phosphorous. Due to its low solubility, TBP addition to the media did not yield a clear solution but resulted in opaque whitish TBP containing suspensions, which were used directly for experiments. The strain was grown either in liquid MMM at 30 °C under shaking (180 rpm) or on solid media supplemented with 1.5 % Difco Bacto agar (MMMA) at 30 °C. All the glassware were soaked in 30 % (*v*/*v*) of nitric acid for 12 h and then washed two times with distilled water to remove inorganic phosphate.

Preinoculum preparation A single colony of RSMS strain was inoculated in MMM supplemented with 0.5 % glucose and 5 mM KH_2PO_4. Exponentially grown cells were harvested by centrifugation at 10,000 rpm for 5 min. The cell pellet was washed three times with equal volumes of MMM to remove residual glucose and phosphate and resuspended in fresh MMM. This cell suspension was used as preinoculum for all the growth and TBP degradation related experiments.

Growth and TBP degradation All growth related experiments in liquid MMM were started with an initial inoculum density of 0.1 OD_{600nm} (~5×10^7 CFU/ml). The ability of the RSMS strain to utilize TBP or DBP was assessed by inoculating the strain into MMM containing desired amounts of TBP or DBP (British Drug Houses, UK) as the sole source of carbon and phosphorous. Cell density required for the optimal rate of DBP or TBP degradation was assessed by inoculating RSMS culture at various cell densities (OD_{600nm}) into MMM supplemented with 20 mM DBP or 7.98 g/L of TBP (equivalent to 30 mM TBP when dissolved). Suppression of DBP or TBP degradation by glucose was studied by inoculating the strain in to MMM containing 10 mM DBP or 2.66 g /L TBP (equivalent to 10 mM TBP

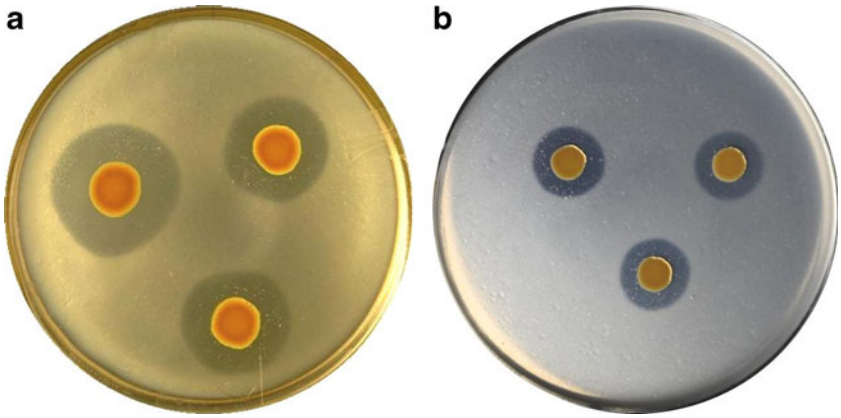

Fig. 1 Zone of clearance formed around the culture spots of RSMS strain due to TBP degradation in **a** Luria Bertani agar (LBA) medium or **b** MMM agar plates both supplemented with 2.66 g/L TBP

when dissolved) supplemented with different concentrations of glucose. Appropriate controls were used in all experiments.

Growth was generally measured as colony forming units (CFUs) at 30 °C after 48 h growth on MMMA plates supplemented either with 0.5 % glucose and 5 mM KH_2PO_4 or with TBP/DBP (added as the sole source of carbon and phosphorous). Growth was also assessed spectrophotometrically as increase in the cell density (OD at 600 nm). TBP and DBP degradation were spectrophotometrically assessed as phosphate released in the medium by phosphomolybdic acid method (Ames 1966).

Analysis of TBP degradation products A gas chromatograph (Schimadzu-GC-2014) equipped with a 10 % XE-60 column (1.5 m×0.32 cm) was used for the analysis of TBP, DBP, and butanol. Helium was used as carrier gas at a flow rate of 40 ml/min. Column temperature was maintained at 120 °C (1 min) to 230 °C (10 min), with an increment of 10 °C/min. The injection port and detector temperatures were maintained at 240 °C and 260 °C, respectively. Thermal conductivity detector was used for the quantitation of organophosphates (TBP, DBP) while butanol was estimated using a flame ionization detector.

Predetermined amounts of TBP (0.532 to 7.93 g/L) or DBP (1–30 mM) were added to MMM and individually acidified with 1/100 volumes of 10 N H_2SO_4. To the acidified samples, 1/4 volume of 10 % benzyl alcohol (in benzene, *v*/*v*) [hereafter referred as benzene benzyl alcohol(BBA)] was added, shaken well for 15 min, and allowed to stand for clear separation of layers. To the top organic layer, four volumes of diazomethane were added for esterification and the esterified samples were injected in to gas chromatograph in appropriate volumes (1–2 μl). Quantification of butanol was carried out by direct injection of MMM containing predetermined amount of butanol. Standard curves for all the analytes were prepared by plotting peak areas against the amount of the analyte injected in to GC.

For the analysis of TBP degradation products by RSMS strain, freshly prepared preinoculum was inoculated at a cell density of 3 OD_{600nm} in to 5 ml of MMM supplemented with 7.98 g/L TBP (equivalent to 30 mM TBP) or 20 mM DBP. The cultures were incubated at 30 °C under shaking (180 rpm). The following controls were used: MMM containing equivalent of 30 mM TBP (7.98 g/L) or 20 mM DBP without cells and MMM containing 3 OD_{600nm} cells without TBP or DBP. Samples were removed each day and subjected to centrifugation at 10,000 rpm for 5 min. A small volume (500 μl) of the supernatant was aliquoted for the analysis of butanol and phosphate. The remaining ~4.5 ml of supernatant was collected, along with traces of TBP present on the walls of the conical flask by rinsing with 1 ml of BBA. These samples were processed for the analysis of TBP, DBP, and butanol by gas chromatography. The concentration of each compound was calculated by using the calibration curve.

Results

Isolation of the TBP degrading strain A single monoxenic bacterial isolate capable of degrading and utilizing TBP as sole source of carbon and phosphorous was isolated and named as RSMS strain. On account of its very low solubility, the TBP containing LBA and MMMA solid media were opaque in nature. A clear transparent distinct zone around the culture spot revealed degradation of TBP. The isolated strain showed a clearly visible phenotype for TBP degradation around the growing colonies (Fig. 1a, b).

Identification of the strain Similarity search for the 16S *rRNA* gene sequence of the isolated strain by BLAST analysis showed that the strain had the highest homology (1,245 identities out of 1,248 nucleotides) with *Sphingobium* sp. strain KK22 (GenBank acc. no. HQ830159.1) (Kunihiro et al. 2013). Analysis of the DNA sequence for the signature nucleotides showed nucleotides T, G, A, T, A, U, G, and T at positions 52, 134, 359, 593, 987, 990, 1,215, and 1,218, respectively, characteristics of the genus *Sphingobium*. The

strain tested negative for nitrate reductase test. A phylogenetic tree was constructed by taking 16s *rRNA* gene sequences of 55 sphingomonads (Fig. S1). The analysis corroborated identification of RSMS strain as a *Sphingobium* species with three other *Sphingobium* species, including *Sphingobium* sp. strain KK22, in the same clad.

Growth and TBP degradation RSMS strain could not grow in MMM containing glucose or KH_2PO_4 alone. However, MMM supplemented with TBP alone supported active growth of the strain, suggesting that the strain utilized TBP as sole source of carbon and phosphorous (Fig. 2a). Growth and TBP degradation by the strain increased with increasing amounts of TBP up to 7.98 g/L (equivalent to 30 mM TBP when dissolved) (Fig. 2a, b). The number of CFUs in MMM suspensions containing TBP increased from 5×10^7/ml at inoculation to 1.3×10^9/ml after 48 h, which is equivalent to >25 times increase in cell number or 4.5 doublings (Fig. 2a). The deduced doubling time for the strain at this TBP concentration was ~11 h. The strain continued to show TBP degradation from TBP suspensions even after attaining stationary phase. Stability of the RSMS strain with respect to TBP degradation and utilization for growth was assessed in 20 repeated consecutive subcultures of 24 h duration each in MMM supplemented with 2.66 g/L of TBP (equivalent to 10 mM TBP when dissolved). In 20 repeated subcultures, the strain exhibited complete stability with respect to the TBP degradation and growth.

The initial inoculum density determined the rate of TBP degradation by RSMS strain (Fig. 2c). Culture incubated in 7.98 g/L TBP suspension at a cell density of 3 OD_{600nm} showed complete degradation of 10 mM TBP in 24 h (Fig. 2c) yielding a degradation rate of ~ 0.42 μmol TBP ml^{-1} h^{-1}. The rate of TBP degradation at 2 mM TBP (concentration used in previously reported studies) concentration was observed to be 0.25± 0.02 μmol ml^{-1} h^{-1} (Table 1). No further increase in the rate of TBP degradation was observed at higher TBP concentration (>7.98 g/L) (data not shown). The RSMS strain degraded 2 mM TBP in 8 h and 30 mM TBP in 3 days (Table 1).

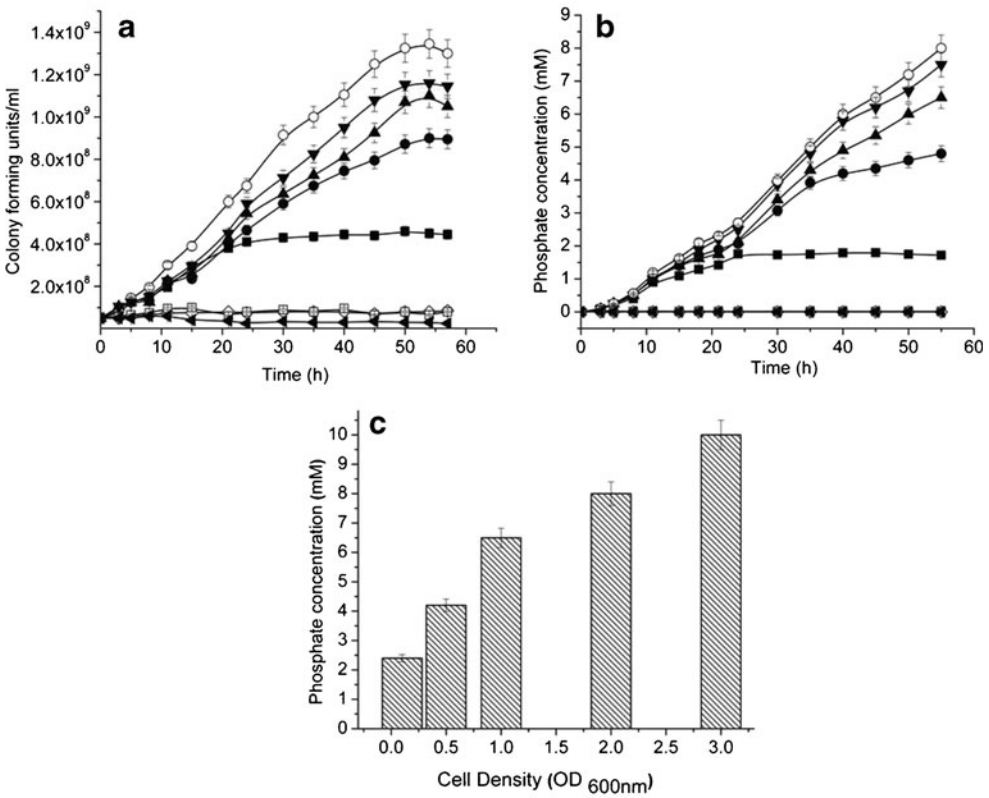

Fig. 2 Growth and TBP degradation kinetics of the RSMS strain. **a** Growth and **b** TBP degradation were studied in MMM supplemented with 0.532 g/L (*black-filled square*), 1.33 g/L (*black-filled circle*), 2.66 g/L (*black-filled up-pointing triangle*), 7.98 g/L (*white circle*), or 13.3 g/L (*black-filled down-pointing triangle*) of TBP, as the sole source of carbon and phosphorous. Initial inoculum density was set at an OD_{600nm} of 0.1 (~5×10^7 CFU/ml). Following controls were also included: TBP supplemented MMM without cells (*black left-pointing triangle*), glucose supplemented culture in MMM without added TBP (*white square*) or KH_2PO_4 supplemented culture in MMM without added TBP (*white diamond*). Growth was assessed as colony forming units (CFU) and TBP degradation was spectrophotometrically estimated as phosphate released upon TBP degradation. **c** Degradation of TBP by RSMS strain inoculated at different cell densities (OD_{600nm}) in MMM supplemented with 7.98 g/L TBP as the sole source of carbon and phosphorous. TBP degradation was spectrophotometrically assessed in terms of phosphate released 24 after inoculation

Table 1 Rate of TBP degradation and time required for the complete degradation of TBP

Amounts of TBP added in mg/ml	0.532	1.33	2.66	5.32	7.98
Rate of degradation (μmole ml^{-1} h^{-1})	0.25±0.02	0.28±0.02	0.32±0.03	0.36±0.03	0.41±0.03
Time (h) taken for the complete degradation of added TBP	8±0.5	18±1	31.5±3	55.5±4.5	73±5.5

The MMM (10 ml) containing different amounts of TBP were inoculated with 3 OD_{600nm} cells. The TBP degradation was monitored as phosphate released at different time intervals. The values reported are average of three replicates

Attempts were also made to see the TBP and DBP degradation in cell free extracts of RSMS strain. While no TBP degradation was seen in cell free extracts, DBP degradation and release of inorganic phosphate could be seen in cell free extracts of both TBP and DBP grown cells (Table. S1). The reasons for the requirement of the intact cells for TBP to DBP conversion are unknown, but may have to do with localization of corresponding triesterase in periplasm/membrane.

Suppression of TBP and DBP degradation in the presence of glucose Possible catabolic repression by glucose was evaluated in MMM suspensions containing 2.66 g/L TBP (equivalent to 10 mM TBP when dissolved) (Fig. 3), wherein the growth was less than that in 7.98 g/L TBP suspension (Fig. 2a). Cultures grown in 2.66 g/L TBP suspension alone showed threefold less growth compared to the cultures grown in such amounts of TBP along with 0.2 or 0.5 % glucose, but the TBP degradation was observed to be similar in all the cases (Fig. 3a, b). Decreased TBP degradation and corresponding growth inhibition (due to unavailability of phosphorous source) were observed with increasing concentrations of glucose above 0.5 % (Fig. 3a, b). No degradation of TBP and correspondingly no growth was observed in cultures supplemented with 5 or 10 % glucose (Fig. 3c). DBP degradation was found to be similarly inhibited by 10 % glucose (Fig. 3c). However, such repression of TBP/DBP degradation by glucose was reversible. TBP degradation and dependent growth was induced upon removal of glucose albeit after a 6 h lag (Fig. S2). DBP degradation in cell free extracts similarly revived after a lag upon removal of glucose (Table. S1).

Analysis of TBP degradation products Gas chromatography procedures were standardized to detect TBP, DBP, and butanol, as well resolved peaks. The sensitivity of detection was 0.1, 0.5, and 0.67 nmol for TBP, DBP, and butanol, respectively. The methods showed linearity up to 30 nmol for all the analytes (data not included).

MMM suspension containing either 7.98 g/L TBP (equivalent to 30 mM TBP when dissolved) or 20 mM DBP alone (without cells) did not show any of their respective intermediates or products of the degradation (data not shown). The RSMS strain completely degraded 30 mM TBP in 3 days when inoculated at a cell density of 3 OD_{600nm}. Both DBP and butanol were detected as products of TBP degradation by gas chromatography (Fig. 4a). The concentration of butanol remained at peak levels in the first 2 days, whereas the DBP reached peak levels at 48 h. Independent measurements showed that the phosphate increased linearly between 0 and 72 h before reaching a peak value. After 3 days, neither TBP, DBP nor butanol was detected in the culture medium suggesting their complete degradation/utilization. Concomitantly, growth of the strain saturated by day 3. The RSMS strain also degraded 20 mM DBP in 2 days with 50 % degradation occurring in the first 24 h (Fig. 4b). Butanol and phosphate were detected as the end products. While butanol was completely used up as carbon source by day 3, phosphate released was in excess of growth requirement and accumulated in the medium. Appearance of butanol (an alcohol) and phosphate among the TBP and DBP degradation products suggests sequential cleavage of all ester bonds, resulting in the conversion of TBP to DBP and then (possibly through MBP) to butanol and phosphate.

Discussion

TBP storage tanks were chosen for the isolation of TBP degrading strain, wherein the TBP concentrations are very high and no other carbon or phosphorous source is available. Such a choice might have facilitated exclusion of organisms incapable of utilizing TBP or unable to survive at such high TBP concentration (1.1 M) (Schultz and Navratil 1984). The RSMS strain showed a clear zone of TBP degradation around the colony (Fig. 1). Such visible phenotype has not been reported earlier for any of the strains reported to degrade TBP. The RSMS strain was identified to belong to the genus *Sphingobium* based on signature nucleotides of 16S *rRNA* gene sequence and the negative nitrate reductase test, which are characteristic features of the genus *Sphingobium* (Takeuchi et al. 2001). Sphingomonads have been known to metabolize several xenobiotics, but this is the first strain of a sphingomonad reported to biodegrade TBP.

The solubility of TBP in water is 400 mg/L (~1.5 mM) (Hernandez, 2001). Most of the previous studies therefore have been restricted to 2 mM TBP concentrations. However, TBP concentration at storage sites is ~1.1 M and any real

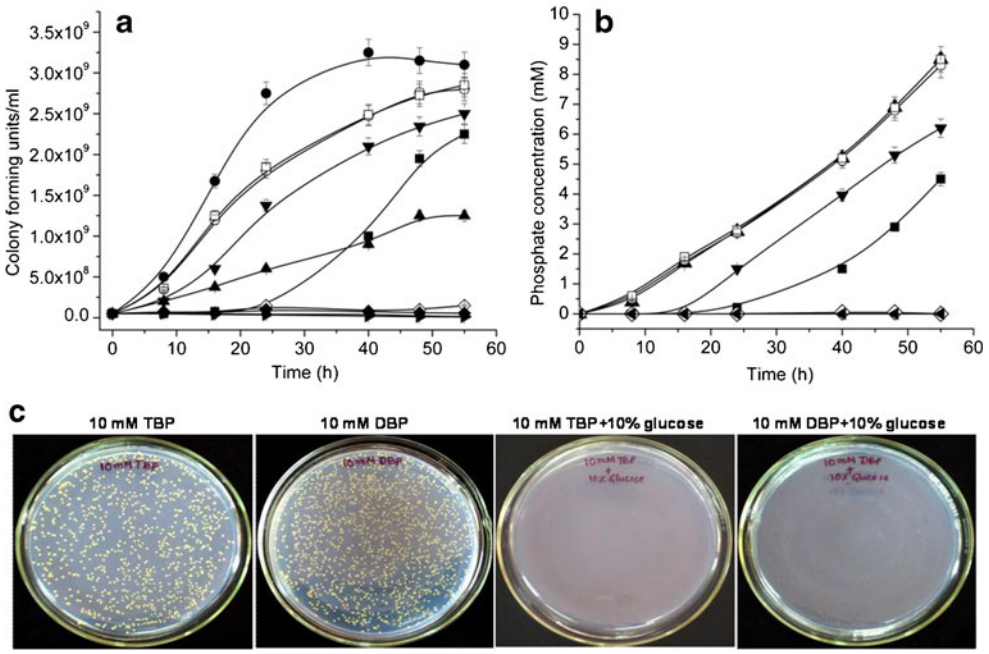

Fig. 3 Inhibition of **a** TBP dependent growth and **b** TBP degradation by glucose. MMM suspension containing 2.66 g/L TBP (*black-filled up-pointing triangle*) was supplemented with glucose either at 0.2 % (*white circle*), 0.5 % (*white square*), 1 % (*black-filled down-pointing triangle*), 2 % (*black-filled square*), 5 % (*white diamond*), or 10 % (*black left-pointing triangle*) concentration. Following controls were also included: cells in MMM (*black right-pointing triangle*), cells in MMM supplemented with 10 % glucose (*black-filled diamond*), or cells in MMM supplemented with 10 % glucose and 5 mM KH_2PO_4 (*black-filled circle*). Other details were as described in legend to Fig. 2. **c** Suppression of TBP or DBP dependent growth by glucose. Growth was assessed as CFUs on MMMA plates containing 2.66 g/L TBP or 10 mM DBP. The plates were supplemented with or without 10 % glucose

biodegrading strain must function at much higher concentration of TBP than what the solubility permits. Addition of >2 mM TBP to the solutions results in opaque liquid or solid media wherein the effective concentration of TBP cannot be estimated due to the immiscible nature of TBP. However, active culture of an efficient TBP degrading strain under strong agitation should quickly degrade the sparingly dissolved TBP forcing fresh solubilization and further degradation. The process can continue until all the added TBP is completely degraded.

We attempted such degradation of TBP from nonhomogeneous liquid suspensions and solid media containing equivalent of up to 30 mM TBP when dissolved. Even from such immiscible media, TBP was rapidly and completely degraded and utilized by RSMS strain. This was evident from (1) formation of a clear, transparent zone of clearance around cells on

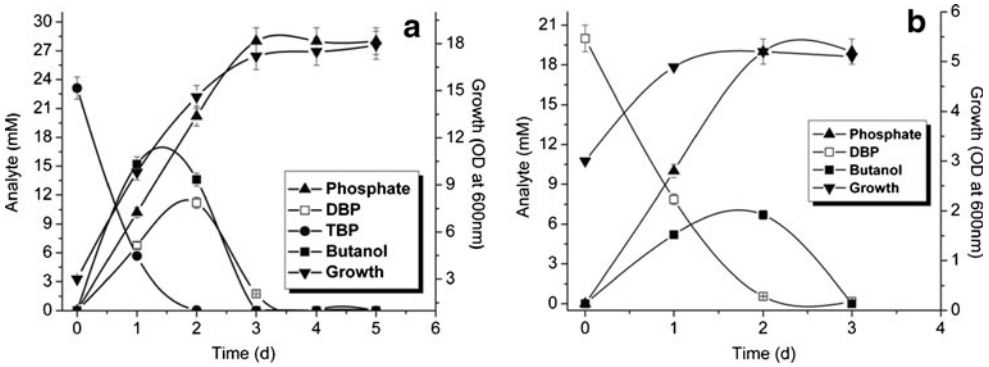

Fig. 4 Analysis of TBP degradation products. The degradation of added analytes by RSMS strain and appearance of degradation products in MMM supplemented with **a** 7.98 g/L TBP or **b** 20 mM DBP. Various symbols indicate levels of TBP (*black-filled circle*), DBP (*white square*), butanol (*black-filled square*), or inorganic phosphate (*black-filled up-pointing triangle*). Growth (*black-filled down-pointing triangle*) of the strain using added compounds as the sole source of carbon and phosphorous is shown for each analyte

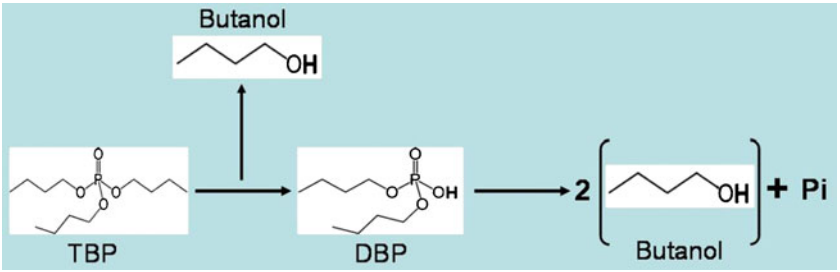

Fig. 5 Proposed biochemical pathway of TBP degradation by the RSMS strain

TBP containing agar plates (Fig. 1), (2) TBP concentration dependent increase in phosphate release (TBP degradation) as well as growth of the RSMS strain (~25 times increase in CFUs) in TBP supplemented suspensions (Fig. 2), and (3) release of ~29 mM inorganic phosphate after complete degradation of 30 mM TBP. The strain continued to show TBP degradation even in stationary phase (Fig. 2b) and also utilized the released butanol (~90 mM) for its growth (Fig. 4). This type of efficient utilization can prevent the accumulation of toxic byproducts such as butanol formed due to TBP degradation in the medium.

TBP degradation results in inorganic phosphate release which can inhibit further TBP degradation and TBP dependent growth as reported earlier (Thomas and Macaskie 1998). However, no adverse effect on TBP dependent growth was seen in the presence of 0–20 mM phosphate (supplied in the form of KH_2PO_4) and TBP degradation was not effected significantly even in the presence of 100 mM phosphate, as adjudged from diameter of the zone of clearance on MMM plates supplemented with 10 mM TBP+0–100 mM KH_2PO_4 (data not included).

TBP degradation rates of various bacterial strains reported earlier range from 1.6 mM in 3 weeks (*R. palustris*) and 2 mM in 3 days (mixed cultures of *Pseudomonas*) (Berne et al. 2005; Thomas et al. 1997) at 2 mM TBP concentration. An earlier study reported 3.05 mM TBP degradation from media suspensions containing equivalent of 5 mM dissolved TBP in 4 days (Ahire et al. 2011). This was claimed to be the highest amount of TBP degradation reported so far. In contrast, the RSMS strain was found to be far superior. TBP degradation by RSMS strain ranged from 0.25–0.45 μmol ml^{-1} h^{-1} and resulted in complete degradation of 2 or 30 mM TBP in 8 or 73 h, respectively.

TBP at 10 to 400 μM concentration inhibits cell division in most bacteria (Nakamura 1991). Few strains have been reported to tolerate 5 mM TBP concentration, which is the highest tolerance to TBP reported so far (Ahire et al. 2011). In contrast, the RSMS strain was found to tolerate equivalent of even 30 mM TBP in suspension without loss of viability in MMM which is truly remarkable. Such high tolerance is needed when dealing with the actual waste where the concentrations of TBP are very high (Schultz and Navratil 1984).

TBP and DBP degradation were strongly inhibited by glucose concentrations above 5 %, indicating possible catabolite repression (Fig. 3b, c). The consequent unavailability of phosphorous (from TBP/DBP degradation) completely inhibited growth in TBP or DBP supplemented cultures containing 5 or 10 % of glucose, indicating complete repression of TBP degradation (Fig. 3a). Such efficient regulation of TBP degradation has also not been reported earlier.

Over the last three decades, mineralization of TBP has been proposed to be mediated by phosphoesterases, wherein the tri-, di-, and monoesterases would sequentially act on TBP to release DBP and monobutyl phosphate (MBP) as intermediates and butanol and phosphate as final products (Macaskie 1991; Owen et al. 1992; Rosenberg and Alexander 1979; Thomas et al. 1997), but no such intermediates or butanol were detected or reported earlier. The present study clearly showed DBP as the most immediate intermediate in TBP degradation and butanol and phosphate as the products of TBP degradation by RSMS strain. Butanol and phosphate were also found to be the products of DBP degradation (Fig. 4b). The scheme of TBP degradation revealed by the present investigation is delineated in Fig. 5. It is possible that DBP degradation to butanol and phosphate proceeds through MBP. However, unavailability of purified MBP precluded experiments which could establish/rule out this possibility.

More than 20 bacterial isolates have been reported so far to degrade TBP. Many of the earlier reported strains were relatively inefficient or unstable (Thomas et al. 1997). For molecular investigation of the TBP degradation, it is necessary to have a stable, single pure isolate which exhibits a visual or easy to score phenotype and survives on high concentrations of TBP, utilizing it as the sole source of carbon and phosphorous. The *Sphingobium* sp. strain RSMS fulfills all these requirements and qualifies as an appropriate strain for further molecular dissection of mechanisms (genes/proteins) underlying TBP biodegradation.

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