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RESEARCH ARTICLE

Genetic improvement of Kraft lignin degrading bacteria *via* mutation induction and protoplast fusion

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Abstract

Pulp and paper manufacturing wastes have been recognized as a significant point source of pollution since they have high levels of lignins and their derivates. Wild type *Bacillus subtilis subsp. Subtilis* and *Citrobacter farmeri* isolated from the pulp and paper manufacturing effluent contaminated soil and water were found to be effective towards lignin degradation. Mutant strains were obtained from the acclimatized species by random mutagenesis using various Ethidium Bromide (EthBr) and the physical mutagen Ultra Violet (UV) and after that mutant strains were tested for lignin degradation. It was found that the maximum degradation rates was achieved by treatment with 100 µg EthBr for strain *B. subtilis* and 150 µg EthBr for strain *C. farmeri*. Genetic Improvement of lignin degradation by these mutant strains was carried out via Protoplast Fusion aiming to isolate new recombinants (fusants) with high capacity of lignin degradation.

Introduction

The environmental pollution due to small and medium scale pulp and paper industries is multidimensional causing serious problems not only to the land mass fertility but also to the natural flora, fauna as well as the aquatic bodies, since they have a high biochemical oxygen demand (BOD), chemical oxygen demand (COD), phenol compounds, high concentrations of chlorinated compounds, suspended solids, fatty acids, tannins, resin acids, lignin and their derivates, sulphur and sulphur compounds, etc. causing brown colour (Ali M. and Sreekrishnan, 2001; Gürs *et al.*, 2002). They block the passage of light to the lower depths of the aquatic system resulting in cessation of photosynthesis, leading to anaerobic conditions, which in turn result in the death of aquatic life causing foul smelling toxic waters (Chandralata *et al.*, 2008).

In biological treatment systems a wide variety of microorganisms including fungi, actinomycetes and unicellular bacteria have been implicated in lignin biodegradation and decolourisation of pulping effluent (Yang *et al.*, 2007). Bacteria, in particular, deserve to be studied for ligninolytic potential because of their immense environmental adaptability and biochemical versatility. In addition the application of fungi in bioleaching of raw pulp is not feasible due to its structure hindrance caused by fungal filament .Several studies have investigated biological treatment of black liquor by using various pure bacterial strains (Chandra *et al.*, 2007)**.**

In recent years, considerable success in studying molecular mechanisms of bacterial mutagenesis induced by physical and chemical factors has been achieved. It has been shown that a key role in this process belongs to the genes which control DNA synthesis on a matrix containing damaged bases (Oleg *et al.*, 2009) Genome shuffling is a recently introduced method that is much more efficient for evolution of strains of microbes with desirable phenotypes. It involves generation of a heterogeneous population of mutants by treatment with a chemical or physical mutagen, followed by recursive fusion of protoplasts to allow recombination. This procedure allows rapid evolution of strains with multiple beneficial mutations (MingHua *et al.*, 2005)

2. MATERIALS AND METHODS:

2.1. Media composition:

Alkali Kraft Lignin (KL) degrading bacteria *Bacillus subtilis subsp. Subtilis* and *Citrobacter farmeri* were isolated from water, soil and the effluent sludge by enrichment culture technique (Morii *et al.* 1995).

2.2. Genetic improvement of the bacterial strains for lignin degradability:

2.2.1. Generation of mutants from wild type isolates:

a) Chemical mutation by Ethidium Bromide (EthBr):

Bacteria were grown in LB medium overnight at 30 ºC. The culture were then centrifuged (5000 rpm, 10 min), and the resulted pellets were re-suspended in solution of 3% NaCl (w/v). Various Ethidium Bromide (EthBr) concentrations (50, 100, 150, 200 µg/ml) were added to bacterial cells. After the examined exposure period (1h), cells were pelleted again, washed by phosphate buffer saline at pH 6.8 and re-suspended in an equal volume of MSM medium , then exposed and control (non-exposed) cell suspensions were diluted and were spread on L-MSM agar containing 1000 mg lignin /l and incubated for six days at 30ºC. After that, colonies were counted and Survival rate was calculated according to Equation (1):

$$\text{Survival rate} = \frac{\text{Survival colonies}}{\text{Total colonies under the control culture}} \times 100\% \qquad (1)$$

The survived cells were then selected and cultivated on LB broth (Katrcolul *et al.*, 2003).

b) Physical mutation by UV:

Bacteria were grown in LB medium overnight at 30 ºC. The culture were then centrifuged (5000 rpm, 10 min), and the resulted pellets were re-suspended in an equal volume of MSM medium. Five ml aliquots from the suspension were then transferred into a polyethylene dish and subjected to UV irradiation at 312 nm in a UV chamber for different times intervals (3, 5, 10, 15 minutes) keeping the distance of the UV source fixed to 20 cm. All these manipulations were carried out in the dark room to avoid any possible photoreaction. After mutagenesis, exposed and control (non-exposed) cell suspensions were serially diluted and were spread on L-MSM agar containing 1000 mg lignin /l and incubated for six days at 30ºC. After that, colonies were counted and Survival rate was calculated according to Equation (1). The survived cells were then selected and cultivated on LB broth medium (Czyz *et al.*, 2000).

2.2.2. Studying the effect of mutation on degradation rates:

Biodegradation experiments were carried out in 100 ml flask containing 50 ml sterile L-MSM (1000 mg KL/ l) at pH 7.6. Culture suspension having an inoculum size (CFU)/ml 105×10^4 of mutants were inoculated into triplicate flasks and were incubated for six days on rotary shaker under aerobic conditions at 30 °C and 120 rpm. Uninoculated medium was used as control in all cases. Samples were withdrawn and analyzed for bacterium growth, reduction of colour and residual KL content.

2.2.2.1. Bacterium growth:

Cell growth was determined by measuring absorbance of inoculated sample at 620 nm (A_{620}) on spectrophotometer **(UV-visible Cintra 40-GBC)** using uninoculated medium as blank.

2.2.2.2. Colour reduction:

The colour reduction of the effluent was determined according to Morii *et al.* (1995).

2.2.2.3. Lignin content:

For the estimation of residual lignin, centrifuged supernatants from control and inoculated were acidified with 12 M HCl to pH 1–2 and then centrifuged at 12,000g for 10 min. Residual KL was obtained after each precipitate had been washed with de-ionised water and dried at 60 °C for 48 h and weighed (Anthony *et al.*, 1986). Kraft lignin loss (%) in the supernatants decolourised by the strain was determined as dry weight (estimating 100% as the KL present in the same volume of uninoculated medium).

2.2.3. Antibiotic resistance:

Each mutant was grown at 30 °C for 48 h on LB agar, LB agar containing Ampicillin (500 µg/ml), LB agar containing Cephalexin (250 µg/ml) and LB agar containing Chloramphenicol (250 µg/ml) separately as markers.

2.2.4. Protoplast fusion:

a) Protoplast formation:

Protoplasts were formed according to method of Weiss (1976).

b) Protoplasts fusion:

Protoplasts were fused using method of Dai and Copley (2004).

c) Protoplast regeneration:

The cells were re-suspended in 0.5 ml LB medium containing 0.5M sucrose and serial dilutions were immediately spread on soft agar LB plates containing 8 g/l agar and 0.5M sucrose using a plastic spreader. After growth on these plates, individual colonies were counted and patched onto plates containing LB agar, Chloramphenicol (250 µg/ml) and Cephalexin (250 µg/ml) as markers to fusants. Colonies growing on these plates were obtained after growth at 30 °C for 48 h, counted and patched onto the same plates twice more to confirm their ability to grow in the presence of the tow antibiotics. Control experiments were carried out using the above procedures but without either addition of PEG6000 or mixing of the protoplasts formed from the two mutants. Fusant ratio was calculated according to Equation 2:

$$\text{Fusant } \% = (X / Y) \times 100\% \qquad (2)$$

Where Y is colonies on soft agar LB plates containing 0.5M sucrose, and X is colonies on soft agar LB plates containing 0.5M sucrose, cephalexin and chloramphenicol.

After that, fusants were selected and cultivated on LB broth to study their ability for biodegradation of Kraft lignin.

2.2.5. Studying the biodegradability of fusants:

Biodegradation experiments were carried out as previously described using fusants.

3. RESULTS AND DISCUSSION:

3.1. Chemical and physical mutation:

3.1.1. Dose–response analysis:

It was clear from **Table 1** that, the survival rates of bacterial strains were decreased with increment of EthBr concentration. Also the same results were obtained with the treatment with UV where the survival rate was declined after exposing the bacterial strains to UV for more time.

Table 1: Survival rates % after treatment the bacterial strains with EthBr and with UV

Survival rate% / Strains	Ethidium Bromide Concentration (µg/ml)					UV Exposure time (min)			
	Control	50 µg	100 µg	150 µg	200 µg	3 min	5 min	10 min	15 min
B. subtilis subsp. Subtilis	100.0	64.2	42.1	34.7	15.8	50.0	27.6	21.8	11.6
C. farmeri	100.0	60.6	37.0	28.3	21.9	54.9	43.1	20.2	12.8

3.2. Studying the effect of mutation on degradation rates:

3.2.1. Cell growth:

The maximum OD was obtained by the treatment with EthBr concentration 100 µg for strain *Bacillus subtilis* and with concentration 150 µg for strain *Citrobacter farmeri*. The maximum OD was obtained after exposing time 15 minutes to UV for the bacterial strain *B. subtilis* and after 5 minutes for strain *C. farmeri*, these results were more than OD of wild types **(Tables 2).**

Table 2: The effect of EthBr concentration and UV on cell growth of the bacterial mutant strains

Optical Density / Strains	Ethidium Bromide Concentration (µg/ml)					UV Exposure time (min)			
	WT	50 µg	100 µg	150 µg	200 µg	3 min	5 min	10 min	15 min
B. subtilis subsp. Subtilis	0.283	0.113	0.406	0.254	0.095	0.260	0.241	0.237	0.363
C. farmeri	0.262	0.325	0.345	0.370	0.352	0.342	0.365	0.335	0.219

3.2.2. Colour reduction:

Colour reduction was increased by treatment *B. subtilis* with 100 µg EthBr and by treatment *C. farmeri* with the four concentrations of EthBr. It was also increased after exposing *B. subtilis* to UV for 15 minutes and *C. farmeri* for 5, 10, 15 minutes as shown in **Tables 3**.

Table 3: The effect of EthBr concentration and UV on lignin colour reduction % by mutant strains

Colour reduction% / Strains	Ethidium Bromide Concentration (µg/ml)					UV Exposure time (min)			
	WT	50 µg	100 µg	150 µg	200 µg	3 min	5 min	10 min	15 min
B. subtilis subsp. Subtilis	85.4	40.3	88.7	68.2	27.9	82.8	78.3	79.4	88.2
C. farmeri	74.4	81.4	82.9	89.8	82.5	82.8	87.3	82.8	57.7

3.2.3. Lignin degradation:

Lignin degradation was increased by treatment *B. subtilis* with 100 µg EthBr and by treatment *C. farmeri* with the four concentrations of EthBr. It was also increased after exposing *B. subtilis* to UV for 15 minutes and *Citrobacter farmeri* for 5, 10, 15 minutes **(Tables 4).**

Table 4: The effect of EthBr concentration and UV on lignin degradation% by mutant strains

Lignin degradation% / Strains	Ethidium Bromide Concentration (µg/ml)					UV Exposure time (min)			
	WT	50 µg	100 µg	150 µg	200 µg	3 min	5 min	10 min	15 min
B. subtilis subsp. Subtilis	71.0	31.5	76.1	57.4	19.8	69.8	65.5	66.4	75.0
C. farmeri	62.1	68.8	69.0	76.7	69.0	69.3	74.0	69.5	51.0

3.3. Antibiotic resistance:

The antibiotic resistance data in **Table 5** show that *B. subtilis* mutated with 100 µg EthBr was resistant to ampicillin and chloramphenicol but sensitive to cephalexin, and *C. farmeri* mutated with 150 µg EthBr was resistant to ampicillin and cephalexin but sensitive to chloramphenicol so, chloramphenicol resistance trait can be used as a marker for *B. subtilis* mutant strain, cephalexin resistance trait can be used as a marker to *C. farmeri* mutant strain, and the two antibiotic can be used as marker to fusant strain resulted from protoplast fusion of the two mutant strains.

Table 5: Antibiotic resistance by *Bacillus subtilis* and *Citrobacter farmeri* mutant strains

Mutant	Antibiotic		
	Ampicillin	Cephalexin	Chloramphenicol
Bacillus subtilis (100 µg EthBr)	+	-	+
Citrobacter farmeri (150 µg EthBr)	+	+	-
Fusant strain	+	+	+

(+) is Resistant and (-) is Sensitive.

3.4. Protoplast fusion:

Genetic Improvement of lignin degradation by mutant strains *B. subtilis* mutated with 100 µg EthBr (chloramphenicol resistant) and *C. farmeri* mutated with 150 µg EthBr (cephalexin resistant), was carried out via Protoplast Fusion aiming to isolate new recombinants (fusants) with high capacity of lignin degradation. Recombination between the genomes of these two strains can lead to fusant strain that grow in the presence of both chloramphenicol and cephalexin (**Table 5**). Protoplast formation ratio was 93% for *B. subtilis* mutant strain and 97% for *C. farmeri* mutant strain**.** After protoplast fusion and regeneration of fusants, fusant ratio was 37% this means that only 37% of protoplasts could be fused and grown in the presence of the two antibiotics because the fusant strain has the antibiotic resistance traits of the two mutants.

3.5. Studying the biodegradability of fusants:

After incubation of the fusant strain at 30 °C and 120 rpm for six days using 1000 mg lignin/l, samples were withdrawn and analysed for the bacterial cell growth, colour reduction and lignin degradation **(Table 6)**. Fusant strain had OD more than parents where it increased from 0.406 and 0.370 to 0.473, colour reduction was enhanced from 88.7, 89.8% for parents to 95.2% for fusant, and lignin degradation was increased from 76.1, 76.6 to 83.2%. Accordingly, the protoplast fusion enhanced growth vigor. In addition, this growth pattern indirectly indicated the enhancement of lignin degradation; this could be directly related to strains improvement.

Table 6: Biodegradation assays by mutant and fusant strains

Strain	OD	Colour reduction %	Lignin degradation %
Bacillus subtilis (mutant strain)	0.406	88.7	76.1
Citrobacter farmeri (mutant strain)	0.370	89.8	76.7
Fusant strain	0.473	95.2	83.2

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