Selection of *Acid Forming Bacteria* Having Decolorization Activity for Removal of Color Substances from Molasses Waste Water

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Abstract

This study focuses on isolation of acid forming bacteria (mostly *Acetobacter spp.*) from vegetable, fruits and fermented food samples. 170 isolates were isolated from samples, they were primary tested on solid medium which contained molasses pigments to select the strains which had decolorization activity. The results showed that 50 isolates had decolorization activity on solid medium (clear zone). Among them, the strains No. BP103 and No.13A gave the highest decolorization activity in the liquid medium which contained molasses pigments. For the effects of carbon sources on decolorization activity of both strains, the decolorization activity of the strains No. 13A and No. BP103 were 61.51%, 78.29%, 81.45%, 78.28%, 78.12%, 72.59% and 23.64% and 56.89%, 72.50%, 77.84%, 68.49%, 79.58%, 87.33% and 33.90 % when the carbon sources were glucose, galactose, fructose, sucrose, ribose, maltose and arabinose, respectively. Fructose was the most suitable carbon source for both strains for decolorizing molasses pigments. The optimal concentration of fructose in the media for highest decolorization activity was 2.0%. For the effects of nitrogen sources on decolorization activity, the organic nitrogen compounds were the best nitrogen sources for both strains. Yeast extract could stimulate the strains No 13A and No. BP103 to give the highest decolorization activity. The decolorization activity of the strains No. 13A and No. BP103 were 80.50% and 82.00%, respectively, when the nitrogen source was yeast extract. At the optimal conditions and medium compositions, the decolorization activity of the strains No. 13A and No. BP103 were 90.54%, 96.75%, respectively. From the results above, we con conclude that the decolorization activities of both strains were dependent on the growth rate (growth associated mechanism). Strain No. 13A and No. BP103 were identified as *Acetobacter aceti.*

Key words: Acid forming bacteria, *Acetobacter aceti*, Decolorization activity, Molasses waste water.

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1. Introduction

Several countries in the world such as Thailand, Brazil, Malaysia and so on produce sugar from sugar cane [1]. Molasses are the by-product from sugar production processes [1]. However, several researchers have tried to use the molasses as animal feed, fertilizer, and raw materials in the industrial sector [2,3,4,5]. For industrial use, molasses are one of the best raw materials as they are cheap and suitable for fermentation processes such as baker's yeast fermentation, amino acid fermentation, alcohol fermentation and so on. Waste water from factories which use molasses as raw materials, contains a large amount of dark brown pigments which is a kind of melanoidin[6] and is hardly decolorized by the usual biochemical treatments such as aerobic and anaerobic treatments[2,3,4]. Therefore, it is a problem for the industrial sector if waste water is discharged to the river without treatment. It would also be a problem for the environment as reduction of transparency and dissolved oxygen would cause danger to aquatic life. Many scientists have tried to conduct decolorization process for treating the molasses waste water by using physical and chemical systems [3,4,5,6]. But many problems arose such as operation costs and down stream processes. However, up to now no suitable method for the treatment of large amounts of molasses waste water has been developed.

Nowadays, several researchers [5,6,7,8,9,10,11] try to use biological processes for treating the molasses waste water for the purpose of removal of organic substances, together with color substances, from the waste water instead of the chemical treatment process. Research into the selection of microorganisms, which have a high ability to decolorize molasses pigments, is going on in several laboratories but no suitable strains which could be applied in waste water treatment plants for the removal of color substances from the molasses waste water have been found.

Genous of *Acetobacter* found in various sources such as vegetables, fruits and fermented food samples is an interesting micro organism group due to its advantages for the vinegar industry, gluconic acid industry, ketogluconic acid industry [12]. And also acetic acid bacteria was one of the main microorganisms in the anaerobic treatment system.

In this study we tried to isolate acetic acid bacteria which had decolorizing activity of molasses pigments. The optimal conditions and nutrients requirement of the isolated strains were investigated for the purpose of high efficiency for removal of color substances from molasses waste water.

2. Materials and methods

Molasses pigments solution preparation: Molasses pigments solution which was used in this study, was prepared from the stillage of an alcohol distillation factory. The stillage from Sang Som alcohol distillation factory, Thailand was centrifuged at 6,000 xg for 15 min. The supernatant was evaporated to 5 times the original concentration in a low temperature vacuum evaporator (Potavapor Buchii, Model RE 120) at 50°C. The concentrated solution was then dialyzed (dialyzing bag type: molecular weight cut off 10,000) against running tap water for 2 days and then against de-ionized water for 1 day. The non-dialyzable molasses pigment solution thus prepared was used in this experiment as an MP solution after dilution with distilled water to a concentration corresponding to an absorbance reading 3.5 at 475 nm (based on the color intensity of the molasses waste water before being sent to the treatment plant [6]).

Medium: There were several kinds of medium [13] used in this experiment as follows:

Acetic acid bacteria isolation medium: The medium, Glucose-Ethanol-Yeast extract Medium: GEYM (13), was used for isolation of acetic acid bacteria, it was composed of glucose 20.0 g, 95% ethanol 50.0 ml and yeast extract 5.0 g in 1,000 ml of distilled water. The medium pH was adjusted to 4.5.

Acetic acid bacteria purification medium: For the single colony isolation and culture purification of acetic acid bacteria, Glucose-Ethanol-Yeast extract-$CaCO_3$ medium (GEY-$CaCO_3$-M) (13) was used. The medium was composed of glucose 20.0g, 95% ethanol 50.0 ml, yeast extract 5.0g, $CaCO_3$ 10.0g and agar 20.0g in 1,000 ml of distilled water.

Stock culture medium: The pure culture which was isolated from fruit, vegetable and fermented food samples were stocked in stock culture medium [13]. The stock culture medium was composed of yeast extract 5.0g, mannitol 25.0g, peptone 3.0g and agar 20.0g in 1,000 ml of distilled water.

Medium for testing the decolorization activity: There were two kinds of media used for isolation and purification of the isolates which had the decolorization activity, molasses pigments agar medium (MPA) and molasses pigments broth medium (MPB). The MPA was composed of glucose 2.0g., KH_2PO_4 0.1g, $MgSO_4.7H_2O$ 0.5g, peptone 0.5g and agar 2.0g in 1000 ml of molasses pigments solution. The MPB was adjusted to pH 6.0. The composition of MPB was the same as MPA except without the agar.

Isolation of acetic acid bacteria: Acetic acid bacteria strains were isolated from vegetable, fruits and fermented food samples which were collected in Thailand. A 10 g of sample was suspended in 90 ml of sterilized distilled water. The 1 ml of suspended solution was inoculated in a 50-ml test-tube, containing 20 ml of GEYM and was incubated at 30°C for 5 days. Then, the strains which grew in the GEYM were isolated by the pour plate method on GEY-$CaCO_3$-M at 30°C. The single colony of acetic acid bacteria which showed the clear zone around the colony was collected (The clear zone showed digestion of $CaCO_3$ by acetic acid which was produced by the strain) The isolated strains were re-isolated again on the same medium as above. Pure isolated strains were stocked in stock culture medium.

Selection of acetic acid bacteria having decolorization activity: The isolated strains were streaked on MPA and incubated at 30° C for 3 days. The strains which showed a clear zone around the colony were collected. Then, the selected colonies were tested to confirm decolorization activity in MPB. The selected colonies were cultivated in a 20-ml tube, containing 10 ml of MPB at 30°c for 0, 3, 4, 5 and 6 days under non-shaking condition. Then, the culture broths were centrifuged at 6,000xg for 10 min for separation of the solid particles and bacterial cells. The supernatants were collected for detection of decolorization activity and pH.

Cultivation of acetic acid bacteria under shaking and non-shaking condition: 10 ml of cell suspension of the strains were inoculated in a 250 ml. shaking flask, containing 50 ml. of MPB. The experiments were done in 2 sets. The first set was incubated under shaking conditions (125 oscillations/min,7 cm stroke) at 30°C for 5 days. The second set was incubated under non-shaking conditions at 30°c for 5 days. A 10 ml of culture broth was collected during cultivation for measuring decolorization activity and pH after centrifugation at 6,000xg for 10 min.

Assays: Decolorization activity was assayed by measuring the decrease in color density as the absorbance at 475 nm after dilution with 0.1 M acetate buffer (pH=6.0). The decolorization yield was expressed as the degree of the decrease in absorbance at 475 nm against the initial absorbance at the

same wavelength. The residual sugar was determined by the Somogyi and Nelson's method. (14,15) The dry weight of mycelia was determined after drying at 105 °C for 24 hrs.

Biochemical tests: The strains which had highest decolorization activity were used in this study. The morphology and Gram stain were observed under light microscope. Biochemical tests of those strains were determined such as catalase test, oxidation of acetate, oxidation of lactase, the carbon assimilation (sucrose, mannitol, sorbitol, methanol and glucose) Ketogenesis test and VR test for classification and taxonomy (15,16).

3. Results

Isolation of acetic acid bacteria: 100 samples of fruit, vegetable and fermented food collected from supermarkets and farms in Thailand were used for isolation of acetic acid bacteria. The 170 isolates were isolated from above samples. All of the isolates showed the clear zone on GEY-$CaCO_3$-M.

Isolation of acetic acid bacteria having decolorization activity: The 170 isolates from above experiments, were determined for decolorization activity by using the agar plate technique. All of the 170 isolates were streaked on the MPA and incubated at 30°C for 3 days. Only 50 isolates showed the clear zone on the agar plate. It meant that these 50 isolates had decolorization activity on the agar plate. We confirmed the decolorization activity of the above strains in MPB. Those isolates were cultivated in the MPB for 5 days. After that the culture broths were collected and centrifugated at 6,000xg for 10 min. The supernatants were determined for the decolorization activity. It was found that 31 isolates gave the high decolorization activity (about 70%). From the secondary screening, only the two isolated strains which showed highest decolorization activity (more than 75%) were collected as No. 13A and No. BP 103.

Comparison between shaking and non-shaking conditions on the decolorization activity: The strains No. 13A and No. BP103 were tested for decolorizing efficiencies in MPB under non-shaking and shaking conditions. The results are shown in table 1. The decolorization activities of both strains under shaking condition were higher than under non-shaking condition. The decolorization activity of strains No.13A and No. BP 103 were 77.04% and 65.30% and 79.41% and 73.00% under shaking and non-shaking conditions, respectively within 5 days cultivation.

Optimization of the medium composition for the highest decolorization activity: The medium composition for decolorization activities of the strains No.13A and No.BP103 were examined as follows.

Carbon sources: The effects of carbon sources on the decolorization activity of the strains No. 13A and No. BP 103 are shown in fig.1 and fig.2. The strain No.13A gave the highest decolorization activity within 5 days cultivation in MPB when fructose and galactose were used as the carbon sources. The decolorization activity of the strain No. 13A were 82.50% and 79.50% when the carbon sources were fructose and galactose, respectively. For the strain No. BP 103, the highest decolorization activity was given after 5 days cultivation when the carbon sources were maltose or fructose, the decolorization activity of strain No. BP 103 were 87.30% and 77.84%, respectively.

Nitrogen sources: The effects of various nitrogen sources (organic and inorganic nitrogen compounds) on decolorization activity are shown in fig.3 and fig.4. Each isolate requires organic nitrogen as the nitrogen source. Both strains No.13A and BP103 gave the highest decolorization activity when yeast extract was used as the

nitrogen source. The decolorization activities of strains No. 13A and No. BP 103 were 80.50% and 82.00%, respectively when the yeast extract was used as nitrogen source within 5 days cultivation .

Fructose concentration: The effects of fructose concentration on decolorization activity is shown in fig. 5. The strains No. 13A and No. BP103 showed the highest decolorization activity when the 2.0% of fructose was supplemented in the MPB. The decolorization activity of strains No. 13A and No. BP103 were 61.20% and 71.00%, respectively.

Time course of the decolorization by strains No. 13A and No. BP103 : The medium composition used in this experiment was similar to MPB but 2.0% of fructose and 0.5% of yeast extract were used as the carbon source and nitrogen source, respectively. Typical culture profiles for strain No. 13A and No. BP103 which were grown under shaking conditions at 30°C are shown in fig.6 and fig.7. The decolorization by both strains was almost coincident with growth of cells. The strain No. 13A gave the maximal decolorization yield (90.00%) and dry weight of cell mass (0.092 g/100ml) within 4 days cultivation. The strain No.BP 103 gave the maximal decolorization yield (96.75%) and dry weight of cell mass (0.092 g/100 ml) within 4 days cultivation.

Identification of strains No. 13A and No. BP 103 : The morphology of strains No. 13A and BP103 were gram negative and short rod. The biochemical assay is shown in table 2, The strains were catalase positive, oxidation of acetate and lactate positive, VR test positive and ketogenesis test positive. For the carbon sources assimilation test, the strains could grow on sucrose, sorbose, glucose. From the morphological and biochemical data above compared to reference strain (ATCC 12876), we suggest that the strains No. 13A and No. BP103 belonged to *Acetobacter aceti*

4. Discussion

For the isolation of acid forming bacteria, 170 isolates were isolated from fruit, vegetable and fermented food samples. Among 170 isolates, 50 strains showed decolorization activity on the solid medium (clear zone). It meant that about 30% of acid forming bacteria had the decolorization activity. But after secondary screening in liquid medium, only 2 strains, strain No. 13A and strain No. BP103, gave high decolorization activity (more than 75%). Both these strains showed the highest decolorization activity under shaking conditions. It meant that dissolved oxygen in the medium was necessary for decolorization mechanisms.

The decolorization activity of strain No.13A and strain No.BP103 were dependent on the nitrogen sources, carbon sources and the concentration of carbon sources. It was similar to the decolorization mechanisms of fungi [7,8,9]. The suitable nitrogen sources for both strains were organic nitrogen such as peptone and yeast extract. The most suitable organic nitrogen compounds for the decolorization activity of both strains was yeast extract because the yeast extract could supply not only the organic nitrogen sources for the strains, but also would supply the growth factors and vitamin for those strains. So, the strains showed a high growth rate and decolorization activity in the medium which contained yeast extract as the nitrogen source. But urea was a bad nitrogen source for decolorization activity both strains. In the case of using urea as the nitrogen source, urea could make the pH of the medium up to the alkaline level (pH about 7 or more). The growth rate of acid forming bacteria under alkaline condition was low [15,16] because the optimal pH range for the optimal growth of acid forming bacteria was acid level (pH about 5.4-6.3) [15,16]. For the effects of

carbon sources on the decolorization activity of strains of No 13A and No.BP103, the results showed that the monosaccharide might be the best carbon source for decolorization activity due to growth rate mechanism, biochemical metabolism and the decolorization mechanisms [5,6,7]. Watanabe et.al. [7] has reported that the enzymes which were concerned in the decolorization activity were sugar oxidase such as sorbose oxidase. The fructose and glucose were the monosaccharide which was easily absorbed into the cell, stimulated growth rate of the cell and induced the sugar oxidase enzyme. But for both strains of No. 13A and No. BP103, the optimal concentration of the fructose for decolorization activity was 2.0%. and Sirianuntapiboon et al (6) reported that the decolorization activity was dependent on the growth rate (growth association mechanism).

From the results above, we believe that the acid former bacteria especially, *Acetobacter aceti* might be a suitable microorganism which could be applied in the conventional waste water treatment plant for the purpose of color removal. For the waste water treatment plant in alcohol factories or the factories which use molasses as the raw material, they always use anaerobic treatment system for removal of the COD as the first step. But anaerobic treatment system consists of an acid forming step and a methane forming step. In the acid forming step, the acid forming bacteria is the dominant group for producing the volatile fatty acid. If we could change the bacterial group in the acid forming step to be the acid forming bacteria which had the decolorization activity (especially, *Acetobacter aceti*). It means that both activities could occur in the acid forming step such as decolorization activity and volatile fatty acid production activity. So, the newly developed anaerobic treatment processes could remove the COD and color substances from the waste water at the same time.

5. Acknowledgements

The author would like to express many thanks to Department of Environmental Technology, School of Energy and Materials, and Department of Microbiology, Faculty of Science of KMUTT for supplying research materials and scientific equipment. The sincere thanks are given to Assist. Prof. Somsri Lee-Wit for her comments. And also deep thanks are given to Miss Chawanit Intarawimolmetha, Miss Pimphaka Phothilangka and Miss Ampun Chaikulsareewath for their research assistantce.

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Table1: Effects of shaking and non-shaking conditions on the decolorization activity of strains No.13A and No. BP103.

The strains were cultivated for 0,3,4 and 5 days in the MPB under shaking and non-shaking conditions as described in the text

Strain	condition	Incubation period							
		0		3		4		5	
		%DA	pH	%DA	pH	%DA	pH	%DA	pH
13A	Shaking	0	6.15	71.86	3.27	77.02	3.26	77.04	3.61
	Non - shaking	0	6.15	63.06	3.25	58.61	3.22	65.30	4.18
BP103	Shaking	0	6.15	83.94	2.20	77.98	3.20	79.41	2.99
	Non - shaking	0	6.16	81.44	2.95	67.72	3.24	73.00	2.99

Remark: % DA = % decolorization activity.

Table. 2: The morphologies and biochemical test of the isolated strains No. 13A and No. BP 103.

The experimental conditions are described in the text.

Properties	Strain No. 13A	Strain No. BP103	Reference strain (ATCC12876)
Morphology	short rod	short rod	short rod
Gram stain	gram negative	gram negative	gram negative
Catalase test	+	+	+
Oxidation of acetate	+	+	+
Oxidation of lactase	+	+	+
VP-test	+	+	+
Carbon assimilation			
-Sucrose	+	+	+
-Mannitol	-	-	-
-Sorbitol	+	+	-
-Methanol	-	-	-
-Glucose	+	+	+
Ketogenesis test	weak	weak	+

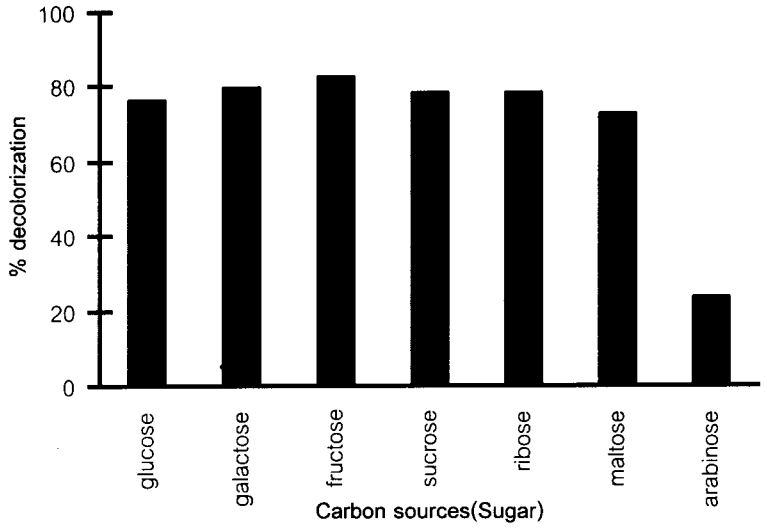

Fig.1: **Effects of carbon sources on the decolorization activity of strain No. 13A.** The strain was cultivated for 5 days at 30°C under shaking conditions in MPB. The 2% of sugars which were used as carbon source in MPB medium were glucose, galactose, fructose, sucrose, ribose, maltose and arabinose. The experimental conditions are described in the text.

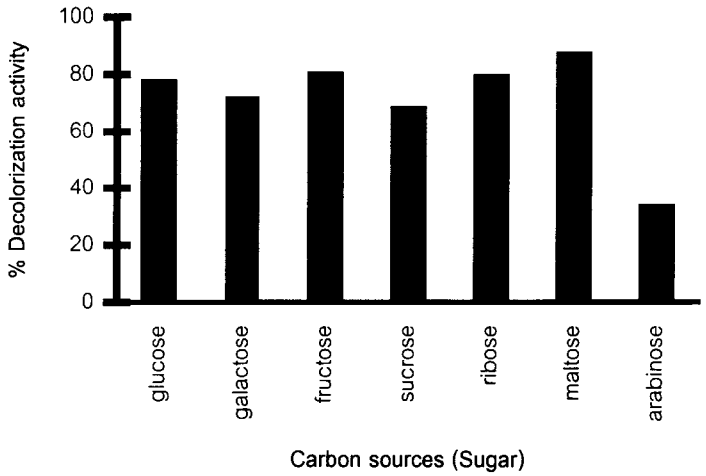

Fig.2: **Effects of carbon sources on the decolorization activity of strain No.BP 103.** The strain was cultivated for 5 days at 30°C under shaking conditions in MPB The 2% of sugars which were used as carbon source in MPB medium were glucose, galactose, fructose, sucrose, ribose, maltose and arabinose. The experimental conditions are described in the text

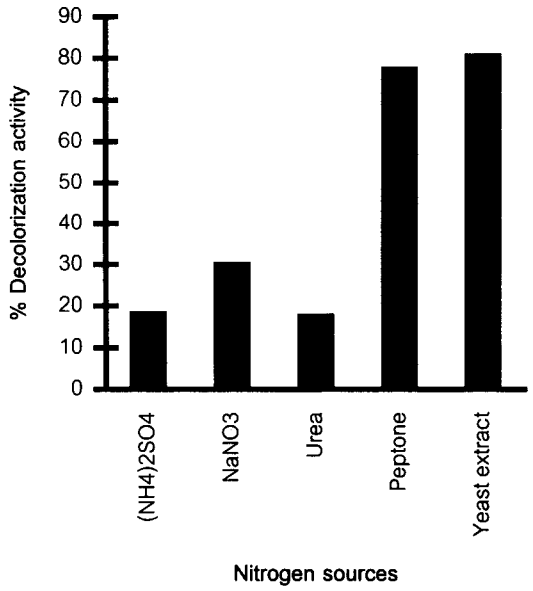

Fig. 3: Effects of nitrogen sources on the decolorization activity of strain No.13A.
The strain was cultivated for 5 days at 30°C under shaking conditions in MPB. The 0.5% of nitrogen compounds which were used as nitrogen source were $(NH_4)_2SO_4$, $NaNO_3$, urea, peptone and yeast extract. The experimental conditions are described in the text.

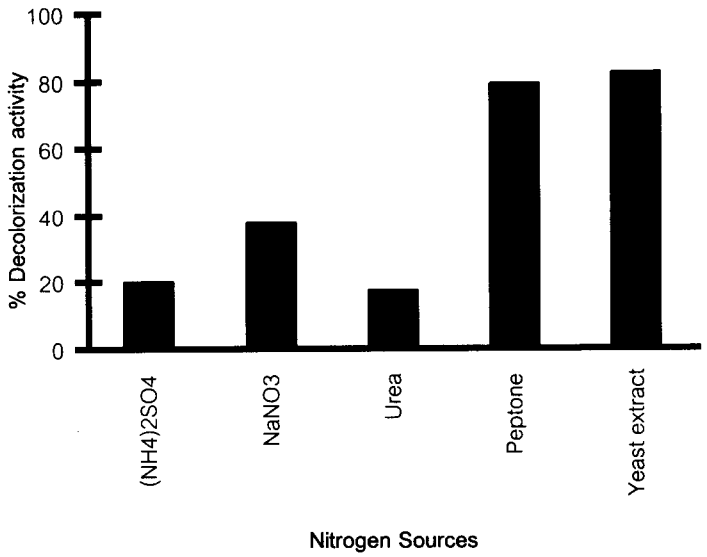

Fig. 4: Effects of nitrogen sources on the decolorization activity of strain No.BP103.
The strain was cultivated for 5 days at 30°C under shaking conditions in MPB. The 0.5% of nitrogen compounds which were used as nitrogen source were $(NH_4)_2SO_4$, $NaNO_3$, urea, peptone and yeast extract. The experimental conditions are described in the text.

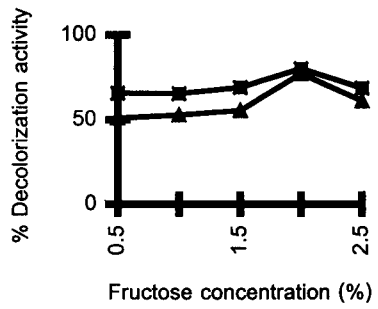

Fig.5 : Effects of fructose concentration on the decolorization activity of strain No.BP103 and strain No. 13A.

The strains were cultivated for 5 days at 30°C under shaking conditions in MPB which used various concentrations of fructose (0.5, 1.0, 1.5, 2.0 and 2.5 %) as the carbon sources. The cultivation condition is described in the text.
Symbol: ■, strain No.BP103; ▲, strain No. 13A.

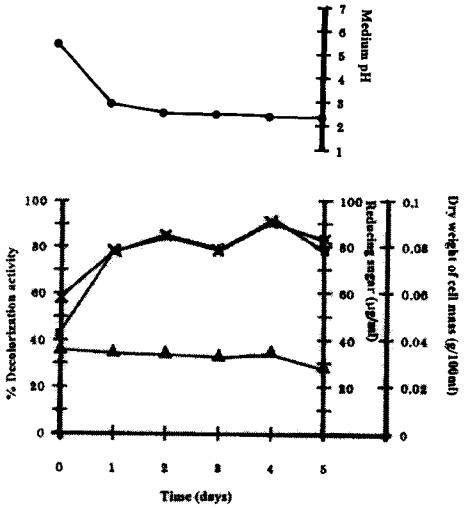

Fig.6: **A typical culture profile for strain No. 13a.**
The strain was cultivated under the optimal conditions as described in the text.
Symbols: ●, medium pH; ■, dry weight of cell mass; ×, %decolorization activity; ▲, reducing sugar.

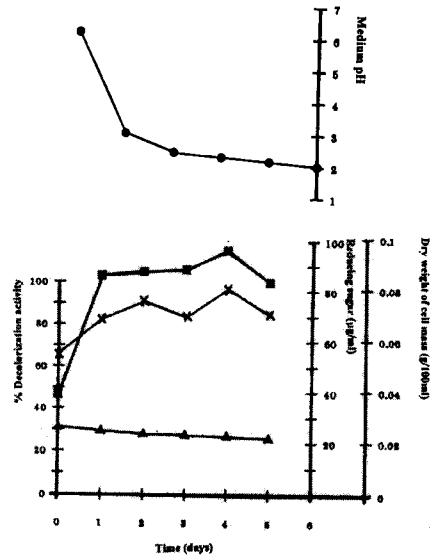

Fig.7: **A typical culture profile for strain No. BP 103.**
The strain was cultivated under the optimal conditions as described in the text.
Symbols: ●, medium pH; ■, dry weight of cell mass; ×, %decolorization activity; ▲, reducing sugar.