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Full Length Research Paper

Isolation of proteolytic, lipolytic, and bioemulsifying bacteria for improvement of the aerobic treatment of poultry processing wastewater

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Proteolysis, lipolysis, as well as, emulsification play important roles for the biological treatment of poultry processing wastewater. Three isolates identified by 16S rRNA gene sequence analysis as belonging to *Bacillus subtilis*, *Bacillus thuringiensis,* and *Lysinibacillus fusiformis*, were then selected. The three combined strains provided a 71.2 ± 0.20% reduction of the chemical oxygen demand (COD) and produced a high biosurfactant 1250 ± 10 mg/L. Moreover, all three bacteria were able to form good biofilm. The combined bacteria strains performed well in terms of COD reduction, protease, and lipase and biosurfactant production at an initial pH of 7.0 and over a wide range (1000 to 5000 mg/L) of organic loads.

Key words: Proteolytic, lipolytic, poultry, wastewater, treatment, biosurfactant.

INTRODUCTION

Poultry processing is one of the major food industries in Thailand. Besides serving local demand, this industry exports chicken products world wide (Sirianuntapiboon and Manoonpong, 2001). Poultry slaughterhouses generate wastewater mainly from the slaughtering process, equipment and washing facilities as well as, from product production. This wastewater is characterized by large quantities of suspended solids, oil and grease, nitrogen and phosphorus, which may vary, depending on the process (Del and Damianovic, 2001).

Biological treatment is one of the best options to remove organic material from such wastewater. Aerobic processes such as activated sludge, rotating biological contactor, trickling filter, and lagoons are suitable for organics removal (Chowdhury et al., 2010). Organic wastewaters containing lipids and proteins are difficult to degrade. In case of lipid, the application of solubilizing agents may be required. Degradation of indigenous microorganisms present in these conventional systems may not be adequate to acquire efficient and reliable treatments (Loperana et al., 2009). Effective microorganisms and their hydrolytic enzymes are sometime applied to enhance treatment efficiency (Masse et al., 2001). Bioaugmentation with indigenous organisms display several advantages over non indigenous organism including; different specific growth rapidity, and ability for a wider number of metabolic pathways (Loperena et al., 2009).

The objectives of this research were to select a combination of bacteria isolated from a poultry processing waste treatment system for enhancing bioaugmentation of such wastewater. A chicken broth production process was selected to represent the poultry processing source of wastewater for this study. Moreover, pH and organic loads affecting proteolytic, lipolytic and emulsifying

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activities of the mixed bacterial inoculant were also investigated.

METHODOLOGY

Samples

Samples of bacteria were collected from the effluent treatment process of an industrial chicken broth plant. Samples were collected throughout the treatment process beginning with raw effluent, followed sequentially by dissolved air flotation (DAF), and sludge from aeration pond and finally a sediment pond. Samples were measured for COD, total solids (TS), total lipids, suspended solids (SS) and total kjeldahl nitrogen (TKN).

Method of bacterial isolation (adapted from Loperana et al., 2009)

Bacterial were isolated from wastewater sample in flask containing 150 ml of tryptone soy broth (TSB, Difco, USA) by shaken at 200 rpm (30°C) for 48 h. Thereafter, the sample was serially diluted, plated onto tryptone soy agar (TSA, Difco, USA) and incubated at 30°C for 96 h. Each bacterial colony was purified and collected according to colony morphology and gram staining. Particles in samples were allowed to settle for 30 min and 30 ml removed and added to 100 ml of mineral medium containing 2 ml/L of chicken oil as a sole carbon and energy source. The mineral medium consisted of NH_4Cl 0.57 g/L, KH_2PO_4 0.43 g/L, K_2HPO_4 1.09 g/L, Na_2HPO_4 1.33 g/L, $MgSO_4.7H_2O$ 0.023 g/L, $CaCl_2$ 0.028 and $FeCl_3.6H_2O$ 0.025 g/L, as described by Saravia et al. (2004). After shaking for 12 h at 200 rpm (30°C) bacterial colonies were collected as described earlier. Bacteria in activated sludge from aerobic pond were increased by shaking in 100 ml of wastewater for 48 h to reduce COD by 70 to 80% before they were isolated and collected.

Screening for proteolytic, lipolytic and emulsifying activities

Proteolytic activity of each bacterial isolate was detected by clear zone formation on M1 agar plate containing skim milk (Loperana, et al., 2009). Lipolytic activity was determined in Rhodamine B agar with 10 ml/L of chicken oil (Gisela and Karl-Erich, 1987) to which was added 10 µl of culture supernatant from a nutrient broth after incubation at 37°C for 24 h. The plate was observed under an UV transilluminator (UVP-M-26, USA) for production of lipase. Emulsifying activity was determined with a modification of a method by Grazyna et al. (2006). Seven milliliters of the supernatant culture in a basal medium were covered with 3 ml kerosene in a test tube. This tube was shaken for 1 min by a vortex mixer. Emulsion stability was measured after 24 h and emulsification index (EI 24) was computed.

Determining biodegradation efficiency and biofilm formation ability

Reduction of COD by bacterial isolates with proteolytic, lipolytic or emulsifying activity was measured for the chicken broth wastewater. Individual or combinations of the inoculums were prepared by cultivating each bacterial isolate in TSB for 8 to 12 h at 30°C and then added to sterile wastewater and shaken for another 8 to 12 h. This culture was adjusted with sterile water and optical density adjusted to 0.2 at 600 nm. Finally, 2 ml of adjusted culture solution was transferred 100 ml of sterile chicken broth wastewater (Yezza et al., 2006) and incubated at 30°C in a rotary shaker (200 rpm) for 48 h after which it was centrifuged at 9000 rpm for 15 min. COD was measured in triplicate from the supernatant and subtracted from the initial to give a residual COD.

Biofilm formed by each isolate was tested after decanting the residual solution according to the method of Maldonado et al. (2007). Biofilms were cultured in Luria Bertoni broth (LB, Difco, USA) for 18 h at 30°C and optical density adjusted to 0.56 to 0.64 at 540 nm. An aliquot (200 µl) was incubated for 6 h at 30°C next, 25 µl of 1% crystal violet was added to each well, mixed and left for 15 min. Planktonic cells and unstained dye were removed from the biofilm by washing with 200 µl sterile phosphate buffered saline (PBS) and twice more with 200 µl of 95% ethanol. An aliquot (400 µl) was removed and added to 1.2 ml of 95% ethanol and absorbance measured at 540 nm.

Effects of pH and organic load

Bacteria were cultured either in wastewater of a constant COD, 1160 ± 14 mg/L, in relation to pHs of 5 to 8 or to wastewater of varying COD, 1000 to 5000 mg/L. pH was adjusted by adding 1 N NaOH or 1 N HCl. COD was adjusted by dilutions of concentrated chicken broth. Bacteria were incubated in the various experimental wastewater solutions for 48 h at 30°C, and supernatants centrifuged at 9000 rpm for 15 min after which COD, proteolytic and lipolytic activities, and biosurfactant were measured.

Analytical methods

The physiochemical characteristics of the wastewater were determined using standard methods of APHA (1995). Biosurfactant production was determined gravimetrically after drying acid precipitation of culture supernatant (Mukherjee et al., 2009). Protease activity was quantified using Azocasein as substrate, according to the method of Park et al. (2003). Lipase activity was determined by a colorimetric method based on cleavage of *p*- nitrophenylpalmitate (*p*-NPP) according to a method of Gaelle and Jacques (1996).

Bacteria were identified by genomic DNA extraction with a nucleic acid extraction kit GF-1 (Vivantis Company, GF-1 Bacterial DNA extraction).

Universal bacterial primers corresponding to *Escherichia coli* position 27 F and 1492 R was used for PCR amplification of 16S rRNA genes. The sequences were analyzed for homology of 16s rRNA gene by alignment with databases of other organisms in GenBank and determined to insistence using BioEdit version 7.00 (Hall, 1999) and BLAST (Altschul et al., 1990).

Statistical analysis

Each experiment was performed in triplicate and the results are presented as the mean value of three replicates. One way analysis of variance (ANOVA) was used to test for statistical significance among treatments. Statistical significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Screening and selection of bacteria

COD, TS and SS of untreated and treated wastewater were 1840 ± 10, 1260 ± 10 and 130 ± 3 mg/L, respectively (Table 1). Untreated wastewater was above concentrations considered acceptable by pollution control

Table 1. Characterization of wastewater and sludge samples used for isolation of bacteria.

Parameter source	COD (mg/L)	TS (mg/L)	Lipid (mg/L)	SS (mg/L)	TKN (mg/L)
Raw wastewater	1840±10.0	1260±10.0	285±4.0	130±3.0	178.1±2.95
DAF	4700±10.0	1000±30.0	378±3.0	60±1.0	163.1±4.05
Activated sludge	9500±20.0	6970±10.0	489±2.0	4440±10.0	508.2±3.95
Sedimentation pond	5000±30.0	1870±20.0	395±3.0	660±5.0	680.4±3.20

Table 2. Number positive of bacterial isolates producing one or more of proteolytic enzymes (P), lipolytic enzymes (L), or emulsifying compounds (E) in the screening test. A skim milk agar plate was used to determine presence of proteolytic enzymes. A rhodamine agar was used to determined presence lipolytic enzyme. Emulsification index at 24 h was used to determined presence emulsifying compounds.

Source	Isolate tested	Activity							Total positive isolates
		P	L	E	P+L	P+E	L+E	P+L+E	
Poultry processing wastewater	14	1	0	0	2	2	0	0	5
DAF	8	0	1	3	2	0	0	2	8
Activated sludge	14	0	4	2	2	3	0	0	11
Sedimentation pond	5	0	1	1	0	2	0	0	4
Enrichment culture	3	0	0	0	1	0	0	0	1
Total	44	1	6	6	7	7	0	2	29

Table 3. Accession numbers of bacterial isolates, their COD reduction efficiencies, expressed as a % of initial and biofilm formation, expressed in optical density units. All values are mean ± S.D. similarities of bacterial strain to closest related species are given as percentages.

Isolate No.	Accession number	Name of closest related species (accession number)	Similarity (%)	COD removal after 48 h (%)	Biofilm formation ($O.D._{540}$)
27	HM047298.1	*Bacillus thuringiensis* strain ZJU03	99	67.5 ± 0.2	0.74 ± 0.008
39	FJ641029.1	*Lysinibacillus fusiformis* IMAUB 1017	98	57.6 ± 0.2	0.96 ± 0.009
43	HM027569.1	*Bacillus subtilis* zj2008	99	43.7 ± 0.1	2.22 ± 0.005

department of Thailand.

A total of 44 bacterial isolates were collected from all sample sources based on colony morphology and microscopic characteristic. After screening for proteolytic, lipolytic and emulsifying activities, 29 isolates contained at least one activity, 14 contained 2 activities and 2 contained 3 activities. Among these positive isolates; 15 contained lipase activity, 17 contained protease activity, and 16 contained emulsifying activity (Table 2).

Isolates were selected for further studies based on activity levels. From the screening tests, three isolates numbers 27, 43 and 39 were selected for further studies with first two isolates (numbers 27 and 43) having relatively high proteolytic and lipolytic activities and one (number 39) with a high emulsifying and proteolytic activity. These isolates were identified as *B. thuringiensis* (27), *L. fusiformis* (39) and *B. subtilis* (43), respectively (Table 3). The 48 h COD reduction efficiencies for chicken broth wastewater of each strain were 67.5 ± 0.2, 57.6± 0.2, and 43.7 ± 0.1%, respectively.

The isolation of *Bacillus* and *Lysinibacillus* from a wastewater treatment system is not surprising since they are ubiquitous and diverse in soil and environment. These genera can catabolize various natural and xenobiotic compounds (Wu et al., 2009). *Lysinibacillus* spp. is able also to survive under extremely harsh conditions, which make them exemplary candidates for bioremediation of contaminated environment (Peng et al., 2009). The strains selected in this study possess proteolytic and/or lipolytic enzymes which account for COD reduction of the chicken broth wastewater. Moreover, *L. fusiformis* produced biosurfactants with emulsifying activity which may also contribute to COD reduction.

Biosurfactant production has been reported for *Bacillus* and *Lysinibacillus*. Josic et al. (2008) found a *L. fusiformis* strain which displayed strong emulsification ability on xylol and moderate activity on mineral oil. *B. subtilis* produced the lipopeptide biosurfactants, surfactin, iturin and fengycin (Nitschke and Costa, 2007). Biosurfactants are beneficial to lipid biodegradability by

Table 4. COD removal and biosurfactant production by the mixed strains cultured in chicken broth wastewater for 48 h.

Isolate	COD removal (%)	Biosurfactant (mg/L)
B. thuringiensis+ *L. fusiformis*	70.4 ± 0.10	0.6 ± 0.11
B. thuringiensis+ *B. subtilis*	67.7 ± 1.56	545 ± 5.00
L. fusiformis+ *B. subtilis*	65.9 ± 1.56	119 ± 1.00
B. thuringiensis+ *L. fusiformis*+ *B. subtilis*	71.2 ± 0.20	1250 ± 10.00

Table 5. Effects of initial pH and organic loads on mean ±S.D. COD removal, hydrolytic activities and biosurfactant production of the combined strains.

Effect of pH and organic loading	COD reduction (%)	Protease (unit/ml)	Lipase (unit/ml)	Biosurfactant (mg/L)
pH				
5	34.50a[1] ± 0.170	0.120a ± 0.002	4.89a ± 0.001	1,110a ± 1.52
6	47.53b ± 0.057	0.120a ± 0.003	4.90b ± 0.001	1,120b ± 2.51
7	70.23c ± 0.570	0.129b ± 0.030	4.95c ± 0.002	1,140c ± 1.15
8	62.03d ± 0.460	0.120a ± 0.002	5.23d ± 0.002	1,130d ± 1.00
Organic load as COD (mg/L)				
1,000	43.00a ± 1.00	0.136a ± 0.002	7.12a ± 0.02	1,250a ± 20.00
2,000	45.16b ± 0.28	0.140b ± 0.004	7.34b ± 0.005	1,310b ± 10.00
3,000	49.40c ± 0.36	0.142c ± 0.00	7.53c ± 0.011	1,340c ± 10.00
4,000	57.20d ± 0.10	0.144d ± 0.00	7.76d ± 0.010	1,380d ± 11.54
5,000	56.39e ± 0.50	0.145e ± 0.001	7.78e ± 0.005	1,400e ± 10.00

[1]Values followed by different letters in same study and column are statistically difference ($p < 0.05$).

increasing interfacial microenvironment of lipid molecules thereby enhancing hydrolysis of lipase (Chatterjee et al., 2009).

Oil and grease are reduced to smaller particle size by biosurfactants, facilitating their metabolism by bacteria and with this a reduction in COD. Thus, in waste containing oil and grease treatment with rhamnolipid produced by *Pseudomonas aeruginosa* zju.um1 removed over 95% of COD compare with < 10% by controls (Zhang et al., 2009). Jacobucci et al. (2009) reported biosurfactants from *Planococcus citreus* and *Pantoea agglomerans* adhered to oil drops.

Biofilm formation was detected in all strains and beneficial to wastewater treatment. The three mixed bacteria in this study formed a thick biofilm when tested with a standard method using polystyrene surface (Table 3). This contributed further to COD reduction.

Formulation of potential combination

Two and three selected bacterial strains were compared for efficiency of COD reduction and biosurfactant production. The general characteristics of the untreated wastewater used in the present study were as follows: pH 6.83 ± 0.04, TS 1,800 ± 50 mg/L, SS 800 ± 10 mg/L and COD 1,160 ± 14 mg/L. After 48 h treatment, the three combinations showed a slightly higher COD reduction than the two combinations (Table 4). Moreover, this three mixed culture also produced highest biosurfactant at 1250±10 mg/L. Biosurfactants were also detected in all two strain combinations including those containing *B. thuringiensis* and *L. fusiformis* which did not show biosurfactant activity. It is suggested that both strains may produce low-molecular mass biosurfactant (de-emulsifier); which could not be detected by the screening method. Chen et al., (2007) reported that biosurfactants may be emulsifiers or de-emulsifiers; therefore, different methods should be applied for the screening test to identify compound with surfactant activities which destabilize emulsions.

Effect of pH and organic loads

Wastewater used in this study was the same as in the previous experiment. Highest COD reduction, proteolytic activity and biosurfactant production by the three combined strains were observed at pH 7 (Table 5). Moreover, lipase enzyme was also relatively high at this pH. Neutral pH favored protease and lipase production during wastewater treatment in *Bacillus* spp. (Mohan et al., 2008). Moreover, emulsifying compound was also influenced by pH (Kokare et al., 2007). The pH of poultry

processing wastewater used in the current study was nearly neutral at 6.83-7.05; and therefore it was not to adjust the pH values prior to bioaugmentation with these inoculants.

The COD reduction increased significantly ($p < 0.05$) with organic load from 1,000 to 4,000 mg/L (Table 5). At an initial load of 4,000 mg/L, a maximum of 57.2 $\pm$ 0.1% COD removal was obtained. However, at an initial organic load of 5,000 mg/L a decrease in COD reduction was found. Protease, lipase and biosurfactant production increased significantly ($p<0.05$) with increasing initial organic load from 1000 to 5000 mg/L. These results indicate a broad degree of application of the combined strains. Bhumibhamon and Phattayakorn (2003) reported a similar trend with COD reduction of kitchen wastewater by *Pseudomonas* sp. Further COD reduction increased with initial kitchen wastewater COD due to elevations in fats and oils. This was attributed to enhanced bacterial growth due to greater availability of nutrients. Pornsunthorntawee et al. (2009) reported that increasing oil loading rate (OLR) from 1 to 10 kg/m^{3}days promoted bacterial growth, since an extension in OLR results in increase nitrogen, phosphorus, and palm oil concentration in the substrate.

Conclusion

A mixture of 3 bacterial strains isolates from a chicken broth waste system expressed potential to reduce of COD under normal pH of the wastewater. Protease, lipase and biosurfactant were produced in the wastewater by the mixed culture and, it is suggested, play important roles in COD removal. These results indicate combined strains can increase the effectiveness of COD reduction from poultry processing wastewater after further scale-up experiments have been conducted.

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