पर्यावरण के रक्षा उपाय

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स्फिंगोबियम प्रजाति आरएसएमएस द्वारा टीबीपी के जैव निम्नन : बृहत अध्ययन

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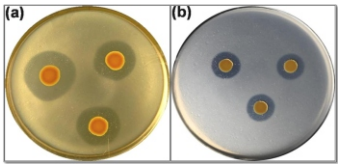

(a) आर. एस. एम. एस. उपभेद द्वारा टी. बी. पी. का क्षरण। जीवाणु संवर्धन (ए) लूरिया बर्टानी अगर माध्यम और (b) एमएमएम अगर प्लेटों पर देखा गया, जो 10 एम. एम. टी. बी. पी. के साथ पूरक थे। निकासी का क्षेत्र ऊष्मायन के 1 सप्ताह के बाद दर्ज किया गया था।

सारांश

कुछ औद्योगिक प्रक्रम बड़ी मात्रा में टीबीपी युक्त अपशिष्ट उत्पन्न करते हैं। टीबीपी के अंतग्रर्हण से समुद्री जीवन और जानवरों में विषाक्तता पैदा होती है। इससे पहले, स्फिंगोबियम प्रजाति आरएसएमएस (आरएसएमएस), भापअकें में आरएसएमएस साइट से विलगित किए गए एक जीवाणु को प्रयोगशाला पैमाने (3 दिनों में 30 mM) पर टीबीपी को प्रभावी रूप से निम्नन करते हुए दिखाया गया। आरएसएमएस स्ट्रेन का उपयोग करके टीबीपी जैवनिम्नन के चरण-वार पैमाने के लिए प्रक्रम विकसित किए गए। 30L पैमाने पर, इष्टतमीकृत परिस्थितियों में, लगभग पूर्ण खनिजीकरण (3 दिनों में 28 mM) प्राप्त किया गया। कम्प्यूटेशनल फ्लूइड डायनामिक्स मॉडलिंग का उपयोग 205L तक के पैमाने के लिए प्रचालन मापदंडों के इष्टतम मानों की पहचान करने के लिए किया गया और 21 mM एमटीबीपी निम्नन 15 दिनों में प्राप्त किया गया जो प्रयोगशाला पैमाने पर देखे गए टीबीपी जैवनिम्नन का लगभग 70% है। ये प्रक्रम टीबीपी जैवनिम्नन के लिए रिपोर्ट किए गए सबसे प्रभावी स्केल अप प्रक्रम हैं।

Safeguarding Environment

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Biodegradation of TBP by *Sphingobium* sp. RSMS: Scale up Studies

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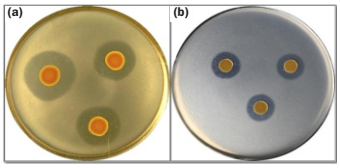

(a) TBP degradation by RSMS strain. Bacterial culture was spotted on (a) Luria Bertani Agar medium and (b) MMM agar plates, supplemented with 10 mM TBP. The zone of clearance was recorded after 1 week of incubation

ABSTRACT

Certain industrial processes generate large volume of TBP-containing wastes. TBP causes toxicity to marine life and to animals upon ingestion. Previously, *Sphingobium* sp. RSMS (RSMS), a bacterium isolated from RSMS site BARC was shown to degrade TBP efficiently at laboratory scale (30 mM in 3 days). Processes for the step-wise scale-up of TBP biodegradation utilizing the RSMS strain were developed. At 30 L scale, under optimised conditions, near complete mineralisation (28 mM in 3 days) was achieved. Computational fluid dynamics modelling was used to identify the optimum values of operating parameters for scale-up to 205 L and 21 mM TBP degradation was achieved over 15 days, which is approximately 70% of the TBP biodegradation observed at laboratory scale. These processes are the most efficient scale up processes reported for TBP biodegradation.

KEYWORDS: *Tributyl phosphate (TBP), Sphingobium sp. RSMS, Biodegradation, Scale up*

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Introduction

Tributyl phosphate (TBP), is an organophosphorus compound which finds applications in various industries as a solvent, complexing agent, plasticizer, herbicide, fungicide, defoamer etc. [1]. TBP is also used as an extractant for recovery of radionuclides from nuclear wastes. TBP finds its way into the environment by leakage from production or usage sites, and leaching from TBP containing waste disposed in landfill sites or aquatic environments [2]. Unaffected by natural photolysis and hydrolysis, TBP is known to be very stable in soil and water. Persistence of untreated TBP in natural environment can cause environmental pollution. Even at low concentration of 4.2-18 mg l^{-1}, TBP can present acute toxicity hazard to organisms in freshwater [3]. In humans, penetration of TBP causes irritation of skin and mucous membranes. Animal studies have also shown toxic effects of TBP [4].

Chemical degradation, incineration or immobilisation methods have been considered for treatment and disposal of TBP bearing radioactive wastes [5]. The conventional physico-chemical processes prove uneconomical when TBP concentrations are low and high volumes of waste are encountered [6]. Moreover, these methods either do not completely destroy the compound or merely convert it to other form of contaminant(s) thereby causing secondary pollution. In such scenario microbial degradation of TBP is considered feasible alternative for detoxification. Unlike traditional methods, microbial degradation is relatively simple not requiring harsh or complex conditions.

A TBP degrading bacterial strain was isolated from the TBP storage tanks at RSMS site, BARC and after identification and characterization named as *Sphingobium* sp. RSMS (RSMS Strain) [7]. Here we discuss the TBP degradation efficiency of RSMS strain and development of processes for scale up of TBP degradation from laboratory scale to 30 L and further to 205 L volumes.

Media, Growth Conditions and Measurement of TBP Degradation

Sphingobium sp. RSMS strain (RSMS strain) was grown either in Luria-Bertani medium (LB) or modified mineral medium (MMM) supplemented with 25-100 mM 3-(N-Morpholino)-propanesulfonic acid (MOPS) as a buffer [7]. The cultures were grown with aeration (160 rpm) at 30°C. For TBP degradation experiments, glucose and phosphate source was omitted and TBP was used as the sole source of carbon and phosphorous. The TBP is partially soluble in the aqueous media. The TBP concentrations mentioned in this article are equivalent concentrations when dissolved. The final product of TBP biodegradation, inorganic phosphate (Pi), was monitored by phosphomolybdic acid method for estimating TBP degradation as per published protocol [7,8]. For growth measurement, 20 ml to 40 ml of culture was centrifuged at 5000 rpm (4470 x g) for 10 min, washed twice with equal volume of MMM and the pellet was resuspended in equal volume of MMM. Cell density was measured at 600 nm using a spectrophotometer.

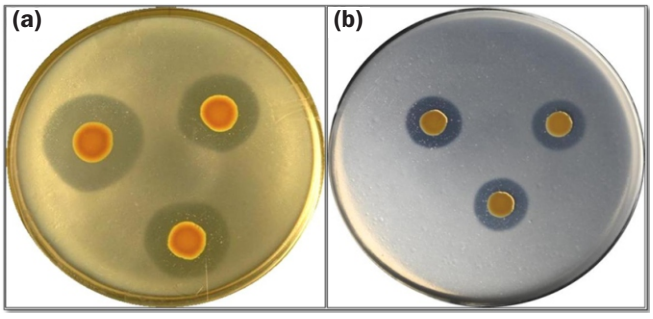

Fig.1: TBP degradation by RSMS strain. Bacterial culture was spotted on (a) Luria Bertani Agar medium and (b) MMM agar plates, supplemented with 10 mM TBP. The zone of clearance was recorded after 1 week of incubation (Source: Rangu SS et al., 2014. Fig.1).

Results and Discussion

Ideally a TBP degrading microorganism should mineralise TBP by using it as the sole source of carbon and phosphorus and degrade high amounts of TBP. As compared to mixed cultures where relative fitness of microbes and growth dynamics make scale up complex, having a pure isolate can simplify the process. The microbes or consortia reported in literature are unlikely to have any practical application as they could degrade only 1-3 mM TBP. [1,9]. In this context *Sphingobium* sp. RSMS a bacterium isolated from storage tanks at RSMS site BARC was evaluated for its TBP degrading potential.

Degradation of TBP by RSMS Strain in Solid and Liquid Medium

RSMS strain was shown to degrade 10 mM TBP on solid medium as evident from the zone of clearance around the grown spot (Fig.1). In liquid medium RSMS strain could degrade 30 mM TBP in 3 days. The strain utilized TBP as sole source of carbon and phosphorous for its own growth. TBP at 10 to 400 µM concentration is known to inhibit cell division in most bacteria. In contrast, the RSMS strain was demonstrated to tolerate ~100 mM TBP when supplemented in solid LB agar media.

TBP Degradation at 30 L Scale

Survey of literature shows very few processes developed for the bio-degradation of TBP. A process reported by Nancharaiah et al., 2015, could degrade 2 mM TBP in 5 h [9]. Two process patents (USA) filed, show 0.6-0.7 mM TBP degradation in 10-30 days using *Acinetobacter* sp. ATCC 55587 while a non-sulphur purple photosynthetic bacterium degraded around 1.6 mM TBP in 3 weeks [10, 11]. As RSMS strain showed excellent TBP degradation at lab-scale, we attempted to develop processes at larger scales using this strain. For scale up of TBP degradation to 30 litres, a fermenter (SU1) made of a stainless-steel vessel with height-to-diameter ratio of 3.2 and with multiple ports on the top was used (Fig. 3). It had an agitator shaft connected to a top driven motor (0.75 hp) and fitted with 3 six-bladed Rushton turbine impellers. Different probes were inserted into the vessel for monitoring pH, DO (dissolved oxygen) and temperature. SU1 fermenter was always maintained in sterile conditions. 400 ml of overnight grown RSMS culture was added to the SU1

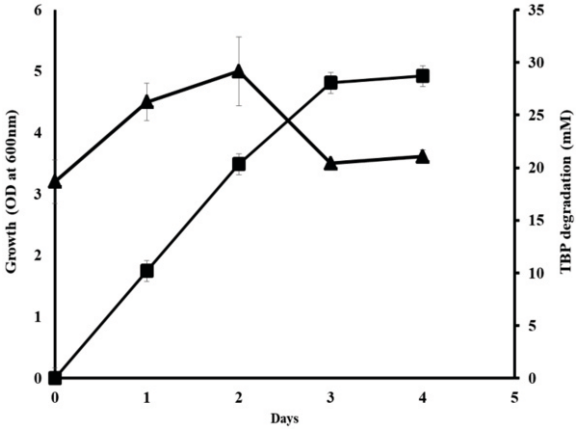

Fig.2: TBP degradation by RSMS strain at 30 litre scale. The growth (▲) in terms of cell density (OD_{600nm}) and degradation of TBP (■) in terms of Pi release is depicted. (Source: Rangu et al., 2022. Fig. 2).

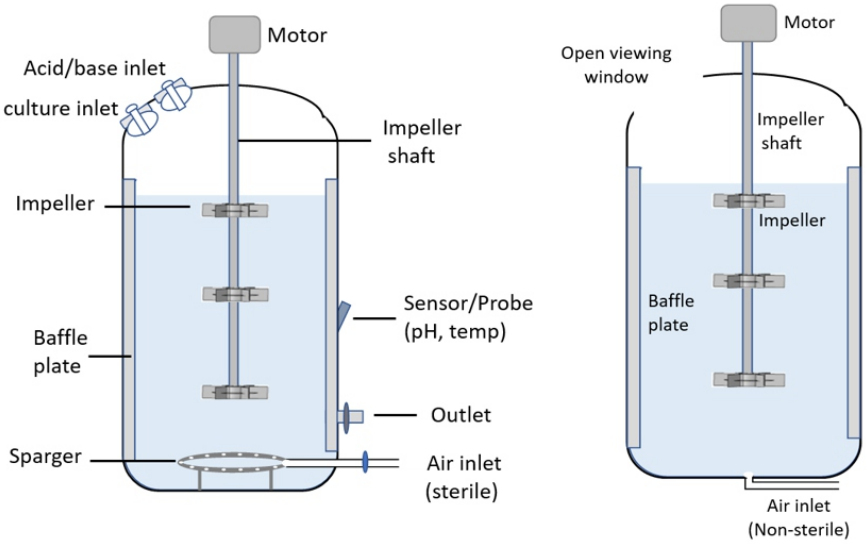

Fig.3: Pictorial depiction of 30 L fermenter (SU1) and 205 L (SU2) fermenter used in the study. (Source: Rangu et al., 2022. Modified Fig. 1).

Table 1: Various parameters of 30 mM TBP degradation at 30 litre scale.

Days	Description	Dissolved oxygen (%)	RPM	Adjusted pH	TBP degradation (mM)	Growth (OD_{600nm})
-1	Inoculation	100	200	7.5 (±) 0.1	NA	0.06 (±) 0.01
0	TBP added	100	150	7.5 (±) 0.1	Nil	3.2 (±) 0.35
1	Day 1 for TBP degradation	100	150	7.5 (±) 0.1	10.2 (±) 0.3	4.5 (±) 0.30
2	Day 2 for TBP degradation	100	150	7.5 (±) 0.1	20.3 (±) 0.8	5.0 (±) 0.56
3	Day 3 for TBP degradation	100	150	7.5 (±) 0.1	28 (±) 0.45	3.5 (±) 0.05
4	Day 4 for TBP degradation	100	150	ND	28.7 (±) 0.2	3.61 (±) 0.11

fermenter containing 30 litres of sterile MMM supplemented with 0.5 % glucose and 2 mM inorganic phosphate using a peristaltic pump. Following conditions were maintained for 20 h of growth period- Agitator speed: 200 rpm; Fermenter temperature: 30^{0}C; Dissolved oxygen: 100 %; Aeration: 10 L min^{-1}. After 20 h of growth, TBP was added to a final concentration of 10, 20 or 30 mM. The growth was continued with a reduced agitator speed of 150 rpm. The pH of the culture was monitored and adjusted to about 7-7.5, if required, by adding NaOH.

The almost complete degradation of 10 mM and 20 mM of TBP was achieved in 30 and 54 hours respectively. In case of 30 mM TBP, a steady rate of TBP degradation was observed over a period of 3 days. After 3 days, ~28 mM of TBP was degraded, which was ~93 % of that seen at laboratory scale. No further degradation of TBP (Fig. 2) was observed. The RSMS strain showed growth as a function of TBP degradation, suggesting utilization of TBP as the source of carbon. Table 1 summarizes the progress of TBP degradation as well as the various parameters used in the standardised experiment. During the growth, a decrease in the pH of the medium to 6.0-6.5 was observed. The pH was adjusted to 7-7.5 by addition of NaOH. The reduction in pH could be attributed to the release of Pi due to TBP degradation.

TBP Degradation at 205 L Scale

After successful scale up at 30 litre, further scale up of the process to 205 litre volume was carried out in a fermenter vessel (SU2) with 1 m height and 0.6 m diameter (Fig. 3). It had three impellers mounted on a single shaft. Agitator shaft was fitted with 3 six-bladed Rushton turbine impellers and connected to a top driven motor. SU2 fermenter had a single open window at the top for the addition of inoculum and reagents. Air was injected from the bottom at the centre of the fermenter. In SU2 probes were not available for monitoring pH, DO, temperate or pressure. To achieve proper mixing, the impeller tip speed was kept the same as in 30 litre reactor. Changes in the fluid dynamics are an important consideration as a process is scaled up. To determine the optimum operating parameters to rule out non-idealities and to visualise the flow patterns, computational fluid dynamics (CFD) modelling of the process was carried out. The cut-section of the computational domain used in CFD simulations, typical spatial variation of velocity magnitude and the velocity vectors in a vertical central plane and the path lines of the liquid in the stirred tank are shown in Fig.4.

Freshly grown overnight culture (900 ml) was added to SU1 fermenter containing 25 litres of sterile MMM supplemented with 5 mM Pi and 1 % glucose. For this initial culture build up, the conditions maintained were identical to the 30-litre experiment. After 20 h of growth, 25 litres of the culture was inoculated into SU2 fermenter containing 180 litres of non-sterile MMM supplemented with 30 mM TBP and glucose. The following conditions were maintained during the experiment (Agitator speed: variable between 44 rpm to 80 rpm; aeration: 26 Nl min^{-1}, non-sterile). The process was

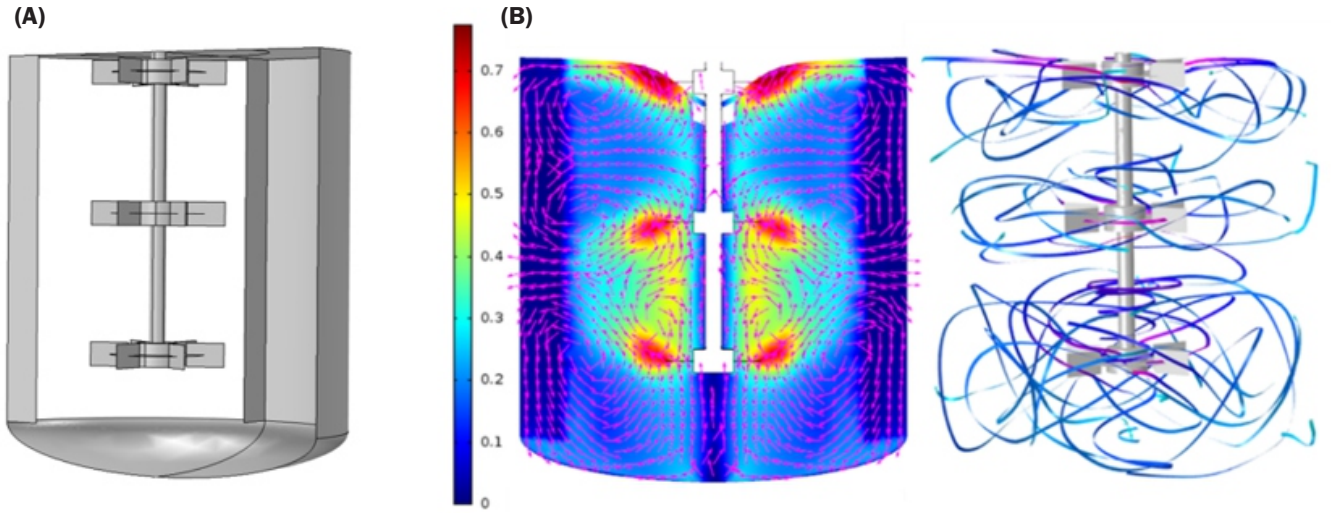

Fig.4: CFD simulation at 205 litre scale. A cut-section of the computational domain representing 205 litre stirred reactor used for CFD simulations (A). Spatial variations of velocity magnitude, velocity vectors in a vertical central plane, path lines of the liquid in 205 litre stirred tank (B). (Source: Rangu et al., 2022. Modified Fig. 2).

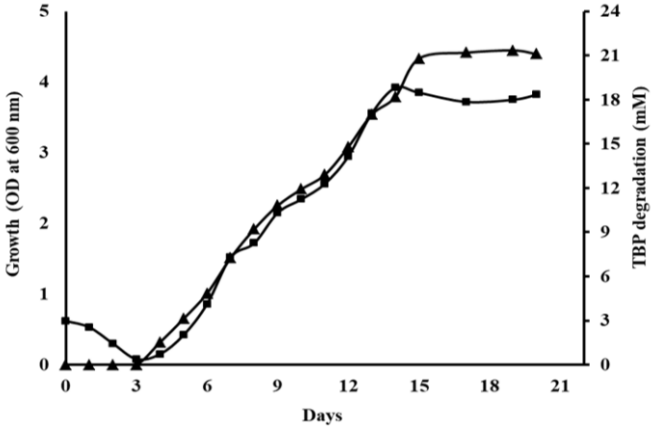

Fig.5: TBP degradation by RSMS strain at 205 litre scale. MMM supplemented with 30 mM TBP was used as medium. TBP degradation (▲) was estimated by measuring Pi by phosphomolybdate method. The culture density (■) was measured spectrophotometrically at 600 nm (OD_{600nm}). (Source: Rangu et al., 2022. Fig. 4).

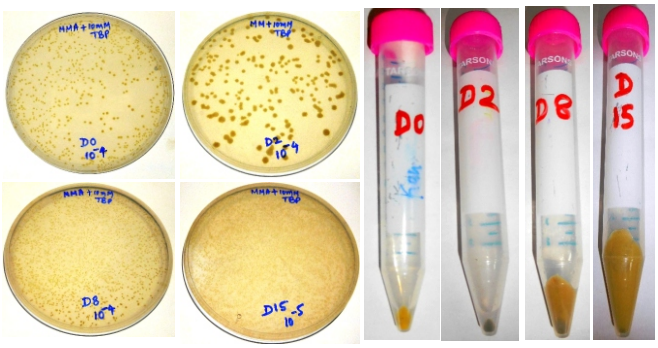

Fig.6: Growth of the Sphingobium sp. RSMS in 205 L tank. Left panel: The colony forming units (cfu count) on Day 0, 2, 8 and 15 respectively. Right panel: A fixed volume of culture was taken from SU2 tank and centrifuged to obtain the cell pellet.

carried out at ambient temperature (24 to 27°C) under non-sterile conditions. TBP degradation (as Pi release), growth of the culture (OD_{600nm}), temperature and pH were monitored over a period of 20 days. The pH of the culture was adjusted to 7-7.5 by adding NaOH as necessary. Earlier attempt to achieve the optimum cell density (OD_{600nm} ~3) by supplementing MMM with glucose led to the contamination of the batch. As glucose is a preferred carbon source for other microbes, non-sterile conditions used would allow their growth. Some of the contaminants were isolated and identified by 16S rDNA gene sequencing (Table 2). Subsequently the process was run without glucose supplementation in SU2.

In SU2 containing medium supplemented with only TBP the degradation could be observed from 4th day onwards at a steady rate up to 15th day. No increase in Pi levels was observed after the 15th day. Between 4th to 15th day, an average TBP degradation of ~1.68 mM/day was measured. Over 15 days, a total of 21 mM TBP degradation was achieved, which was approximately 70 % of the TBP degradation observed at laboratory scale. The period of the active growth of the culture coincided with the degradation of TBP (Fig. 5).

Increase in accumulation of bacterial mass during the course of the experiment is shown in Fig. 5 & 6. A decrease in pH each day from the fourth day onwards was observed and this correlated with the release of Pi (Table 3). The pH of the culture was maintained at 7-7.5 by adding NaOH. Details of the conditions and parameters during the course of the experiment is presented in Table 3.

As the 205-litre batch process was carried out under non-sterile conditions and without regulation of the temperature, it made the process less energy intensive and hence more economical. In future, utilization of environmental waste or industrial waste to generate biomass of RSMS strain, can be explored to further economise the process. Recently, we have assembled the complete genome sequence of this bacterium [12] which will aid in identifying the genetic pathway of TBP bio-degradation and in turn may open up the possibility of

Table 2: Contaminant strains identified in the 205 tank when glucose was added along with TBP.

Strain No.	Strain identified	Percent identity
1	*Paenibacillus* sp. strain	99
2	*Paenibacillus xylanilyticus* strain	93
3	Uncultured bacterium clone 069088	92
4	*Pseudomonas* sp. strain RP4	98
5	Uncultured gamma proteobacterium	83
6	Not identified	No significant identity

Table 3: Various parameters of TBP degradation at 205 litre scale.

Days	Observed pH	Aeration	Observed Temperature	TBP degradation (Pi released mM)	Culture density (OD_{600nm})
		Nl min^{-1}	(^oC)		
0	7.22	26	24	0	0.62
1	6.93	26	24	0	0.53
2	7.2	26	24.5	0	0.3
3	7.3	26	23.5	0	0.08
4	7	26	26.5	1.52	0.15
5	6.89	26	26.5	3.12	0.42
6	6.6	26	26.5	4.85	0.86
7	6.4	26	25	7.25	1.52
8	5.8	26	25	9.21	1.72
9	6.9	26	25.5	10.81	2.15
10	6.9	26	26	11.92	2.34
11	6.9	26	26	12.9	2.56
12	6.9	26	25	14.8	2.95
13	6.6	26	25.5	17	3.56
14	6.68	26	27	18.2	3.92
15	6.63	26	26.5	20.8	3.85
17	6.61	26	26	21.2	3.72
20	6.6	26	26	21.1	3.82

metabolic engineering of the strain to further enhance its TBP biodegradation efficiency.

Conclusion

TBP biodegradation processes reported earlier were inefficient (degradation of 1-3 mM TBP). *Sphingobium* sp. RSMS, a bacterium isolated from RSMS site BARC, showed efficient degradation of TBP at lab scale (30 mM in 3 days). A 30 L scale-up process was developed and optimised to achieve 28 mM TBP degradation in 3 days. Further scale-up to 205 L volume was done and a more economic process was developed. These processes are much more efficient than the patented processes for TBP biodegradation.

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