

## Decolourization and Degradation of Textile Azo Dyes by *Corynebacterium* sp. Isolated From Industrial Effluent

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**Abstract.-** The present study is aimed at assessing the ability of *Corynebacterium* sp. to decolorize and degrade dyes into non-toxic form. *Corynebacterium* sp. could tolerate Reactive Black5 and Yellow15 up to 10 mg/ml. The maximum growth of bacterium was observed at 37°C and at pH 7. *Corynebacterium* sp. showed high azoreductase activity against Reactive Black5 (68%) and Reactive Yellow15 (80%). *Corynebacterium* sp. could decolourize 60% (Reactive Black5) and 76% (Reactive Yellow15) from the medium containing 100 µg/ml after 2 and 4 days, respectively. The additional bands in TLC chromatograms after decolourization of the dyes showed that the dyes were degraded by the bacterium when compared with the undecolourized dyes. The presence of extra bands in TLC in decolourized samples indicates the ability of the bacterium to degrade dyes enzymatically and can be exploited for bioremediation of azo dyes and their derivatives containing wastes, since it seem to have the potential to degrade the toxic azo dyes into non-toxic product form.

**Keywords:** Azo dyes, decolourization, *Corynebacterium* sp., bioremediation.

### INTRODUCTION

Textile dyes are chemicals with complex aromatic structures designed to resist the effects of laundering and sunshine, for example (Nigam *et al.*, 2000). A great number of dyes and other chemicals are used in textile wet processing. There are more than 105 commercially available dyes with over  $1 \times 10^6$  ton of dyestuff produced annually world-wide (Pandey *et al.*, 2007). Among these available dyes, azo dyes constitute about 70% of all known dyestuffs in the world and represent 70% of total dyes produced per year, thus making them the largest and the most important group of synthetic colorants released into the environment (Carliell *et al.*, 1998; Robinson *et al.*, 2001; Selvam *et al.*, 2003; Ambrosio and Campos-Takaki, 2004).

Dyes-particularly reactive dyes-usually have a synthetic origin and complex aromatic molecular structures, which make them stable and difficult to biodegrade. Reactive dyes differ from all other dye classes in that they bind to textile fibers, such as cellulose and cotton, through covalent bonds (O'Mahony *et al.*, 2002). Reactive dyes are typically

azo-based chromophores combined with various types of reactive groups, which show different reactivity. The recalcitrance of azo dyes has been attributed to the presence of sulfonate groups and azo bonds, two features generally considered as xenobiotic (Rieger *et al.*, 2002; Lee and Pavlostathis, 2004).

The industrial manufacturing of azo dyes and textile finishing processes generate wastewater streams contaminated with azo dyes. Some of the azo dyes are difficult to treat by conventional wastewater treatment methods. Compared with physical and chemical methods, biological techniques are preferable because of economical advantages and low possibility of by products production. Screening potential microorganisms is a critical step in the construction of an effective remediation system.

At present, a number of studies have focused on microorganisms, which are able to decolourize and biodegrade these dyes. Several combined anaerobic and aerobic microbial treatments have been suggested to enhance the degradation of azo dyes (O'Neill *et al.*, 2000). Alternatively, dye decolorization using microbial enzymes has received great attention in recent years due to its efficient application (Abadulla *et al.*, 2000; Claus *et al.*, 2002; Zille *et al.*, 2003; Couto *et al.*, 2005).

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0030-9923/2011/0001-0001 \$ 8.00/0  
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Color removal processes with active microorganisms have two different simultaneous steps: an adsorption of dyes on the surface of the organisms and a degradation of dyes by the enzymes produced by these organisms (Fu and Viraraghavan, 2001; Ozer *et al.*, 2005; Khalaf, 2008).

The present study deals with the isolation of azo dyes degrading bacterium from a dyes contaminated environment, its ability to degrade reactive dyes into non-toxic product and optimization of temperature and pH for maximum dyes degradation.

## MATERIALS AND METHODS

### Sample collection

Wastewater samples were collected in screw capped sterilized bottles from Klas Textile industrial effluents (Fig. 1), Lahore (Pakistan). Some physicochemical parameters of wastewater *viz.*, temperature, pH, (APHA, 1989) colour and smell were measured.



Fig. 1. Sites of textile industrial effluents (a; Red dye and b; Black dye) from where the wastewater samples were collected.

### Isolation of Azo dyes degrading bacteria

For isolation of azo dyes degrading bacteria, 100  $\mu$ l of the wastewater sample was spread on Luria-Bertani (LB) agar plates containing 0.5% Reactive Black 5 and Yellow 15/100 ml of the medium. LB agar plates were prepared by dissolving 1 g NaCl, 1 g tryptone and 0.5 g yeast extract in 100/ml distilled water, pH adjusted at 7 to 7.2 and then 1.5 g agar was added in the 250 ml flasks. The medium was autoclaved at 121°C and 15 lbs/inch<sup>2</sup> pressure for 15 min. The growth of the bacterial colonies was observed after 24 hours of incubation at 37°C. Effect of dyes on the growth of bacterial isolates was determined in Minimal Salt Medium (MSM) which contained (mg/l): (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 500; CaCl<sub>2</sub>, 14; MgSO<sub>4</sub>.7H<sub>2</sub>O, 120; FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.13; ZnSO<sub>4</sub>.7H<sub>2</sub>O 5.0; Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O, 2.5; Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O, 700, 0.5; KH<sub>2</sub>PO<sub>4</sub>, 400 (pH 7) with minor modifications (NaCl 1g/l and glucose 10g/l were not given in the medium) and supplemented with azo dyes (Pesce and Wunderlin, 2004). It was again incubated at 37°C for 24 hours. This process was repeated with successively higher concentrations of dyes until the minimum inhibitory concentration (MIC) of the bacterial isolate was obtained.

### Physical and biochemical characterization

The isolate was Gram stained. For biochemical characterization the isolate was tested for catalase activity, motility, oxidase activity, nitrate reduction, and hydrolysis of casein. The procedures of these biochemical tests were taken from Cappuccino and Sherman (2001).

### Catalase test

Catalase test was performed by preparing 3% hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>, solution (3 ml H<sub>2</sub>O<sub>2</sub> in 100 ml distilled water). A drop of 3% H<sub>2</sub>O<sub>2</sub> was taken on a clean glass slide and with the help of inoculating loop colony of the test organism was mixed in the drop. Bubble production is indicative of positive reaction meaning presence of catalase enzyme, while no bubble production is a negative reaction.

### Oxidase test

To perform the oxidase test few drops of

freshly prepared oxidase reagent (0.1g tetramethyl-p-phenylene-diamine dihydrochloride in 10 ml distilled water) were poured on a piece of filter paper. Using a sterile loop colony of the test organism was put on the filter paper having oxidase reagent immediately. Purple colour production within 10 seconds indicates a positive oxidase reaction.

#### *Motility test*

For the preparation of motility test medium, 3 g of the motility agar was added in 100 ml distilled water. PH was adjusted to  $7.5 \pm 0.2$ . The medium was sterilized by autoclaving at  $121^\circ\text{C}$  and  $15 \text{ lb/inch}^2$  for 15 minutes, cooled to  $50\text{-}55^\circ\text{C}$  and poured under aseptic conditions in sterilized test tubes. The medium was inoculated with a straight wire, making a single stab down the center of the tube to about half depth of the medium. It was incubated at  $37^\circ\text{C}$  for 24 hours. Non-motile organism's growth was restricted along the stab line. Motile organisms swarm and gave diffuse spreading growth extending as a zone of turbidity from the stab line.

#### *Nitrate reduction test*

This test was performed to analyze the nitrate reducing ability of the bacterial isolate. Nitrate reduction medium was prepared by adding 0.5g peptone, 0.3g beef extract and 1 ml 2%  $\text{KNO}_3$  (1 ml  $\text{KNO}_3$  in 100 ml distilled water) in 100 ml distilled water. The pH of the medium was adjusted to 7. Medium (5 ml) was dispensed in each test tube and was autoclaved. Inoculation was done by adding 200  $\mu\text{l}$  of log phase of actively growing bacterial isolates in the test tubes. After 24 hours of incubation, 2-3 drops of reagent 1 (0.8 % sulfanilic acid prepared in 5N acetic acid) was added. Then 2-3 drops of reagent 2 (0.6 % Dimethyl-alpha-naphthylamine prepared in 5N acetic acid) were added to the test tubes. Red colour indicates the positive reaction while the colourless reaction is considered as negative.

#### *Hydrolysis of casein*

Both skim milk (10g /100 ml) and agar (2g/100 ml) were prepared separately and autoclaved at  $121^\circ\text{C}$  for 20 min. After cooling to  $45^\circ\text{C}$  both solutions were mixed and poured into

sterile Petri plates. Bacterial isolate was seeded on the skim milk agar by point inoculation, incubated for 24 hours at  $37^\circ\text{C}$  and finally examined for evidence of casein hydrolysis.

#### *Determination of optimum growth conditions*

For optimum growth of the bacterial isolate, two parameters *i.e.*, temperature and pH were considered. For determination of optimum temperature, 5 ml LB broth was added in 4 sets, each of three test tubes, autoclaved and inoculated with 50  $\mu\text{l}$  of freshly prepared culture of each bacterial isolate by overnight growth at  $37^\circ\text{C}$  in LB broth. The four sets of tubes were incubated at  $20^\circ\text{C}$ ,  $30^\circ\text{C}$ ,  $37^\circ\text{C}$  and  $45^\circ\text{C}$ . After an incubation period of 12 h, their absorbance was measured at 600 nm using a  $\lambda$  650 UV/Vis Spectrophotometer (PerkinElmer, USA). For determination of optimum pH, test tubes having 5 ml LB broth were prepared in 9 sets, each containing 3 test tubes and their pH was adjusted at 5.0, 6.0, 7.0, 8.0, 9.0 and 10 then autoclaved. These tubes were inoculated with 50  $\mu\text{l}$  freshly prepared culture of bacterial isolate. After an incubation period of 12 h, their absorbance was measured at 600 nm.

#### *Effect of dyes on bacterial growth*

Growth curves of bacterial isolate were determined in minimal salt medium with (100  $\mu\text{g/ml}$  of Reactive Black5 and Yellow15) and without dyes (control). For bacterial isolate 50 ml medium was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 50  $\mu\text{l}$  of the freshly prepared inoculum. The cultures were incubated at  $37^\circ\text{C}$  in a shaker at 100 rpm. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 0, 3, 6, 9, 12, 18, 21 and 24 h. Absorbance was measured at 600 nm. Growth was plotted graphically.

#### *Crude cell extract*

To prepare the crude cell free extract, the bacterial cultures were grown with azo dyes (100  $\mu\text{g/ml}$ ) in 100 ml minimal salt medium for 96 h at  $37^\circ\text{C}$ . Cells were harvested by centrifugation at 9000 ( $3800 \times g$ ) for 15 min. Pellets were washed twice with 0.1 mM phosphate buffer (pH 7) and were suspended in 1 ml of the same buffer. Cells

were disrupted by sonication for 5 min (Sonics VC 500 USA) in cold condition. The resultant homogenate was centrifuged at 8000 (3300 x g) for 20 min at 4°C; the supernatant was used as a crude extract. The activity of azoreductase was determined by slightly modified method of Moutaouakkil *et al.* (2003) by measuring optical density of a reaction mixture containing 200 µl of 25µM dye, 200 µl of 1mM NADH, 2.07 ml of PBS buffer (pH 7.0) and 300 µl of the crude enzyme extraction. The reaction was allowed to proceed for 30 min at 37°C, and measured at 558 nm and 410 nm for Reactive Black5 and Yellow15, respectively (Kalyani *et al.*, 2008) by the LAMBDA 650 UV/Vis Spectrophotometer (PerkinElmer, USA). The crude extracts that were heated at 100°C for 30 min acted as control.

#### Dyes decolourization

Decolourization experiment was performed in 250 ml Erlenmeyer flasks containing 100 ml minimal medium and was inoculated with 2% inoculum. Cultures were incubated at 37°C. The dyes were added after 24 h of growth at 100 µg/ml separately and culture media was withdrawn after 2 and 4 days, centrifuged at 6000 rpm for 15 min to separate the bacterial cell mass. Decolourization was determined by measuring the absorbance of decolourization medium at 558 nm and 410 nm for Reactive Black5 and Yellow15, respectively (Kalyani *et al.*, 2008). Percentage decolourization was calculated as follows:

$$\text{Decolourization (\%)} = \frac{(\text{Initial absorbance} - \text{Observed absorbance})}{(\text{Initial absorbance})} \times 100$$

#### Thin layer chromatography

The metabolites produced during the biodegradation of azo dyes after decolourization of the medium were extracted with equal volume of chloroform. The supernatant was concentrated with anhydrous Na<sub>2</sub>SO<sub>4</sub>. TLC analysis was carried out according to Kalyani *et al.* (2008) on silica gel using mobile phase solvent system *n*-propanol, methanol, ethyl acetate, water and glacial acetic acid (3:2:2:1:0.5) and results were observed under UV illuminator.

#### Toxicity assay

The biodegraded products were tested for their toxic effect on the agriculturally important soil bacterial flora (Mali *et al.*, 2000; Gottlieb *et al.*, 2003). *Azotobacter* sp. and *Bacillus cerus* were inoculated on minimal salt medium containing agar. Two wells were made on the respective media containing plates and filled with decolourized centrifuged broth. The plates were incubated at 30°C for 48 hours. Zone of inhibition surrounding the well represented the index of toxicity.

#### Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

## RESULTS AND DISCUSSION

#### Physicochemical characteristics of wastewater

Table I shows the some physicochemical characteristics of industrial wastewater from where azo dyes degrading bacterium was isolated.

**Table I.- Physical characteristics of the textile industrial effluent from where wastewater samples were collected.**

Samples	Temperature	pH	Color	Smell
A	40°C	10.0	Dark blue	Earthy smell
B	42°C	8.5	Reddish pink	Rotten egg
C	26°C	7.0	Light yellow	No smell

#### Isolation and identification of the bacterial isolate

A total of 18 cultures of bacteria were isolated, purified and screened for the degradation of azo dyes. Of all the cultures tested, 4 bacterial isolates were further screened for dyes degradation and finally one bacterial isolate was selected on the basis of dyes tolerance *i.e.*, resistance in minimal medium containing 2% of reactive red and yellow. Table II shows

physical and biochemical characteristics of the bacterial isolate. On the basis of these characteristics bacterial isolate has been identified as *Corynebacterium* sp. Kalyani *et al.* (2008) reported that *Pseudomonas* sp. SUK1 decolourized Red BL1 (50 µg/ml) 99% within 1 h under static anoxic condition at pH range from 6.5 to 7.0 and 30°C.

**Table II.- Morphological and physiological characteristics of the bacterial isolate.**

Characteristics	<i>Corynebacterium</i> sp.
Gram stain	Positive
Shape	Rod
Arrangement	Single
Motility	Positive
Sporulation	Negative
Behavior of oxygen	Aerobic
Catalase	Positive
Reduction of nitrate to nitrite	Positive
Starch hydrolysis	Positive

*Optimum growth conditions*

The most suitable temperature for dyes decolourizing bacterial isolate was found to be 37°C. The bacterium also showed maximum growth at pH 7. The growth curve pattern was studied by growing the organism in the presence of dyes (100 µg/ml) and comparing with the control culture in which no dyes were added. The growth pattern of *Corynebacterium* sp. was significantly different from those of control and the lag phase delayed up to 12 h in the presence of Reactive Black5 when compared with control. The maximum growth was observed after 15 and 21 h in the presence of Reactive Black5 and Reactive Yellow15, respectively. Growth rate was lower in the presence of Reactive Black5 as compared to Reactive Yellow15 and control culture where no dyes were added into the culture medium. The growth pattern is shown in figure 2.

*Dyes degradation and crude enzyme assay*

Decolourization of azo dyes by bacterial strains has been reported by many researchers (Khalid *et al.*, 2008; Hong *et al.*, 2007; Xu *et al.*, 2007). Bacterial degradation of azo dyes is generally considered a specific reaction by azoreductase under

aerobic conditions or a nonspecific reduction process under anaerobic conditions. Maier *et al.* (2004) reported that a thermoalkalophilic *Bacillus* sp. was able to reduce a large structural variety of azo dyes. David and Steven (1994) reported that azo-dyes are also degraded efficiently under aerobic conditions by wood rotting fungi which are in nature responsible for the degradation of lignin. In the present study cell free extract of *Corynebacterium* sp. illustrated degradation of Reactive Black5 and Reactive Yellow15 68% and 80%, respectively.

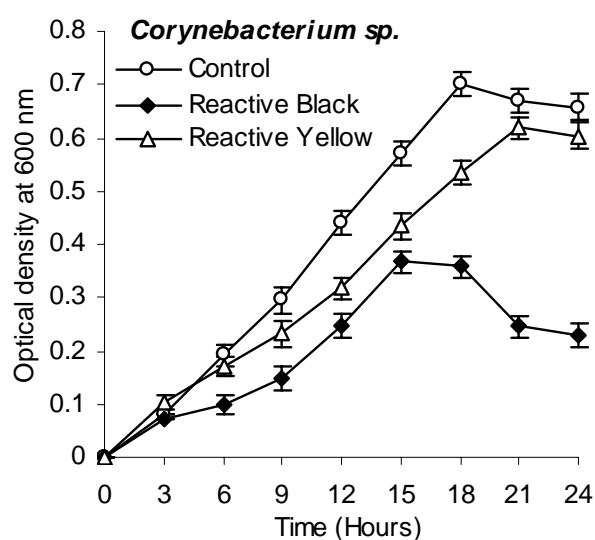


Fig. 2. Growth curves of dyes degrading *Corynebacterium* sp. in minimal salt medium containing dyes (100 µg/ml) and without dyes after incubation at 37°C.

*Dyes decolourization by bacterial isolate*

The efficient removal of these dyes is necessary and significant for environmental protection. In the present investigation cell free extract of *Corynebacterium* sp. illustrated decolourization of Reactive Black5 up to 32 and 60% at 100 µg dye/ml after 2 and 4 days of incubation, respectively. The bacterium was also able to decolourize the Reactive Yellow15 (100 µg dye/ml) up to 40 and 76% after 2 and 4 days of incubation, respectively (Fig. 3). Sheth and Dave (2009) reported that *Pseudomonas aeruginosa* exhibited 91% decolourization of Reactive Red BS (300 ppm) within 5.5 h over a wide pH range from

5.0 to 10.5 and temperature ranging from 30 to 40°C. The bacterium was able to decolourize more than 91% of Reactive Red BS under static conditions in presence of glucose, peptone or yeast extract.

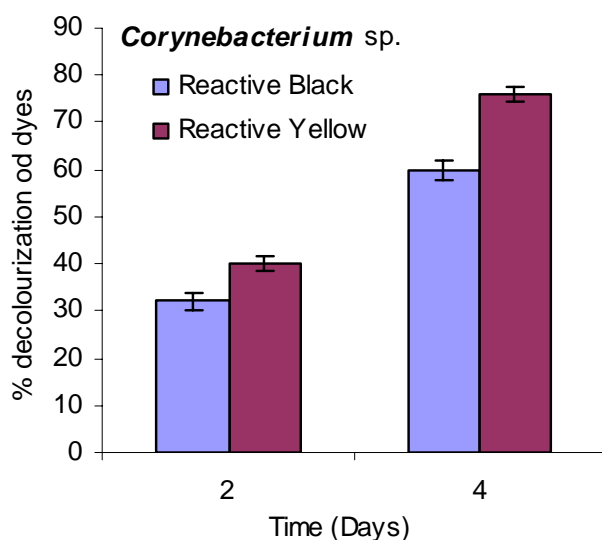


Fig. 3. Decolourization of azo dyes (100 µg/ml) by *Corynebacterium* sp. in minimal salt medium after 2 and 4 days of incubation at 37°C.

Wu *et al.* (2009) reported that *Shewanella oneidensis* WL-7 was capable to decolourize 95% of the Reactive Black5 from the medium containing 100 µM after 12 hours. Azo dyes are widely distributed as a characteristic pollutant in effluents of many industrial processes due to its common use and are recalcitrant to conventional sewage treatment systems (Banat *et al.*, 1996). Kalyani *et al.* (2008) reported that *Pseudomonas* sp. SUK1 decolourized Red BL1 (50 µg/ml) 99% within 1 h under static anoxic condition at pH range from 6.5 to 7.0 and 30°C.

#### Toxicity assay

No zone of inhibition observed in surrounding the wells containing decolourized dye water, indicated that the biodegraded or decolourized product was non-toxic to beneficial soil bacteria. In one of the investigation from this laboratory reveals that no toxic effects were

observed when fungal decolourized dye wastewater was used to grow *Vigna radiata* (Unpublished data). The toxic effect of untreated Malachite green, Brilliant green, Fast green, Methylene blue and Congo red and removal of their toxicity after biological treatment has been reported by Mali *et al.* (2000).

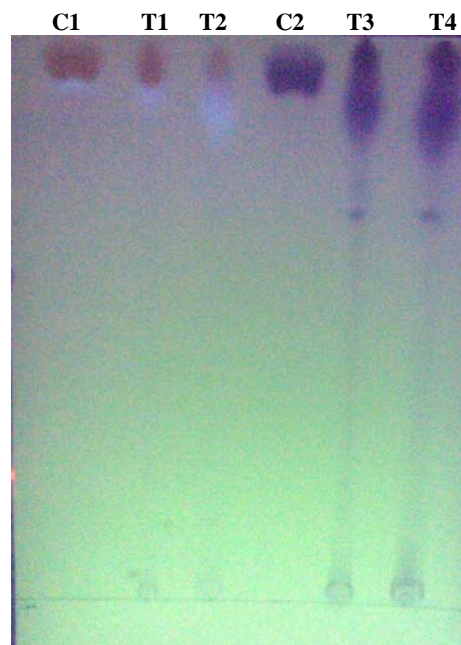


Fig. 4. TLC pattern of reactive dyes after enzymatic treatment of *Corynebacterium* sp.. C1, Control (Yellow dye); T1 and T2, treated (Yellow dye); C2, Control (Black dye); T3 and T4, treated (Black dye). The results are shown in UV radiation.

#### TLC

Azo dyes degradation by microorganisms may utilize many enzymes such as lignin peroxidase, tyrosinase and laccase. The comparison of TLC chromatograms before and after decolourization by the *Corynebacterium* sp. under UV light showed that the decolourized samples had one additional band (Lanes 2-3 in Fig. 4) in Reactive Yellow15 treated samples and two additional bands (Lanes 5-6 in Fig. 4) in Reactive Black 5 treated samples, which might have originated from the dye metabolites. The TLC results suggested that *Corynebacterium* sp. was able to degrade reactive dyes and most of the enzymes

could be induced by its degradable substrates. Sheth and Dave (2009) also confirmed the degradation of Reactive Red BS on TLC and no spot was observed in visible light whereas spots with different  $R_f$  values were observed as compared to untreated dye in UV light. Similar TLC results against *Remazol Black B* treated with halotolerant bacteria were reported by Asad *et al.* (2007).

## CONCLUSIONS

*Corynebacterium* sp. resisted reactive dyes up to 30 mg/ml and showed fairly high azoreductase activity against Reactive Black5 (68%) and Reactive Yellow15 (80%). *Corynebacterium* sp. also showed excellent ability to decolourize azo dyes *i.e.*, 60% (Reactive Black5) and 76% (Reactive Yellow) in the presence of 100 µg dyes/ml. The bacterial isolate can be exploited for bioremediation of azo dyes containing wastes, since it seems to have the potential to degrade the toxic reactive dyes into non-toxic product form. Knowledge of biodegradation is important for the evaluation of the persistence of organic pollutants and the design of biodegradation facilities. Therefore, further detailed research is needed to quantify these substrates interactions in the degradation of dyes and its derivatives.

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(Received 17 May 2010, revised 2 July 2010)