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Studies on Stability of a Six Membered Bacterial Consortium Degrading Amoxicillin

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A B S T R A C T

A six member microbial consortium consisting of six amoxicillin-degrading strains of bacteria was developed and maintained in a fed-batch reactor by feeding 250 mg/l amoxicillin at 28 ± 3°C. The consortium could degrade 95% of 250 mg/l amoxicillin after 24 hours incubation with a biomass increase of 3.5 x 10^7 to 4.6 x 10^8 CFU ml/l. Characterization of the members revealed that it consisted of six bacterium *Streptomyces Steonii, Bacillus subtilis, Pseudomonas aeruginosa, Bacillus cereus, Pseudomonas putida, Klebisella pneumonae*. Amoxicillin degradation by the mixed culture and *Bacillus subtilis*, an isolate from the consortium was compared using a range of amoxicillin concentrations (50 to 250 mg/l). The ability of a large membered microbial consortia to maintain its stability with respect to its composition and effectiveness in amoxicillin degradation indicated its suitability for bioremediation applications.

Introduction

Microbial consortia are considered to have several advantages over a pure cultures, such as greater stability and increased metabolic capabilities for bioremediation applications. These characteristics enable the consortium to overcome limitations for the complete biodegradation of toxic compounds. The degradative efficacy of a microbial community depends on the stability It is important from the perspective of future applications to identify bacteria in the consortium which are responsible for degradation as this information could lead to optimization of degradative processes as well as development of monitoring tools. To date, however, there are only a few reports are available on the nature of defined microbial consortia capable of degrading toxic organic compounds.

Antibiotics have been widely used as an effective class of effective drugs, and their presence has been reported in sewage treatment plant effluent, sewage treatment plant biosolids, surface water, groundwater, and drinking water (Barancheshme and Munir, 2018; Zhang *et al.*, 2018). Such antibiotic contamination has posed a major global

threat. Some scholars claim that future bioremediation work will focus on enzymatic remediation, and biotechnology should be prioritized over chemical treatment to minimize contamination after treatment (Kumar *et al.,* 2019). Microbial consortia showed excellent degradability in studies on the biodegradation of antibiotics. There are many types of antibiotics, and each antibiotic biodegrades in different ways. Some antibiotics are so complex that they require the cooperation of several strains to be completely degraded. Thus, microbial consortia also have advantages in degrading antibiotics.

The objective of the present study was to identify the different species in a large membered degrading consortia and to determine the role that each species plays in the degradative process. Amoxicillin removal efficacy of the microbial consortium was compared with reconstituted mixtures of bacterial isolates from the consortium. The performance of consortium and its best amoxicillin degrading isolate was also evaluated under varing concentrations (50 to 250 mg/l).

Materials and Methods

Isolation

Amoxicillin-degrading consortium was developed and maintained in a fed-batch reactor employing the enrichment-culture technique that described earlier (Ambujom and Manilal, 1995). The reactor was fed daily with enrichment media consisting of (g/l)K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.01;NH_4NO_3, 3.0; $FeSO_4 \cdot 7H_2O$, 0.01; and 1 ml of trace elements solution (containing per liter: $MnSO_4 \cdot H_2O$,0.5 mg; $ZnSO_4$, 0.5 mg; $CuSO_4 \cdot 5H_2O$, 5 µg and $CoCl_2 \cdot 6\ H_2O$, 5 µg) and antibiotic as the sole carbon and energy source. The fed-batch reactor was maintained for 4 years under the following conditions: working volume 14 litre; stirring with air flow greater than 4 Lpm; temperature, 28 ± 3 °C; dissolved oxygen, 1.4 mg/l; pH 6–7; hydraulic retention time, 14 days; dilution factor, 0.04h; influent chemical oxygen demand, 1487 mg/l; effluent chemical oxygen demand, 297 mg/l and the biomass concentration in the range of 2700–3000 mg/l.

Microorganisms and growth conditions

Amoxicillin degrading bacterial strains of the microbial consortium were isolated by plating on antibiotic agar medium consisting of the enrichment media supplemented with 0.5 g/l of antibiotic and 1.5% agar. Non phenol degrading organisms were isolated by the spread plate method on nutrient agar medium. The Biological microbial identification system (Biolog Inc. Hayward, CA. USA) was used to identify the isolates following the procedure described by Klinger *et al.,* (1992).

Inoculum preparation

Individual isolates from the consortium were grown separately in 500 ml Erlenmeyer flasks containing 100 ml enrichment media supplemented with 250 mg/l of amoxicillin for antibiotic degraders. For non-antibiotic degrading isolates, organisms were cultured in Nutrient broth (100 ml). Cells were grown for 72 hours, then separated and washed with phosphate saline buffer solution (NaCl, 8 g//l; K_2HPO_4, 1.21 g/l; KH_2PO_4, 0.3 g/l pH 7.3) by centrifugation at 10,000 g for 15 minutes at 4 °C. The bacterial suspensions (approximately 107 CFU/ml) were aseptically transferred to experimental flasks for further studies.

Quantification of amoxicillin (AC) and Its Sample Pretreatment

AC concentration in soil was detected by HPLC (Hitachi 8DD-0801) with a C18 reversed-phase column (Agilent TC-C18(2) 150 mm × 4.6 mm). Instrument parameter setting, operation, and pretreatment of liquid samples were performed according to the previous study [14]. Besides, soil samples were treated as follows: In a 10 mL centrifuge tube, 5 mL of methanol, and EDTA (1/1,*v*/*v*) buffer were added to 2 g of soil samples, then oscillated at 25 ◦C for 10 min, ultrasonicated

for 10 min and centrifuged at 4500 rpm/min for 10 min. The obtained solid samples were dissolved with 1 mL of methanol and water solution (3/2, *v*/*v*), filtered through a 0.22 µm membrane filter and stored in a 1.5 mL amber vials at -20 ◦C prior to HPLC analysis.

Degradation experiments

The bacterial isolates were inoculated separately or in combination based on their physiological state. Cultures consisting of 2 members, 3 members and 4 members were inoculated into 500 ml Erlenmeyer flasks containing 100 ml enrichment media added with 50, 150, 200 and 250 mg/l antibiotic as sole carbon source. Concentration of inoculum used in each flask was made up to 0.6% (w/v) with all strains. Inoculated flasks were incubated on an orbital shaker adjusted to 200 rpm at 28 ± 3 °C. Data presented herein are the averages of triplicates. The concentration of antibiotic was successively quantified via HPLC at different cultivation timing (i.e., 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 10th, 12th, 14th day), and optical density (OD) values were also recorded at 600 nm to investigate the bacterial concentration. After comparing the antibiotic degradation efficiency and growth of each bacterial consortium, the best antibiotic-degrading bacterial consortium was selected for subsequent experiments.

Results and Discussion

Characteristics of microbial consortium

The microbial consortium enriched on 250 mg/l of antibiotic consisted of six bacterial degraders (nominate 1 to 6). The composition of the microbial consortium remained stable under a variety of incubation conditions and retained the ability to degrade antibiotic. The identities of the bacterial isolates as determined using the Biolog system, are given in Table 1. Different trophic groups in the bacterial consortia were evaluated by growing isolates of consortium in the presence or absence of antibiotic as the sole carbon source.

AC degradation by individual members of the consortium

Over a 24 hour growth period on antibiotic, isolate 2 had a faster growth rate compared to other antibiotic degraders: microbial numbers increased from an initial concentration of 3.5 x $10^7$to 4.6 x 10^8CFU/ml (Table 2). Rate of AC degradation was varied with each individual member of the consortium in shaken flasks containing 250 mg/l antibiotic as shown in Fig. 1 and 2. The amount of AC degraded by isolate 2 (*Bacillus subtilis*) was greater (205.6 mg/l) than all the experimented isolates. The degradative performances of individual isolates were also varied when assessed in samples containing range of antibiotic feed ranging from 50 to 250 mg/l (Fig. 3). The isolate 4 and 5 showed an increase in degradation with an elevated concentration of antibiotic (Table 3 and 4), but most of the other cultures failed to attain higher rate beyond antibiotic concentration.

AC degradation by reconstituted mixtures of bacterial isolates

Table 5 and Figure 4 shows the AC degradation pattern of the consortium and reconstituted mixtures of its isolates during different period of incubation. there are combination of bacterial consortia (named as A, B, C and D) 1 member (*Bacillus subtilis*), 2 members (*Bacillus cereus, Bacillus subtilis*), 3 members (*Streptomyces Steonii, Pseudomonas aeruginosa, Bacillus cereus, Pseudomonas putida*) and 4 members (*Bacillus subtilis, Bacillus cereus, Pseudomonas putida, Klebisella pneumonae*) used in the present study were enriched on the basis of their ability to degrade AC, with all combination included the best antibiotic degrading 4 member of the community.

About 240.4.5 mg/l of 250 mg/l antibiotic was degraded by the 4 member consortium while the 2 member consortia could degrade only 220.2 mg/l during 24 hours of incubation. Degradation activity increased gradually with an increase in the number of members.

Table.1 Morphological and biochemical characteristics of members of the consortium

Test	1	2	3	4	5	6
Colony Morphology	Discrete	Circular	Discrete	Circular	Circular	Ireegular
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Gram reaction	-	+	-	+	-	-
Motility	+	+	-	+	+	+
Catalase	+	+	+	+	+	+
Urease	+	+	-	+	+	-
Oxidase	+	-	-	-	+	-
Isolate name	***Streptomyces Steonii***	***Bacillus subtilis***	***Pseudomonas Aeruginosa***	***Bacillus cereus***	***Pseudomonas putida***	***Klebisellapneumonae***

Table.2 Profile of growth of bacterial isolates of the consortium at 250 mg/l antibiotic during 24 hours of incubation

Strain	Initial concentration of CFU/ml	Final concentration of CFU/ml
1	4×10^7	5×10^7
2	3.5×10^7	4.6×10^8
3	3.1×10^7	4.2×10^7
4	3×10^7	4×10^7
5	3.1×10^7	4.6×10^7
6	3.5×10^7	4.3×10^7

Table.3 Antibiotic degraded by individual members of consortium at 250 mg/l during 24 hours of incubation

Time (h)	Isolate-1	Isolate-2	Isolate-3	Isolate-4	Isolate-5	Isolate-6
04	100.5	115.5	90.5	105.7	105.7	99.7
08	140.4	170.4	125.7	120.3	130.3	120.3
16	150.4	185.6	140.6	138.4	144.4	139.4
24	160.2	205.6	150.3	155.2	165.2	162.2

Table.4 AC degraded by individual members of consortium at varying concentration

Conc.	Isolate-1	Isolate-2	Isolate-3	Isolate-4	Isolate-5	Isolate-6
50	110.4	115.5	110.5	105.7	106.3	105.4
100	130.2	140.4	145.7	160.3	125.4	120.2
150	180.4	190.6	180.6	188.4	140.4	155.4
200	200.2	225.6	210.3	205.2	172.3	184.3
250	260.3	350.6	270.4	250.3	215.2	218.3

Table.5 AC degraded by various consortia reconstituted with isolated members of microbial consortium

Conc.	A	B	C	D
50	100.0	102.3	100.6	104.5
150	130.3	128.4	130.4	140.3
200	184.3	170.5	180.6	190.9
250	215.1	220.2	225.5	240.4

Fig.1 Amoxicillin degraded by individual members of consortium at 250 mg/l during 24 hours of incubation

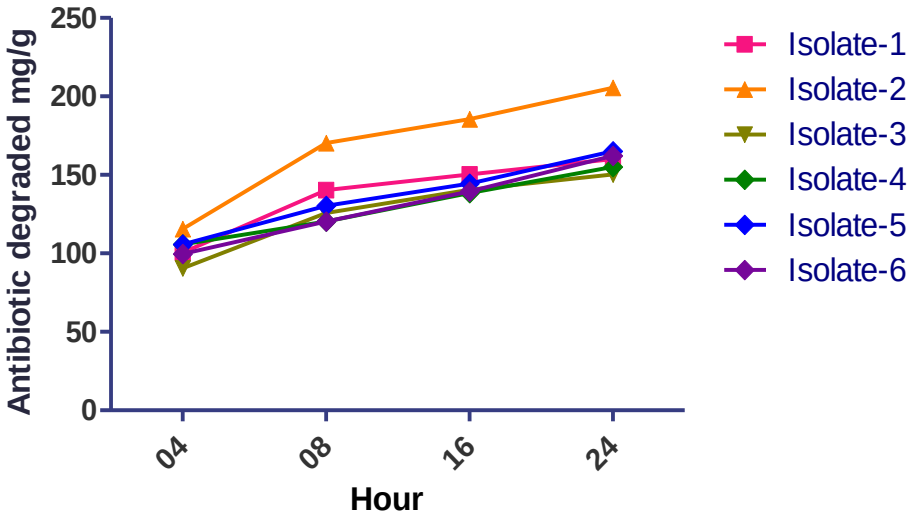


Fig.2 Amoxicillin chromatogram

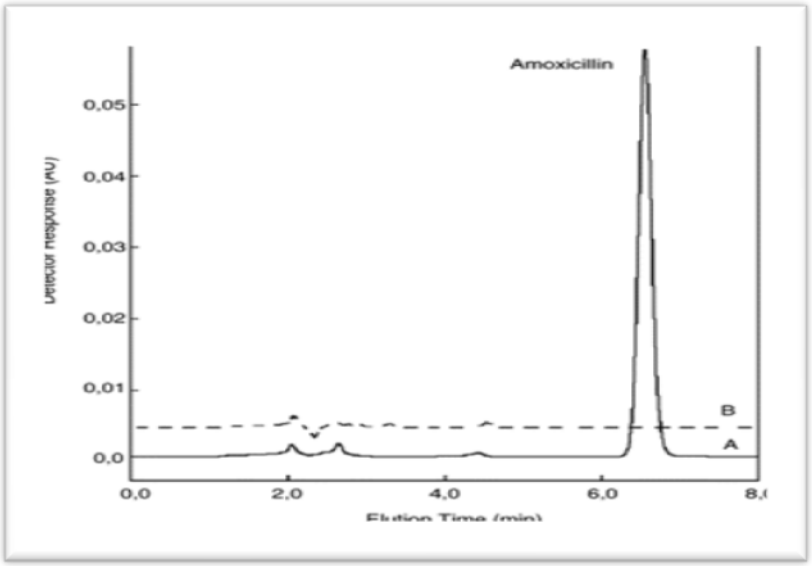


Fig.3 AC degraded by individual members of consortium at varying initial concentration during 24 hours of incubation.

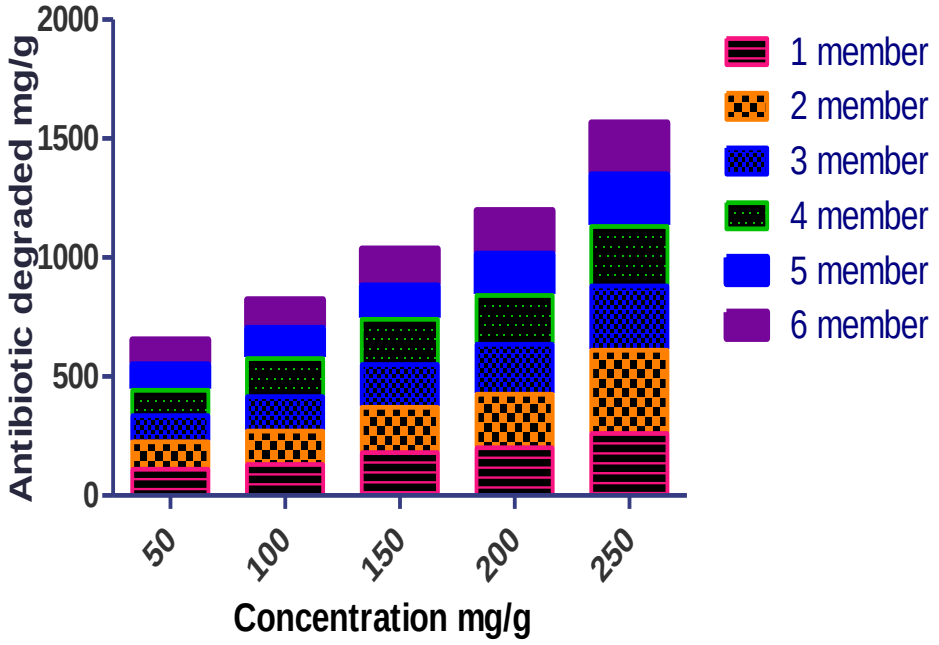
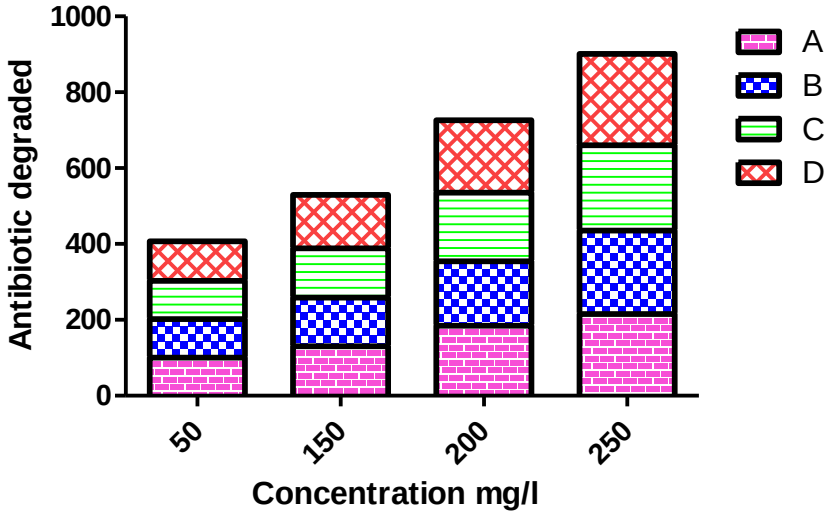


Fig.4 AC degraded by various consortia reconstituted with isolated members



Results of the batch experiments conducted with various reconstituted consortia at varying initial concentration of antibiotic are presented in Fig. 4. The amount of AC degraded by the consortium was 104.5, 140.3, 190.9and 240.4 mg at antibiotic concentration of 50, 150, 200 and 250 mg/l respectively. In contrast, single member system could degrade smaller amounts of antibiotic (100,

130.3, 184.3 and 215.1mg/l) over a 24 hour period when it was supplied at 50, 150, 200 and 250 mg/l AC respectively.

Many studies have shown that it is difficult to achieve the complete degradation of pollutants by a single strain. As different strains have different metabolic pathways, the bacteria with different removal abilities are mixed, and the microbial consortium can integrate each strain's advantages to achieve the efficient degradation of pollutants.

Mixed microbial consortia exhibited good performance in substrate tolerance and enhanced pollutant degradation (Bhatt *et al.,* 2021; Varjani *et al.,* 2021; Kang *et al.,* 2021; Ali *et al.,* 2020; Vieira *et al.,* 2021; Liu *et al.,* 2021; Abou Khalil *et al.,* 2021). Compared to the culture of a single strain, the performance of the consortium of microorganisms is better. The microbial consortium showed apparent effects in the degradation of pollutants (Bhatt *et al.,* 2021).

Some existing microbial strains isolated from the intestinal flora and natural floras have the inherent ability to degrade pollutants (Bhatt *et al.,* 2021; Liu *et al.,* 2021). *Lactobacilli, Actinobacteria, Pseudomonas, Clostridium, Salmonella,* and *Escherichia coli* have been found to have the inherent ability to degrade pollutants. These strains are suitable for the bioremediation of pollutants (Bhatt *et al.,* 2021).

The microbial consortium has become an important technology because it degrades pollutants more effectively than a single strain (Bhatt *et al.,* 2021). Bioremediation is usually carried out by the microbial consortium rather than by individual species in the natural environment, and different strains or species play different functional roles (Zhang *et al.,* 2021).

The co-cultivation of the microbial consortium is more effective than single bacteria, degrades pollutants faster, and can significantly enhance the biodegradation of pollutants in the soil.

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