

Full Length Research Paper

Biodegradation of aliphatic, aromatic, resinic and asphaltic fractions of crude oil contaminated soils by *Pleurotus tuber-regium* Fr. Singer - a white rot fungus

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The use of chemical fertilizer and animal manure on crude oil reduction during biodegradation with *Pleurotus tuber-regium* was investigated. The co-substrates and inocula types used for the investigation were shredded banana leaf blades and sawdust of *Albizia* and sclerotium and spawn of the fungus, respectively. The reduction of total petroleum hydrocarbons was higher in treatments with a combination of fertilizers and co-substrates. Degradation of total petroleum content was higher in poultry litter treatment than the NPK treatments. The degradation of aliphatics in substrates with co-substrates was higher than those without co-substrates. Reduction of aromatics was appreciable in all treatments with the least been ~40% in banana leaf blades + contaminated soil and contaminated soil only substrates. Degradation of resins was higher in contaminated soils with co-substrates only than those with fertilizers + co-substrates. The reduction of asphaltenes was low and some substrates instead of a decrease, recorded increase in the asphaltic fraction. Phytoassessment tests show that the addition of poultry litter and sawdust and banana leaf blades to fungal remediation restored the contaminated soil. The addition of NPK to crude oil contaminated soil in the presence of *P. tuber-regium* affected the soil negatively as it could not support plant growth anymore. *P. tuber-regium* is better able to remove hydrocarbons from soil with the aid of poultry litter and co-substrates.

Key words: Biodegradation, petroleum hydrocarbons, co-substrates, phyto-assessment, fertilizers.

INTRODUCTION

Crude oil is a major contaminant of soil and water in oil producing countries as a result of extraction and processing of the oil. Crude oil spills from pipelines and

refineries cause damage to the environment. The contamination changes the physicochemical and biological properties of the soil because the oil may be toxic to some soil microorganisms and plants. Depending on the crude oil composition and concentration, its effects on living organisms and its fate in environment vary (Minai-Tehrani and Herfatmanesh, 2007). Petroleum hydrocarbons that constitute crude oil are categorized into four fractions: saturates (aliphatics), aromatics, resins and asphaltenes (Singh, 2006). These fractions and their derivatives constitute environmental pollutants when released into the environment. They are a serious concern worldwide because of the hazards they pose to the health of humans and animals (Reddy and Mathew, 2001). Various bacteria and fungi use some crude oil fractions as a sole carbon source and change them to non-toxic compounds such as CO₂ (Cerniglia, 1992). The aliphatic and some aromatic fractions are the most bio-

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Abbreviations: DDT- pentachlorophenol; TNT- 2, 4, 6-trinitrotoluene; PCBs- pentachlorophenyl; PAHs- polycyclic aromatic hydrocarbon, NPK- nitrogen, phosphorus and potassium; cso- contaminated soil only; sds- sawdust + contaminated soil; sdsnpk- sawdust + NPK + contaminated soil; pls- poultry litter + contaminated soil; sdspl- sawdust + poultry litter + contaminated soil; npks- NPK + contaminated soil; sdsnpk- sawdust + NPK + contaminated soil; bls- banana leaf blades + contaminated soil; blsnpk- banana leaf blades + NPK + contaminated soil.

degradable components and the resins and asphaltenes are believed to be resistant to biodegradation (Atlas, 1981; Oudot et al., 1993). Bioventing and land farming are common methods used in field bio-treatment of contaminated soils and recently mycoremediation. Factors such as temperature, moisture, oxygen accessibility and nutrients influence the rate of biodegradation of crude oil in soil. Many reports indicate the importance of nutrient addition to contaminated soil to enhance biodegradation (Braddock et al., 1997; Lindstrom et al., 1991). Phosphates and nitrate salts are the most common nutrient additives (Wrenn et al., 1994). Mycoremediation which involves the use of white rot fungi for the cleaning up of contaminated soils is widely reported in literature but little is said about the use of fertilizers in conjunction with these fungi. White rot fungi secrete non-specific extracellular enzymes, which are involved in the degradation of lignin (Barr and Aust, 1994). The same mechanisms that give these fungi the ability to degrade lignin are also used to degrade a wide range of pollutants such as DDT, TNT, PCBs and PAHs. This study therefore focuses on the remediation of crude oil contaminated soils using *P. tuber-regium* a white rot fungus amidst different substrate combinations. The use of chemical and organic fertilizer is compared and also the combination of cellulosic wastes and the fertilizers is also studied. The use of cellulosic wastes is investigated also because literature records show that degradation of high molecular weight compounds by white rot fungi requires a suitable carbon co-substrate to be successful. Examples of such co-substrates are potato pulp, wheat straw, peat, bark and wood chips (Lamar and Glaser, 1994; Zeddel et al., 1993).

P. tuber-regium is a tropical sclerotial mushroom. It is the only species of *Pleurotus* known to produce fruit bodies from a globose true sclerotium. The sclerotium is spherical to ovoid and can be quite large up to 30 cm or larger in diameter (Isikhuemhen et al., 1999).

MATERIALS AND METHODS

Inoculations

Sclerotium of *P. tuber-regium* was bought from a local market in Benin City, Nigeria. Pure culture isolates were got from the sclerotium induction and cultured using potato dextrose agar in Petri dishes. Un-threshed rice bought from a local market in Gwagwalada Abuja, Nigeria was used to prepare the grain mother spawn following the method adopted from Chang and Miles, (2004). Sawdust spawn was prepared from the grain mother spawn using the method also outlined in Chang and Miles, (2004). Sawdust used was that of *Albizia* wood collected from a Sawmill at Amukpe near Sapele, Delta State Nigeria. The spawn run lasted for 14 to 17 days.

Collection of crude oil contaminated soil, soil amendment and analysis

The polluted soils were collected at a spill site Uvwianmughe near Ughelli in Delta State. The site is a flow station called Ughelli Pump-

ing Station owned by Shell Petroleum Development Company Nigeria Limited. The spill was from a rupture at wellhead 27. Soil samples were collected at 10 sites 1 meter apart using a core borer. Top soil to the depth of 5 to 10 cm was collected and composite mixture was made of the ten different samples. All debris, wood pieces, plant roots and stones removed. The soil was air dried and then filtered through a sieve made of strong wire gauze with mesh size 2 mm. Particle size analysis of sand, silt and clay in soil was got using the Hydrometer (ASTM 152H type) by Sheldrick and Wang (1993). The particle size analysis of the contaminated soil shows that the percentage clay was 11.33%, silt was 33.67% and sand 55.00%. The permeability of the soil samples was 0.068 mm.

Crude oil contaminated soil (1.7 kg) and uncontaminated soil (300 g) made up the contaminated soil only treatment. NPK 15:15:15 (40 g) + 1.7 kg of crude oil contaminated soil and 300 g of uncontaminated soil made the NPK + contaminated soil treatment. Poultry litter (40 g) + 1.7 kg of crude oil contaminated soil + 300 g of uncontaminated soil made up the poultry litter + contaminated soil treatment. Poultry litter 12 weeks old was collected from a farm at Eku, Delta State. NPK 15:15:15 was purchased from Edo State Agriculture Development Programme at Oko, Edo State. Sawdust and shredded dry banana leaf blades 300 g each were added to crude oil contaminated soils (1.7 kg) as treatment options to get sawdust + contaminated soil and banana leaves + contaminated soil treatments, respectively. Another set of treatment had banana leaf blades (300 g) + NPK (40 g) + 1.7 kg of crude oil contaminated soil to give the banana leaf blades + NPK + contaminated soil treatment. Sawdust (300 g) + NPK (40 g) + crude oil contaminated soil were added up to make up the sawdust + NPK + contaminated soil treatment. The last treatment had sawdust (300 g), poultry manure (2%) and contaminated soil (sawdust + poultry manure + contaminated soil).

There were two controls: Control A was contaminated soil to which no inoculum (*P. tuber-regium*) was added. Control B was soil from a site not impacted/ polluted. Soil for control B was collected from Abraka on the banks of River Ethiope, Delta State and was inoculated with the fungus.

Innoculation of soil samples

There were two types of inocula; these were sawdust spawn and sclerotium of *P. tuber-regium*. Sclerotium and sawdust spawn both weighing 300 g were used for the inoculation. The soil was moistened with 500 ml of de-ionized water before inoculation was carried out. Six replicates were made for each treatment. The bags were closed up by tying up after inoculation. The mushroom was grown in the soil for a period of six months and fruiting bodies were harvested from substrates that showed fructifications.

Gravimetric determination of hydrocarbon content of remediated soil

The gravimetric determination of petroleum hydrocarbons in contaminated soils was carried out using a modification of the method of Oudot and Dutrieux, (1989). Soil weighing 100 g was taken from each treatment as follows; contaminated soil only, sawdust + contaminated soil, banana leaf blades + contaminated soil, sawdust + poultry manure + contaminated soil, sawdust + NPK + contaminated soil, banana leaf blades + NPK + contaminated soil, poultry manure + contaminated soil, contaminated soil + NPK, control A - contaminated soil (no inoculation of *P. tuber-regium*). The soil samples were air-dried, ground with mortar and pestle to remove clumps that had formed. Residual petroleum hydrocarbons were extracted through soxhlet extraction with chloroform for 6 h. Fractionation into the four main molecular classes was carried out using column chromatography. After precipitation of asphaltenes in

hexane and filtration, the maltenes were separated into saturates, aromatics, and resins by successive elution with n-hexane, benzene and methanol respectively on a silica gel (60 - 100 mesh) column (Oudot and Dutrieux, 1989). After evaporation of the solvents the percentage biodegradation (%B) of each fraction was determined as $%B = 100 [(MI - MC)/MI]$, in which MI is the mass of the fraction in the control and MC is the mass of the fraction in the treatments or other substrates.

Phytoassessment of remediated soils

Phyto-assessment of remediated soil was done using a modification of the methods adopted by Baud-Gasset et al. (1993) for evaluation of remediation in contaminated soil. Soil (10 g) from all treatments used for the research was weighed and sieved to remove debris. The soil samples were then poured into Petri dishes. Three replicates were made for each treatment. Ten seeds of *Vigna unguiculata* were planted into the soils in the Petri dishes after they had been made moist with 4 ml of de-ionized water. Percentage germination was calculated by counting the number of seeds that sprouted in each plate. The plants were allowed to grow for 7 days. Plant height was determined by using a meter rule from the soil level to the tip of the youngest leaf. Leaf area was got by applying the traditional short cut field method by first getting the actual leaf area through taking the entire leaf perimeter and plotting this against leaf length x leaf breath readings. The slope was used as the multiplying factor for subsequent leaf breath x leaf length readings. This gave the leaf area for all leaves that sprouted (Pearce et al., 1975).

Statistical analysis

This was done using a randomized block design; the treatments were ten with two variables. The treatments were the various substrate compositions and the variables were the inocula types. The results obtained were analyzed statistically using the SPSS 13.00 for windows package. The means were compared using one-way ANOVA to get the LSD and also Duncan's multiple range tests was used to indicate where there was significant differences between treatments and the variables.

RESULTS

The type of inocula used and the substrate composition affected the removal of total petroleum content, saturated hydrocarbons (aliphatics), aromatics, resins and asphalt-tenes from the contaminated soils. The performance of the fungus inoculum type varied with the different substrate composition. In the sawdust + poultry litter + contaminated soil matrix, the spawn option performed better than the sclerotium option removing over 70% of the total petroleum hydrocarbons while the reverse was the case in the banana leaf blades + NPK + contaminated soil matrix (Figure 1).

This trend was also shown in the degradation or removal of the saturated hydrocarbons from the soil with the spawn performing better than the sclerotium in the sawdust treatments. The least degradation of saturates was recorded in NPK + contaminated soil treatments (Figure 2). The degradation of aromatics in contaminated soil only and NPK + contaminated soil treatments were

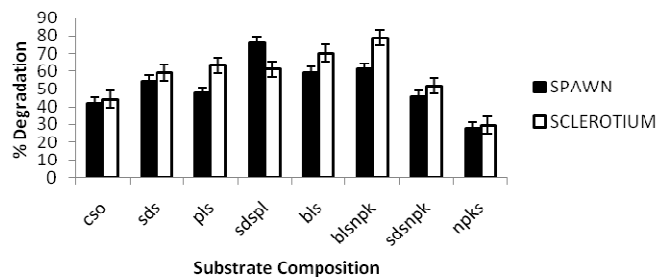


Figure 1. Percentage degradation of total petroleum content of crude oil polluted soils by *Pleurotus tuberregium*.

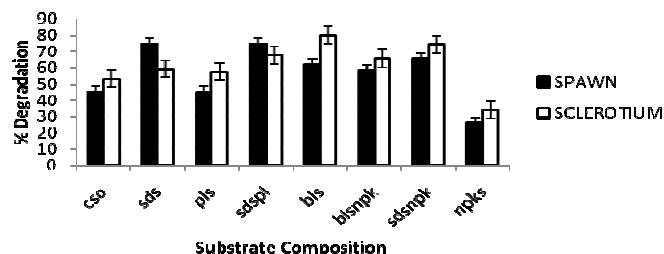


Figure 2. Percentage degradation of saturated hydrocarbons in crude oil contaminated soils by *Pleurotus ruber-regium*.

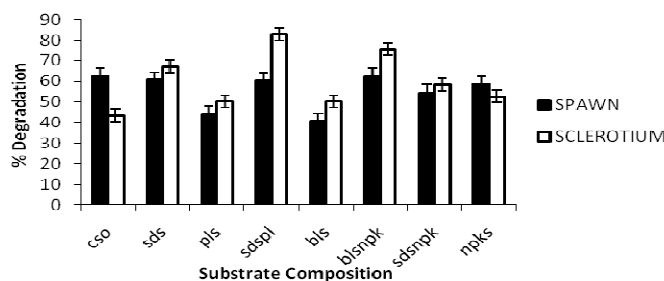


Figure 3. Percentage degradation of aromatics in crude oil contaminated soil by *P. tuber-regium*.

comparable with that in all other treatments (Figure 3).

The spawn option in contaminated soil only treatment removed more of the aromatics than the sclerotium option. In sawdust + poultry litter + contaminated soil and banana leaf blades + NPK + contaminated soil, the sclerotium option removed more aromatics than the spawn option removing over 70% of the aromatic hydrocarbons (Figure 3). The fungus was able to remove up to 60% of the total resins in sawdust + contaminated soil and banana leaf blades + contaminated soil (Figure 4). The sclerotium removed more resins in contaminated soil only, sawdust + contaminated soil and sawdust + NPK + contaminated soil. The reverse was the case in all other treatments with the spawn removing more of the resinic fraction. There was no appreciable degradation of this fraction in the contaminated soil only treatments

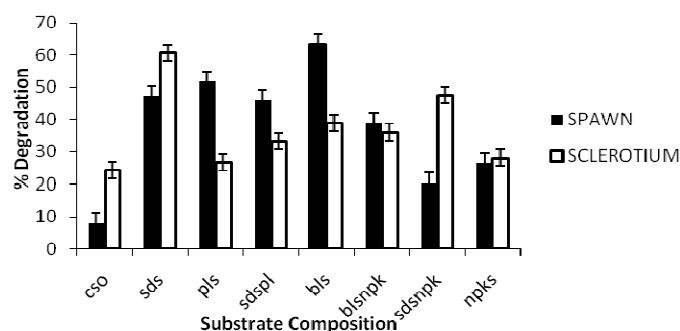
Table 1. Percentage germination (%) for *Vigna unguiculata* grown in soil remediated with *P. tuber-regium*.

Treatment	Inocula option	
	Spawn	Sclerotium
Control A (contaminated)	23.30±0.29 ^a	23.30±0.29 ^a
Control B (no contamination)	90.00±0.50 ^b	92.10±0.16 ^b
Contaminated soil only	73.30±0.77 ^c	46.70±0.77 ^d
Poultry litter + contaminated soil	56.60±0.85 ^d	76.70±0.29 ^c
Sawdust + poultry litter + contaminated soil	80.00±1.00 ^b	63.30±0.29 ^c
NPK + contaminated soil	00.00±0.00	00.00±0.00
Sawdust + NPK + contaminated soil	13.30±1.16 ^d	10.00±0.50 ^d
Sawdust + contaminated soil	90.00±0.50 ^b	83.3±0.29 ^b
Banana leaf blades + contaminated soil	83.30±1.04 ^b	40.00±0.00 ^d
Banana leaf blades + NPK + contaminated soil	83.30±0.29 ^b	53.3±1.53 ^c

Superscripts with same letters on the same column are not significantly different using LSD and Duncan's multiple range tests.

(Figure 4). There was no appreciable degradation of the asphaltenes in all substrate combinations. Degradation was less than 40% in most treatments. An increase was noticed in the asphaltic fraction in contaminated soil only, sawdust + poultry litter + contaminated soil, banana leaf blades + contaminated soil and NPK + contaminated soil (Figure 5). The negative degradation was highest in contaminated soil only treatments indicating that the addition of plant materials reduces the formation of these metabolites.

Phytoassessment with *V. unguiculata* using germination rates indicates that the treatment of contaminated soils with *P. tuber-regium* using NPK is counterproductive as the substrate will not support plant growth showing there must have been formation of harmful metabolites. The crude oil contamination caused a reduction in the germination of the test plant as it reduced germination from 90% in control B to 23% in control A. The addition of animal manure and co-substrates reduced the toxicity of the contaminated soil as there was improved germination (Table 1). After germination, continued support of plant growth was better in treatments like sawdust + poultry litter + contaminated soil, sawdust + contaminated soil than even the control that had no crude oil contamination indicating that there was improvement of soil nutrient and status (Table 2). There was no growth in NPK + contaminated soil substrates and much reduced growth in sawdust + NPK + contaminated soil. There was no significant difference between control without remediation and banana leaf blades + NPK + contaminated soil treatments with the spawn option (Table 2). Using leaf area as an index for the proof of remediation shows that the substrate sawdust + poultry manure + contaminated soil was the best substrate combination as it had the highest leaf area (Table 3). The NPK combinations were confirmed here as not good for the remediation of soils using the fungus *P. tuber-regium*. The cellulosic wastes

**Figure 4.** Percentage degradation of resins in crude oil contaminated soil by *Pleurotus tuberregium*

were also better than the poultry litter + contaminated soil treatments as leaf area was significantly higher in them (Table 3).

DISCUSSION

The successful growth and reduction of total petroleum in crude oil contaminated soil by *P. tuber-regium* has been reported by Isikhuemhen et al. (2003). This study agrees with them and in addition shows that the fungus is able to degrade effectively both aliphatics and aromatics to varying degrees. The resins and asphaltenes were not appreciably degraded although the fungus was active against them. The addition poultry litter and co-substrates (sawdust and shredded banana leaf blades) improved the degradative potential of the fungus- *P. tuber-regium*. The removal of the four fractions from the contaminated soil matrix was higher in treatments with fertilizers and co-substrates. The combination of the fertilizer and the co-substrates was better as degradation was higher in them

Table 2. Vine height (cm) of *Vigna unguiculata* grown in soil remediated with *P. tuber-regium*.

Treatment	Inocula option	
	Spawn	Sclerotium
Control A (contaminated)	2.05±0.84 ^a	2.05±0.84 ^a
Control B (no contamination)	7.02±0.71 ^b	9.05±0.41 ^e
Contaminated soil only	7.61±1.42 ^b	5.29±1.05 ^c
Poultry litter + contaminated soil	6.82±1.28 ^b	6.34±1.14 ^b
Sawdust + poultry litter + contaminated soil	10.27±1.24 ^e	7.83±0.70 ^b
NPK + contaminated soil	-	-
Sawdust + NPK + contaminated soil	-	0.2±0.032 ^d
Sawdust + contaminated soil	8.58±1.12 ^b	12.44±0.57 ^e
Banana leaf blades + contaminated soil	8.55±2.16 ^b	5.89±1.03 ^c
Banana leaf blades + NPK + contaminated soil	2.20±1.22 ^a	4.75±1.35 ^c

Superscripts with same letters on the same column are not significantly different using LSD and Duncan's multiple range tests.

Table 3. Total leaf area (cm²) of *Vigna unguiculata* grown in soil remediated with *P. tuber-regium*.

Treatment	Inocula option	
	Spawn	Sclerotium
Control A (contaminated)	6.55±0.04 ^a	6.55±0.04 ^a
Control B (no contamination)	51.68±3.08 ^b	55.03±4.42 ^b
Contaminated soil only	21.99±1.46 ^c	15.36±0.69 ^c
Poultry litter + contaminated soil	32.70±2.74 ^d	21.48±1.34 ^c
Sawdust + poultry litter + contaminated soil	48.35±0.08 ^b	68.36±1.47 ^e
NPK + contaminated soil	-	-
Sawdust + NPK + contaminated soil	-	0.35±0.20 ^e
Sawdust + contaminated soil	33.32±1.43 ^d	50.34±2.16 ^b
Banana leaf blades + contaminated soil	52.86±1.07 ^b	27.03±0.24 ^c
Banana leaf blades + NPK + contaminated soil	7.00±2.03 ^a	10.62±2.19 ^a

Superscripts with same letters on the same column are not significantly different using LSD and Duncan's multiple range tests.

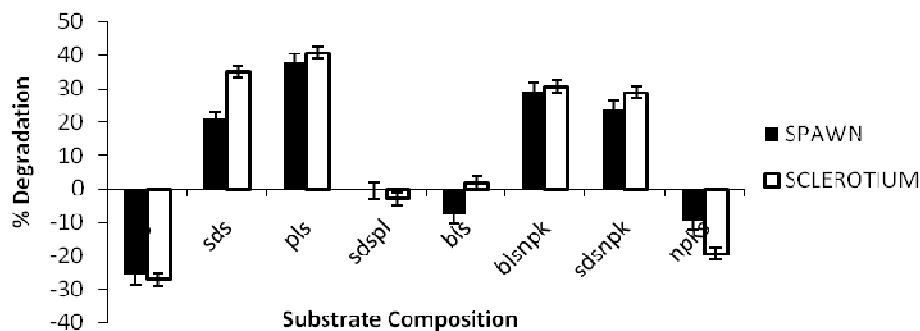


Figure 5. percentage degradation of asphaltene in crude oil contaminated soils by *Pleurotus tuberregium*.

than the contaminated soil only treatments and contaminated soil + fertilizer treatments.

The use of chemical fertilizers for the decontamination

of soils during land-farming processes have been successful and also in the use of plants for remediation purposes in previous researches (Ogbo et al., 2004;

Liebeg and Cutright, 1999; Gogoi et al., 2003; Greer et al., 2003; Molina-Barahona et al., 2005). Minai-Tehrani and Herfatmanesh (2007) recorded reduction in the aliphatic and aromatic fractions with the use of chemical fertilizers for the decontamination of crude oil contaminated soils. This is not the case now with fungal remediation. It recorded a higher level of toxicity compared to the contaminated soil that was not treated with the fungus (Table 1). This shows that there must have been the formation of harmful metabolites or incomplete mineralization which produced more toxic compounds. According to Strauss (1997), this is one of the drawbacks of bioremediation that is the possibility of potentially more harmful compounds been formed. Eggen and Sveum (1999) also reported that NPK did not have a positive effect on the degradation of PAH when they used *Pleurotus ostreatus* for decontamination. This study however agrees with Minai-Tehrani and Herfatmanesh (2007), that animal manure improves the removal of aliphatics and aromatics from crude oil contaminated soils. They also recorded that the combination of the fertilizers with co-substrates (wood chips) was better than the fertilizer alone in the contaminated soil matrix as is shown in this study. This study also agrees with them on the fact that animal manure brought about higher reductions of hydrocarbons than chemical fertilizers. Greer et al. (2003) had previously recorded that the use of fertilizers enhances remediation especially when encouraging indigenous flora during remediation and in their study more aliphatics and aromatics were also removed from the contaminated soil. Chemical fertilizers like NPK have been shown to reduce the growth of *P. tuber regium* in growth studies by Isikhuemhen et al., 1999. They recorded however that a 2% concentration improved the yield hence in this study, 2% NPK application was employed. This was okay for the aromatics as removal was above 50%, but not so for saturates or aliphatics as removal was less than 30% and less for resins with much lower values recorded for asphaltenes. Clearly poultry litter or animal manure is preferred by this fungus than chemical fertilizers. Poultry litter have also been shown to improve the growth of plants in crude oil contaminated soils by Ogbogodo et al. (2004).

The use of organic materials like sawdust, straw as co-substrates has been shown to improve the degradation of hydrocarbons in contaminated soils by fungi (Otte et al., 1999). This explains the success of the fungus in substrates that have sawdust and shredded banana leaf blades in the removal of petroleum hydrocarbons from the soil. The decontamination of crude oil contaminated soils can be best achieved when a combination of animal manure and co-substrates is used.

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