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BIODEGRADATION OF AZO DYES BY *PSEUDOMONAS*, *BACILLUS*, *STAPHYLOCOCCUS* AND *MICROCOCCUS* STRAINS

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ABSTRACT

Degradation of azo dyes by pure and mixed bacterial strains of *Pseudomonas*, *Bacillus*, *Staphylococcus* and *Micrococcus* strain was studied. These strains grew in nutrient broth supplemented with Indosole Yellow BG, Indosole Black G and Indosole Brilliant Red BAwith specific growth rates of 0.28 h^{-1}, 0.31 $h^{-1}$and 0.44 h^{-1}, respectively. Very low percentage (i.e. < 5%) of azo dyes was removed in mineral salts medium (MM) by pure bacterial strains. Mixed culture degraded azo dyes not more than 20% in MM medium. The degradation was enhanced when a cosubstrate (i.e. glucose) was supplemented in the MM medium containing azo dyes with bacterial consortia. Decolorisation of over 95% of azo dyes with mixed culture was observed when glucose was added with azo dyes in MM medium. Different concentrations of azo dyes (i.e. 10 mg/L and 20 mg/L) were degraded in MM medium supplemented with the same concentration of glucose with almost similar degradation rates.

Key words: Azo dyes, biodegradation, decolorisation, cosubstrate, mixed culture, kinetics

INTRODUCTION

Humans are using colorants and dyes for over a hundred years. Currently, over 700,000 tons of colorants are being produced on yearly basis (Zollinger 1987). It has been estimated that over 10,000 different dyes and pigments are used in making different products in textile and paint industries (McMullan *et al.,* 2001). Because of changing nature and huge volume of textile effluents, dyes are very difficult to remove with a single conventional treatment technology (O'Neill *et al.,* 1999). Azo dyes are the largest group of dyes used in textile industries, mainly due to the simple synthesis (Jianbo *et al.,* 2010; Pandey *et al.,* 2007). Therefore, removal of azo dyes from the industrial effluents is necessary for preventing contamination of soil and water.

Previously, various methods have been adapted to remove azo dyes with physical technologies (i.e. adsorption, chemical precipitation, membrane separation (Carriere*et al.,* 1993; McKay 1980), coagulation/flocculation, and ozonation (van der Zee 2002) and chemical treatment (i.e. oxidation, reduction, and adsorption by activated charcoal) (Li and Bishop 2004). These methods are not only expensive but also generate toxic wastes that are more difficult to treat and dispose (Moutaouakkil *et al.,* 2004).

A wide variety of microbial species such as bacteria and fungi are found to remove azo dyes from textile effluents during waste treatment. Fungal strains isolated from textile effluents are reported to degrade azo dyes. For example, *Aspergillus niger* SA1 decolorized over 98% Acid Red 151 (Ali *et al.,* 2008) and *Aspergillus terreus* SA3 efficiently utilized Sulfur Black by removing 85% of color and 75% COD (Andleeb *et al.,* 2010) by fungal growth and hyphal uptake mechanism under different physicochemical conditions. There are only a few bacteria that are able to grow on azo dyes as the sole carbon source. However, mixed bacterial strains can completely remove azo dyes (Liu *et al.,* 2007; Patil *et al*., 2008; Tony *et al*., 2009). In this study we have isolated four different types of bacterial strains and tested pure and mixed culture for their potential to degrade azo dyes in synthetic media.

MATERIALS AND METHODS

Chemicals

Azo dyes (Indosole Yellow BG, Indosole black G and Indosole brilliant red BA) were kindly provided by Clariant Pakistan (Pvt.) Ltd. All other chemicals were of analytical grade and purchased from Merck Pakistan.

Culture medium and growth conditions

All bacterial isolations were cultivated at room temperature (28-35°C) on nutrient broth (Merck, Germany) and mineral salts medium (MM) with composition (g/L) as shown in Table 1.

Table 1. Composition of mineral salts medium.

S. No.	Ingredients	Concentration (g/L)
1	$Na_2HPO_4.12H_2O$	5.3
2	KH_2PO_4	1.4
3	$(NH_4)_2SO_4$	0.5
4	$MgSO_4.7H_2O$	0.2
5	Trace elements solution	5 ml/l

Trace elements solution contained per litre 780 mg of $Ca(NO_3)_2.4H_2O$, 200 mg of $FeSO_4.7H_2O$, 20 mg of $Na_2SeO_4.10H_2O$, 10 mg of $ZnSO_4.7H_2O$, 10 mg of H_3BO_3, 10 mg of $CoCl_2.6H_2O$, 10 mg of $CuSO_4.5H_2O$, 4 mg of $MnSO_4.H_2O$, 3 mg of $Na_2MoO_4.2H_2O$ and 2 mg of $NiCl_2.6H_2O$. The pH of MM medium was adjusted at 7.0.

Enrichment and isolation of azo dye degrading strains

In order to isolate potential azo dye degraders, wastewater samples were collected from primary sedimentation tank and activated sludge of a treatment plant of a textile company situated at Korangi Industrial Area, Karachi. Bacterial strains able to grow on azo dyes were isolated from the liquid sludge made by mixing wastewater and activated sludge samples in MM medium. Enrichments of dye degrading microorganisms were done in serum bottles (250 ml) by mixing 50 ml liquid sludge in 50 ml MM containing 500 mg/L of azo dye under non-shaking conditions at room temperature. Three different dyes (i.e. Indosole Yellow BG, Indosole black G and Indosole brilliant red BA) were selected in this study.

Each serum bottle contained a single dye kept at room temperature. After every 15-20 days 10 ml samples of the suspensions were transferred to new flasks containing fresh MM medium with 500 ppm of respective azo dye. Growth of azo dye degraders was followed by visual observation of dye removal. After 2-3 serial transfers, approximately 50-60% dye removal was observed in selected dyes. The decrease in color intensity indicated the ability of strains to grow on azo dyes. Pure bacterial cultures were obtained by repetitive streaking onto nutrient broth (NB) plates with growth checks in MM medium with respective dyes.

Identification of isolated strains

Five morphologically different isolates were obtained. The isolates were identified on the basis of cell shape, cell arrangement, gram staining, relation to oxygen, nutritional characteristics, physiological and biochemical characteristics (Sivaraj *et al.*, 2011; John *et al.*, 2000). The isolated strains were maintained on nutrient agar slants and stab cultures were preserved at 4^{o}C.

Azo dyes measurements

The decolorisation of azo dyes in MM medium was monitored by measuring the optical densities (ODs) of the dye solutions at their respective absorbance maxima (i.e., Indosole Yellow BG, 560 nm; Indosole black G, 480 nm and Indosole brilliant red BA, 530 nm) using spectrophotometer. A loopful of culture from each plate specific for the dye was inoculated in serum bottles containing 100 ml MM medium and 10mg/L of dye. The bottles were kept at room temperature and observed for color reduction by withdrawing samples after every 24 h. The samples (1-1.5ml) were centrifuged at 13,000 rpm for 3 min. The distilled water was used as a blank. The pellets were resuspended in distilled water and their ODs were monitored at 600nm.

The efficiency of color removal was expressed as the percentage of the decolorized dye concentration to that of the initial one. Decolorisation activity was calculated as follows:

$$Decolorisation\ (\%) = \frac{(A_i - A_f)}{A_i} \times 100 \qquad \text{(Eq. 1)}$$

Where, A_i = initial absorbance, A_f = final absorbance

Cosubstrate as a parallel carbon source

Biodegradation of azo dyes by pure and mixed cultures was also studied by adding glucose as a cosubstrate in the MM medium. Glucose was added into the mineral media at the concentrations equal or double the concentrations to the azo-dyes in parallel experimental setup. In all cases, other growth conditions like temperature, pH and micro-aerophilic conditions were kept constant. The medium was overlaid with paraffin oil in order to create partially anaerobic conditions. The flasks were incubated at 35°C. Samples were drawn after every 24 h to observed ecolorisation by reading absorbance at λ_{max} for each dye.

RESULTS AND DISCUSSION

Biodegradation of azo dyes

Biodegradation of three different azo dyes (Indosole Black G, Indosole Red BA, and Indosole Yellow BG) by exploring the optimizing conditions (i.e., type of cosubstrate supplemented with azo dyes, different concentrations of cosubstrate and temperature variations) for these dyes is studied. The effects of mixed and pure culture on azo dye degradation were also explored.

Identification and characterization of isolated azo-dye degraders

Identification of the azo dye degrading bacterial isolates was done up to the level of genus by performing Gram staining, motility test, biochemical tests (i.e. Indole, Methyl Red, Voges-Proskeur, citrate, catalase and nitrate reduction tests)as described in the Burges's manual of determinative bacteriology. Assimilation of various sugars such as lactose, glucose, sucrose and maltose was determined by inoculating the isolates into sugar broth supplemented with respective sugar for 24 hours at 37°C.

The isolates were identified as *Pseudomonas sp.* (AZ-1, AZ-4 and AZ-5*), Staphylococcus* sp. (AZ-2) and*Bacillus*sp. (AZ-3) (Fig. 1). Pure and mixed cultures of these isolated strains were used to study the growth and degradation kinetics of azo dyes.*Pseudomonas* and *Bacillus* species are well known for their ability to decolorize azo dyes.*Pseudomonas* S-42 was capable of decolorizing Drimerin Brilliant Orange RR (DBO-RR), Direct Brown M (DBM), Eriochrome Brown R (EBR) and many more (Sheng and Bao 1989). *P. putida* MET94 removed more than 80% of the structurally diverse azodye in 24 h under anaerobic conditions (Mendes *et al*.,2011). Acid Orange 52, which is extensively used in textile industries, was decolorized by *Pseudomonas putida* mt-2 (Mansour *et al*., 2011). Rapid biodegradation and decolorisation of Direct Orange 39 was observed by *Pseudomonas aeruginosa* strain BCH (Jadhav *et al*., 2010). Decolorisation of orange MR was carried out by *Micrococcus* sp. DBS 2 (Rajee and Patterson 2011). Degradation of azo dyes with an NADH-dependent azoreductase of *Bacillus* sp. strain SF has also been reported (Maier *et al*., 2004).

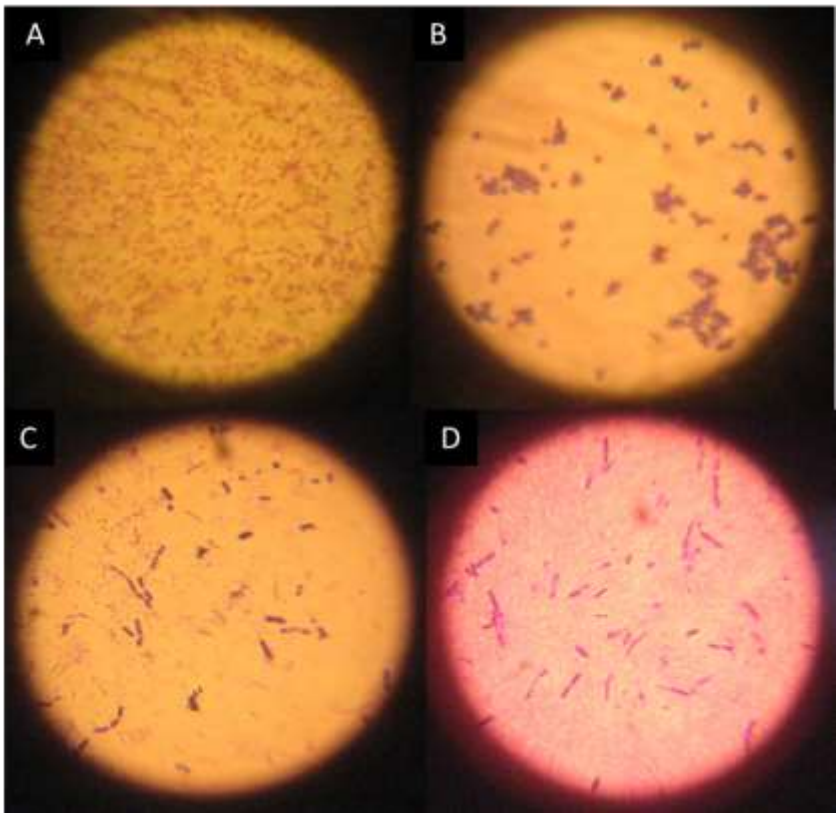

Fig. 1. Gram straining of pure and mixed bacterial strains degrading azo dyes, Indosole Yellow BG (**A,***Pseudomonas* sp. AZ-1 *and* **B**, *Staphylococcus* sp. AZ-2), Indosole Black G (**C**, *Bacillus* sp. AZ-3 and *Pseudomonas* sp. AZ-4), Indosole Brilliant Red BA (**D,** *Bacillus* sp. AZ-3 and *Pseudomonas* sp. AZ-5).

Biodegradation of azo dyes using pure culture

The pure cultures of isolated strains were separately inoculated into 120 ml flasks containing 50 ml MM medium supplemented with azo dyes and incubated at optimum growth conditions (temperature 35-37°C and p^H 7±2). Over a period of 15 days, slight decrease in color intensity (up to 2%) was observed. The decrease in dye concentration was not significant (i.e., <5%) when flasks were incubated for 45 days in the same physical conditions.

Very few researchers have observed that pure culture was able to bring about a high level of decolorisation of azo dyes. However, in many bioremediation studies, mixed bacterial cultures have proved to be superior to single pure cultures (Khalid *et al*., 2010). The reason being the nature of azo dyes in which first step of degradation requires anaerobic and then aerobic conditions for the stepwise degradation.

Biodegradation of azo dyes using mixed culture in nutrient broth

Growth kinetics of mixed cultures of five differentcultures degrading its respective dyes was studied by inoculating the strains in flasks containing nutrient broth supplemented with azo dyes. All mixed cultures showed different growth patterns in nutrient broth. Cultures for dyes IndosoleBlack G and Indosole Yellow BG had steady growth curves showing a stable log phase with specific growth rates of 0.28 h^{-1} and 0.31 h^{-1}, respectively. While cultures degradedIndosoleBrilliant Red BA had rapid and short log phase with specific growth rate of 0.44 h^{-1} (Fig. 2).

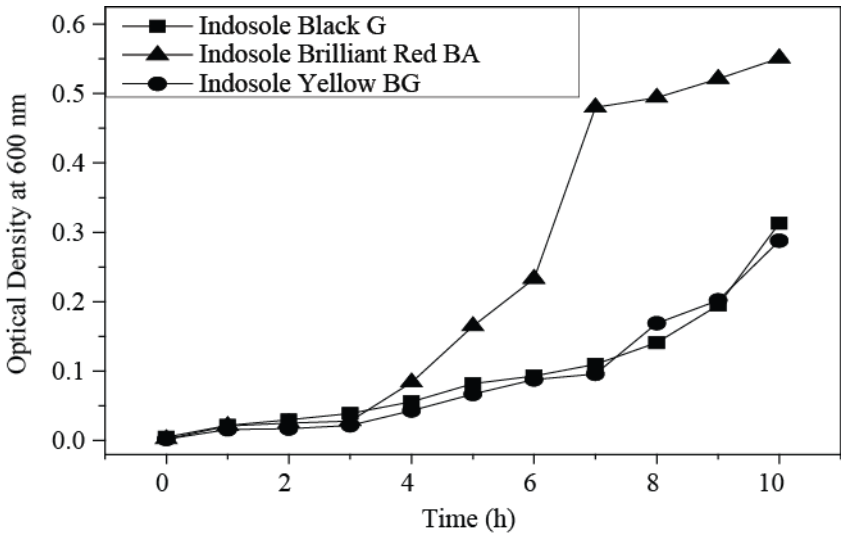

Fig. 2. Growth kinetics of mixed culture in nutrient broth.

Biodegradation of azo dyes using mixed culture in mineral salts medium (MM)

Biodegradation of azo dyes was also studied by mixed culture in MM medium. Samples were drawn after every 24 h for monitoring growth and degradation. During the first stage of biodegradation, azo dyes alone are a very difficult substrate for microorganisms to utilize as carbon source unless there is another carbon source present in the medium. This is due to the complex structure of the azo dyes. Azo dyes often have a very complex structure and due to this structural complexity, they are resistant to attack by microbial enzymes and hence are recalcitrant. Not more than 20% decrease in color intensity was observed (Table 2) indicating that inoculated bacterial strains were unable to completely degrade azo dyes.

Table 2. Degradation of azo dyes by mixed culture with and without supplementation of glucose.

S. No.	Azo dyes	Decolorisation without cosubstrate(%)	Decolorisation with glucose(%)	
		10 mg/l	10 mg/l	20 mg/l
1	Indosole Black G	20	> 99	>99
3	Indosole Brilliant Red BA	5	95	96
4	Indosole Yellow BG	6	95	95

Biodegradation of azo dyes in MM medium supplemented with co-substrates using mixed culture

Complete biodegradation of azo dyes was not observed unless a cosubstrate was added with the azo dyes in MM medium (Fig. 3). Similar observations have been reported previously by other researchers that a very little decolorisation of azo dyes was observed when cosubstrate was not added in MM medium (Kuhn and Suflita 1989; Tan 2001 and Stolz 2001).It was reported that complete removal of Basic Red 46 and Methylene Blue without supplementation of a cosubstrate took a longer time (2 h) and degraded partially (10-15%) (Sarioglu and Bisgin 2009).

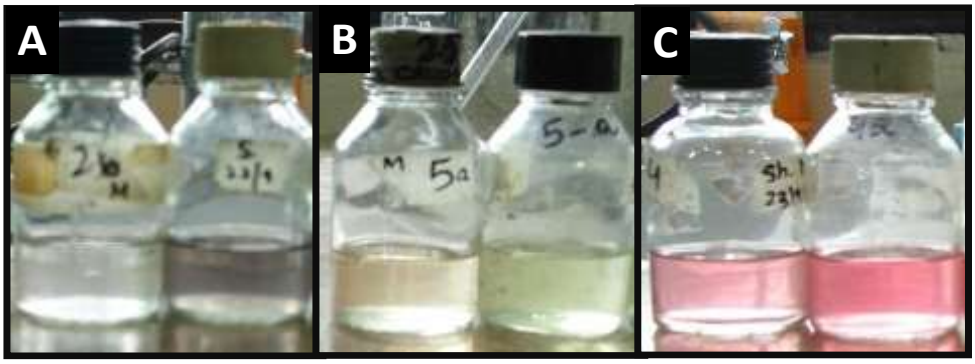

Fig. 3. Flasks showing the degradation of A) Indosole Black G, B) Indosole Yellow BG and C) Indosole Brilliant Red BA at some point in MM medium with and without supplementation of cosubstrate. High intensityof the dye colors indicatedegradation without cosubstrate and light color indicates with cosubstrate

Addition of glucose as a cosubstrate

Respective mixed cultures degradedup to 95% of 10 mg/l (Fig. 4A) and 20 mg/l (Fig. 4B) of Indosole Black G in 100 h, 85% of 10 mg/l (Fig. 4C) and 20 mg/l (Fig. 4D) of Indosole Brilliant Red BAin 200 h and 90% of 10 mg/l (Fig. 4E) and 20 mg/l (Fig. 4F) of Indosole Yellow BGin 100 hin separate flasks in MM medium supplemented with glucose as much as azo dyes. Enhanced biodegradation of azo dyes in the presence of cosubstrate has been reported frequently by many researchers (Field *et al*., 1995; Sarioglu 2009; Taj 2009; Maddhinni *et al*., 2006; Telke*et al*., 2008; Khalid*et al*., 2011). Using glucose as cosubstrate, 90% of Methylene Blue (Field *et al*., 1995; Sarioglu and Bisgin, 2009), 94% of Orange II in 45 min (Taj, 2009), up to 80% of Remazol Black-B (Khalid *et al*., 2011), 70% Direct yellow 12 in 105 min (Maddhinni *et al*., 2006), 70% Triphenylmethane dyes (Field *et al*., 1995) and complete biodegradation of Basic Red 46 in 25 hours (Sarioglu and Bisgin, 2009) were degraded. This indicates that an additional carbon source is required in the medium to accelerate the degradation of azo dyes.

The concentration of cosubstrate is also an important factor which affects the biodegradation of azo dyes. Therefore, different concentrations of glucose were added to the MM medium to study the biodegradation of azo dyes. Similar degradation rates were observed when 20 mg/l each of Indosole Black G, Indosole Brilliant Red BA and Indosole Yellow BG were degraded in MM medium supplemented with equal concentrations of glucose (Fig. 4) by maintaining similar growth conditions. Hence, increased cosubstrate concentration reduced the chances of growth inhibition that is usually caused due to increased concentration of a carbon source. This could be reasoned that the presence of anaerobic environment during the first stage of biodegradation is very important. In most of the cases of biodegradation of azo dyes studied so far, azo dye reduction is very difficult to occur in an aerobic environment. A cosubstrate helps degrading azo dyes by i) releasing electrons that are used by azo reductases for cleavage of azo bond (Hong *et al*., 2007) and ii) creating anaerobic micro niches in the medium, thereby accelerating the degradation process under anaerobic conditions (Tan, 2001). Therefore, paraffin oil was used to coat the surface of the medium to establish and maintain anaerobic environment.

During this research, some inadequacies also appeared. One of the limitations was that the information about the structure of dyes studied was not provided. This created problem in the study of the aromatic amines produced during anaerobic degradation (first step of azo dye degradation). The aromatic amines could not be identified and the mineralization of these metabolites also could not be followed. Also the residual concentration of the dye after treatment could not be determined. However, the approach of biodegradation of the azo dyes was successful. All dyes were degraded up to 80-100% but further study of the metabolites of the dye and their mineralization is only possible if the dye structure is known.

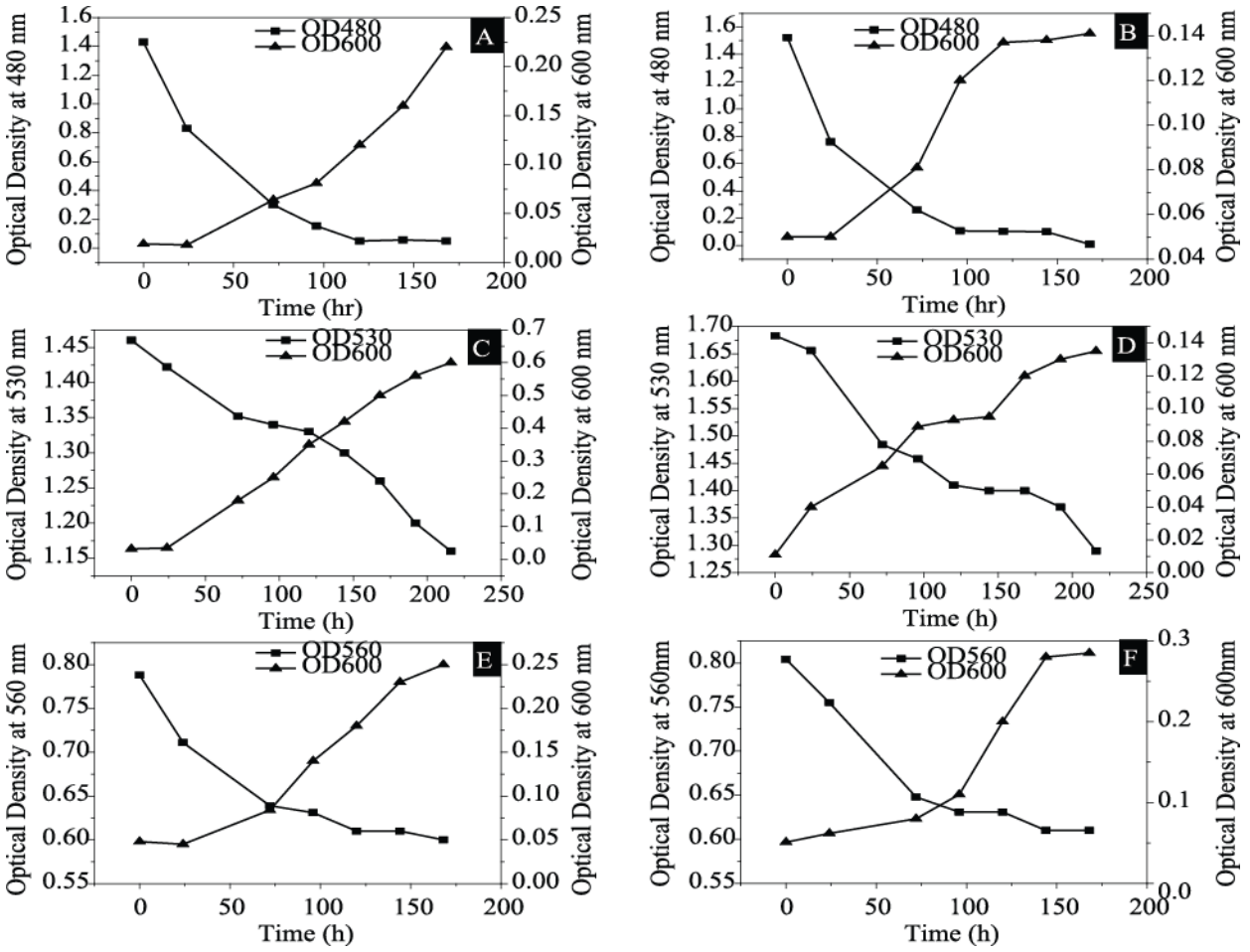

Fig. 4. Growth of mixed-culture in MM medium containing Indosole black G; **A)** 10 mg/l, **B)** 20 ;mg/l; Indosole Red BA; **C)** 10 mg/l, **D)** 20 mg/l and Indosole yellow BG; **E)** 10 mg/l, **F)** 20 mg/lsupplemented each with equal concentration of glucose.

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