Thesis for the Master's degree

Biodegradation of azo dye using beneficial microorganisms and transcriptomic analysis of the degradation pathway

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유용미생물을 활용한 아조염료의 생분해 및 전사체 분석을 통한 분해경로 탐색

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Abstract

The goal of this study was to select the most appropriate microbial consortium for efficient bioremediation of azo dye wastewater. A consortium composed of two bacterial cultures (*Mesorhizobium* sp. and *Sphingomonas melonis*) and two yeast cultures (*Apiotrichum mycotoxinivarans* and *Meyerozyma guilliermondi*) achieved more than 80% decolorization within 24 h (50 and 100 mg/L dye). The chemical oxygen demand (COD) removal rate for the bacterial consortium (B-C) reached 97% in 72 h while the yeast consortium (Y-C) and the total microbial consortium (T-C; bacterial and yeast consortia combined) achieved 98.0% and 97.5%, respectively, in 24 h, indicating potential mineralization of the azo dye Acid Blue 113. Moreover, there was a positive relationship between cell growth and the azo dye degradation rate in all consortia. The Fourier transform infrared (FT-IR) spectra profiles for

yeast-containing consortia showed a rapid disappearance of absorbance at the azo bond specific wavenumber (1455 cm^{-1}) (24 h), while the B-C showed disappearance within 72 h. Metabolic products containing $-NH_2$ groups were also detected based on the absorbance at the 1300 cm^{-1} wavenumber, reflecting an occurrence of azo bond cleavage. It was concluded that the data for decolorization, COD removal, cell growth, and FT-IR spectra collectively provide evidence for azo dye decolorization and potential mineralization of the dye by the bacterial and yeast consortium. Moreover, transcriptomic analysis using RNA-sequencing further explained the potential mechanisms of azo dye biodegradation by *Sphingomonas melonis*. NAD(P)-dependent oxidoreductase and type 1 glutamine amidotransferase were differentially expressed in the dye treatment, indicating that degradations of the azo bond and the aromatic compounds could be catalyzed by these enzymes. The selected microbial consortia could be applied for the bioremediation of azo dye wastewater at the industrial scale of varying environmental conditions.

Key words: Azo dye; Acid Blue 113; Decolorization; Biodegradation; Wastewater treatment; transcriptomic analysis; RNA-sequencing

유용미생물을 활용한 아조염료의 생분해 및 전사체 분석을 통한 분해경로 탐색

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초록

이 연구의 목적은 아조 염료 폐수의 효율적인 생물학적 분해를 위한 가장 적절한 미생물 컨소시엄을 선택하는 것이다. 2 종의 박테리아(*Mesorhizobium sp.* 및 *Sphingomonas melonis*)와 2 종의 효모(*Apiotrichum mycotoxinivarans* 및 *Meyerozyma guilliermondi*)로 구성된 컨소시엄은 24 시간만에 50 또는 100 mg/L의 azo 염료(Acid Bule 113)를 80 % 이상 탈색을 달성했습니다. 화학적 산소 요구량 (COD) 제거율은 24 시간 후 효모 컨소시엄 (Y-C)과 총 미생물 컨소시엄 (T-C, 박테리아 및 효모 컨소시엄 결합)이 각각 98.0 %와 97.5 %를 달성하는 반면, 박테리아 컨소시엄 (B-C)은 72 시간 후 97 % 달성하였다. 또한, 모든 컨소시엄에서 세포 성장과 azo 염료 분해율은 비례관계를 가지고 있다. Fourier transform infrared (FT-IR) 스펙트럼 분석 결과, Y-C는 아조 결합을 나타내는 특정 주파수

(1455 cm^{-1})에서 24 시간 후 흡광도의 급격한 감소를 보인 반면, BC는 72 시간 후 소멸을 보였다. 또한, 아조 결합의 절단을 의미하는 $-NH_2$ 그룹의 주파수 (1300 cm-1)에서도 azo 염료가 분해되는 시간에 따라서 검출되었다. 탈색 실험, COD 제거, 세포 성장 및 FT-IR 스펙트럼에 대한 결과를 종합해보면, 박테리아 및 효모 컨소시엄이 의한 아조 염료의 잠재적인 무기화 작용에 대한 증거를 제공한다고 평가되었다. 또한, RNA 시퀀싱을 통한 전사체 분석은 *Sphingomonas melonis*의 잠재적 아조 염료 분해 매커니즘을 밝혀내었다. 즉, NAD(P)-dependent oxidoreductase 및 type 1 glutamine amidotransferase는 염색물질의 처리구에 차별적으로 많이 발현된 것으로 보아, 이러한 효소가 염색물질의 아조결합구조 및 그 분해산물인 방향족화합물의 분해에 관여한 것으로 판단되었다. 선발 된 미생물 컨소시엄은 다양한 환경적 요인에 노출되어 있는 산업규모의 아조 염료 폐수 처리에 생물학적처리를 위해 적용될 수 있을 것으로 보인다.

주제어: 아조 염료, Acid Blue 113, 탈색, 생분해, 폐수처리, 전사체 분석

Chapter 1. Introduction

Different classes of dyes are used in numerous industries including the rubber, textile, cosmetic, plastic, leather, food, and paper manufacturing industries. Various dyes are seen in wastewater discharged from these industries [1]. Among these, the largest contributor to dye wastewater is the textile industry, which is responsible for two-thirds of the total production of dye wastes due to the high quantities of water used in the dyeing processes [2-4]. The major problem is the unfixed dyes that remain in the wastewater after textile processing [5]. During dyeing processes, approximately 15 to 30% of the dyestuff fails to bind to the fibers and is therefore released into the environment [6].

Dyes are usually recalcitrant and may be toxic to organisms [7]. Therefore, the release of wastewater containing dyes is quite harmful to the environment. Their presence in an aquatic ecosystem reduces the penetration of sunlight to benthic organisms, thus limiting the process of photosynthesis [1, 8]. Furthermore, dyes reduce the solubility of oxygen in water. Dyes also affect the aesthetic value of an aquatic ecosystem due to the coloration of water resources.The key concern in the treatment of wastewater is the release of dyes and their metabolites into the environment, as some may be mutagens and carcinogens [9].

Azo dyes, being the largest group of synthetic dyes, constitute up to 70% of all known commercial dyes produced [10,11]. Their chemical structure is characterized by highly substituted aromatic rings joined by one or more azo groups (-N=N-) [7]. This double bond structure is a chromophore that makes the color visible, and thus cleavage of the azo bond will eventually decolorize the dye.Furthermore, some of the

decolorization products are even more toxic than their parent compounds, and therefore, it is desirable to degrade and remove the dyes in an eco-friendly manner—one that will affect neither the environment nor human health.In particular, azo dyes are regarded as xenobiotic in nature and recalcitrant to biodegradation (Guptaetal.,2013). For this reason, Acid Blue113 (AB113), one of the azo dyes, was targeted for degradation in this research.

Chemical and physical treatment technologies have been well developed and are widely used to treat dye wastewater. However, these approaches have not been economically feasible due to a low rate of color removal, high cost, the production of a large amount of sludge, ineffectiveness in chemical oxygen demand (COD) removal, and production of secondary wastes [3, 9, 12]. In contrast, the use of microbial treatment technologies has advantages in treating azo dye wastewater because they are environmentally friendly, cost-competitive, and can produce less sludge and yield end-products that are non-toxic or can mineralize the target chemicals and require less water consumption compared to physicochemical methods [13].

Therefore, bioaugmentation, a biological treatment technology, has the potential to sustainably and efficiently bioremediate dye wastewater [14]. The addition of specific microorganisms has been shown to be more effective than using a consortium of non-specific functions because they are selectively designed for individual degradative bioprocesses [15]. Bacterial and yeast decolorization methods are widely used due to their high activity and adaptability [16].

Various types of enzymes, including bacterial azoreductases, lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) from

yeast, are able to effectively degrade xenobiotics such as azocompounds [17,18]. However, decolorization intermediates, such as aromatic amines, can inhibit the biodegradation activity of bacteria [19]. Yeast, however, can efficiently degrade recalcitrant organics using the enzymes mentioned above [20]. They also have been found to be efficient in treating high-strength organic wastewater [21]. It is therefore assumed that treatment systems with mixed microbial populations could be more effective because of the concerted metabolic activities of the microbial community [22-24].

The goal of this study was to evaluate the decolorization efficiency of candidate microbial cultures for effective bioremediation of azo dye wastewater. Herein, two bacterial cultures and two yeast cultures were isolated from a commercial microbial consortium acclimated with the azo dye AB113. It was hypothesized that bacteria and yeast efficiently degrade AB113 under aerobic conditions and their enzymes either directly or indirectly affect the biodegradation process. Also, it was assumed that presence of azo dye affect the microbial gene expression. Results showed that a consortium composed of bacterial and yeast cultures appeared to more efficiently and stably degrade AB113, indicating possibility of application for bioremediation of azo dye wastewaters on an industrial scale. Therefore, this study suggested the potential biodegradation pathway of Acid Blue 113 and its mechanism was explained through transcriptomic analysis using RNA-sequencing.

Chapter 2. Literature Review

2.1 Principles of color chemistry

Dyes have a unique characteristic (i.e. the color) that are different from the most organic compounds. They absorb light in the visible spectrum (400-700 nm), have at least one chromophore (color-bearing group), have a conjugated system, i.e. a structure with alternating double and single bonds, and exhibit resonance of electrons, which is a stabilizing force in organic compounds [25]. All conditions must be satisfied for the molecular structure to have color. In other words, if any of these features is not satisfied, the color will be lost. In addition to chromophores, most dyes also contain auxochromes (color helping supplements), for instance, carboxylic acid, sulfonic acid, amino and hydroxyl groups. These are not responsible for color; however, their presence can shift the color of a colorant and they are most often used to adjust dye solubility. Table 2.1 shows the ranges of wavelength absorbed and respective color absorbed/observed.

Table 2.1 Wavelength of absorption versus color in organic dyes [25]

Wavelength Absorbed (nm)	Color Absorbed	Color Observed
400 – 435	Violet	Yellow – Green
435 – 480	Blue	Yellow
480 – 490	Green – Blue	Orange
490 – 500	Blue – Green	Red
500 – 560	Green	Purple
560 – 580	Yellow – Green	Violet
580 – 595	Yellow	Blue
595 – 605	Orange	Green – Blue
605 – 700	Red	Blue – Green

Azo dyes, which contain one or more azo groups, comprise the largest group of organic dyes. Prominent types are acid dyes for polyamide and protein substrates such as nylon, wool, and silk; disperse dyes for hydrophobic substrates such as polyester and acetate, and direct and reactive dyes for cellulosic substrates such as cotton, rayon, linen, and paper [26].

2.2 Azo dye decolorization and degradation

2.2.1 Azo dye treatment

Azo dyes are known as electron deficient xenobiotic compounds because they possess electron withdrawing groups, generating electron deficiency in the molecule (dyes) which makes them resistant to degradation [27]. Approximately 80% of azo dyes are used in the dyeing process of textile industries [26]. The presence of these dyes in the water ecosystem is the cause of serious environmental and health problems [28,29].

Despite of numerous physico-chemical approaches for azo dye decolorization such as adsorption, chemical treatment and ion pair extractions, these methods were not sustainable due to their high cost and large amounts of sludge produced after treatment. Also, coagulation or flocculation techniques that are widely used create massive amounts of sludge, which requires further process for safe disposal. Adsorption and membrane filtration techniques lead to secondary waste streams as well [26].

2.2.2 Biological treatment of azo dyes

Biological approaches are widely used for azo dye treatment due to their cost effectiveness, ability to produce less sludge, and eco-friendly nature [30,31]. Various taxonomic groups of microorganisms like bacteria, fungi, yeast, and algae have been reported for their capability of degrading azo dyes under anaerobic and aerobic conditions [32]. In most cases, presence of oxygen prevents the cleavage of azo dyes in conventional sewage treatment plants while anaerobic conditions are preferred by various microorganisms to reduce the azo dyes into their corresponding aromatic amines, which are further degraded in presence of oxygen [33,34]. Azo dyes easily accumulate in the environment up to very high extent due to highly resistant nature to cleavage of azo bonds (-N=N-). Under aerobic condition, bacterial treatment of azo dyes usually shows low efficiencies since oxygen is a stronger electron acceptor than azo dyes. It has been reported that anaerobic and static condition increases the color removal efficiency of azo dyes. Anaerobic treatment, regardless of types of microorganisms involved in the process, contributes to the reduction of dyes [33]. On the other hand, aerobic decolorization may be more cost-effective as it can degrade the secondary metabolites at the same time.

2.2.3 Enzymatic decolorization and degradation of azo dyes

The primary step in bacterial decolorization of azo dyes, in both anaerobic and aerobic conditions, is the reduction of the azo bond (-N=N-). This reduction may be achieved by various mechanisms, such as enzymes, low molecular weight redox mediators, chemical reduction

by biogenic reductants like sulfide, or a combination of these. Also, the location of the reactions may be either intracellular or extracellular sites as (**Fig. 2.1**). According to previous researches, two enzyme families, azoreductase and Laccases have shown remarkable potential in enzymatic decolorization and degradation of azo dye. Laccases, especially, have great potential to decolorize a wide range of known industrial dyes [35,36]. Recently, promising enzymes like manganese peroxidase (MnP), lignin peroxidase (LiP) and polyphenol oxidase (PPO) have been also reported to be involved in the decolorization and degradation of azo dyes. Azoreductase is the major group of enzymes expressed in azo dye degrading bacteria and fungi. It cleaves the azo bonds of dyes, thus, reduces the dye into their corresponding colorless aromatic amines [37]. Azoreductases catalyze the reaction with the presence of reducing agents such as NADH, NADPH and $FADH_2$. These agents act as an electron donor and are involve in the cleavage of azo bond at intracellular or extracellular site of the bacterial cell membrane [38,39].

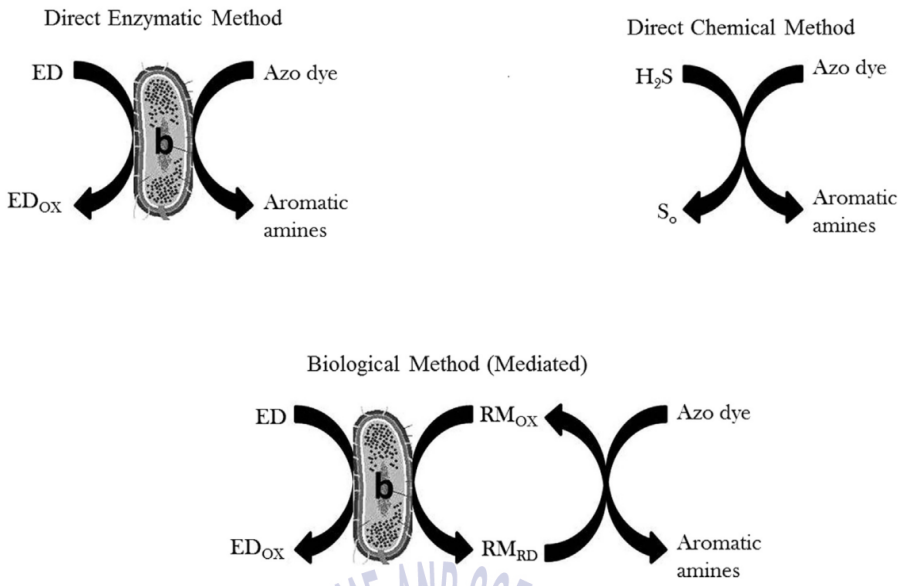

Figure 2.1 Various methods of degradation of azo dyes (RM = Redox mediator; ED = Electron donor; b = bacteria)

In the past decade, several bacteria that produce azoreductase were reported (**Table 2.2**). Catalytic proteins that possess azoreductase activity have been identified in various bacterial groups such as *Xenophilus azovorans KF46F*, *Pigmentiphaga kullae K24*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus sp. OY1-2*, and *Rhodobacter sphaeroides* [40-46]. However, actual involvement or efficiency of intracellular azoreductase in bacterial decolorization has been doubted in recent years since most of azo dyes have complex structures and high polarities which make them difficult to diffuse through cell membranes for reducing process.

Table 2.2 Decolorization of various azo dyes by Azoreductase producing bacterial culture

No.	Bacterial culture	Name of dye	Time (h)	Decolorization (%)	Ref.
1	*Acinetobacter radioresistens*	Acid Red	48	>70	[47]
2	*Alcaligenes* sp. *AA09*	Reactive Red BL	24	100	[48]
3	*Bacillus lentus BI377*	Reactive Red 141	6	99.11	[49]
4	*Bacillus megaterium*	Red 2G	NA	64.89	[50]
5	*Bacillus strain SF*	Reactive Black 5 Mordant Black 9	24	86 38	[51]
6	*Bacillus subtilis ORB7106*	Methyl Red	48	40-98	[52]
7	*Brevibacterium sp. strain VN-15*	RY107	96	98	[53]
8	*Escherichia coli JM109 (pGEX-AZR)*	Direct Blue 71	12	100	[54]
9	*Enterococcus gallinarum*	Direct Black 38	20	100	[55]
10	*Mutant Bacillus sp. ACT2*	Congo Red	37-48	12-30	[56]
11	*Pseudomonas aeruginosa*	Remazol Orange	24	94	[57]
12	*Proteus* sp.	Congo Red	48	67	[58]

The typical mechanism of bacterial cells for the redox mediator dependent reduction of azo dyes under anaerobic conditions is shown in Fig. 2.2. In the cell supernatant, however; the outcome of the reduction of the azo dyes is a mostly chemical redox reaction and mediators rely on cytoplasmic enzymes to supply electrons [59]. It is also reported that this chemical redox reaction works together with a direct enzymatic reaction involving an azoreductase, possibly a cytoplasmic dehydrogenase enzyme that is synthesized and secreted without intracellular accumulation [60].

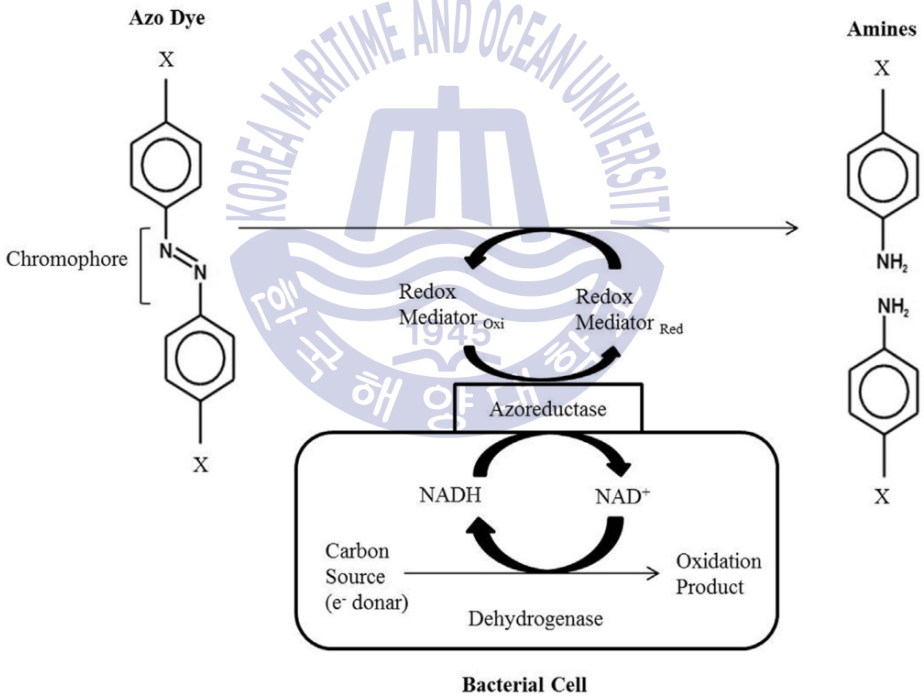

Figure 2.2 Proposed mechanism for degradation of azo dyes by azoreductase [61]

2.3 RNA sequencing for transcriptomic analysis

The transcriptome is the complete set of transcripts in a cell for a specific stage or physiological condition. It is essential to understand the transcriptome in order to interpret the functional elements of the genome and reveal the molecular constituents of cells and tissues, and also to understand the development and disease. The transcriptomics mainly aims to catalogue all species of transcript, including mRNAs, non-coding RNAs and small RNAs; for determination of the transcriptional structure of genes, in terms of their start sites, 5′ and 3′ ends, splicing patterns and post-transcriptional modifications. Also, it is to quantify the changes in expression levels of each transcript during development and under specific conditions [62].

Recently, 'omics' analysis has revealed the mechanisms of various biological processes. Especially, technology like proteomics enabled better understanding of fungal degradation of hazardous compounds including azo dyes [63,64]. However, it was still difficult to detect how those identified enzymes work each other in certain circumstance. Thus, there have been numerous attempts to monitor the expression of the some of the specific genes during azo dye degradation [65]. In that sense, RNA sequencing is a promising technology that will allow overall understanding of the entire genes expressed during azo dye degradation and their relationships.

Chapter 3. Materials and methods

3.1 Isolation of efficient microorganisms for azo dye biodegradation and their identification

In order to select cultures that would be effective for dye degradation, a commercial microbial consortium product for general wastewater treatment from Bayo, Inc., Jinju, South Korea was acclimated to the azo dye AB113 (200 mg/L). Upon noticeable decolorization while shaking at 120 rpm at room temperature for 6 days, various microorganisms were isolated on spread plates of tryptic soy agar (TSA). Initially, two dominant bacterial cultures (B-1 and B-2) and two yeast cultures (Y-1 and Y-2) were isolated and further tested for decolorization effectiveness compared with the parental consortium culture itself.

The four different cultures were taxonomically identified based on sequencing of the 16S or 26S rRNA genes, as shown in Fig. 1. The two bacterial cultures (B-1 and B-2) were identified using the 16S rRNA gene sequencing method. The target gene fragments were obtained using a modified colony PCR method in which the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1492R (5'-GGTTACCTTGTTACGACT T-3'), 518R, and 785F were used in PCR amplification. The amplified genes were purified using the PCR Product Purification Kit (Qiagen, Boston, MA, USA) and gene sequencing was performed using the Genetic Analyzer 3730xl (Applied Biosystems, USA).A homology search for the analyzed sequences within the NCBI Genebank database was conducted, and their phylogenetic positions were identified using CLUSTAL-W and MEGA6 software.According to the NCBI BLAST Search, B-1 was identified as Mesorhizobium sp. NBIMC_P2 (KF040403) with 99%

similarity. B-2 was identified as *Sphingomonas melonis* DAPP-PG 224T (KB900605) according to the EzbioCloud database (Chunlab, Seoul, South Korea).The two yeast cultures (Y-1 and Y-2) were identified using the 26S rRNA sequencing method.The primers Y-F (5'-GCATATCAATAAGCGGAGGAAAAG-3') and Y-R (5'-GGTCCGTGTTTC AAGACG-3') were used in PCR amplification. The amplification, purification, and homology search processes were performed following the same procedures used for bacterial identification. According to the NCBI BLAST Search, Y-1 and Y-2 were identified as *Apiotrichum mycotoxinivarans* CBS: 10094 (KB900605) and *Meyerozyma guilliermondi* GJ8-2 (KU316708), both with 99% homology.

All individual cultures were incubated on TSA, and pure cultures were preserved in phosphate buffer with glycerol (15%) at 80 ℃. Prior to the experiment, frozen cultures were recovered on TSA, and then inoculated into mineral salts medium (MSM) and incubated in a shaking incubator at 27 ℃ and 150 rpm for 24 hours.

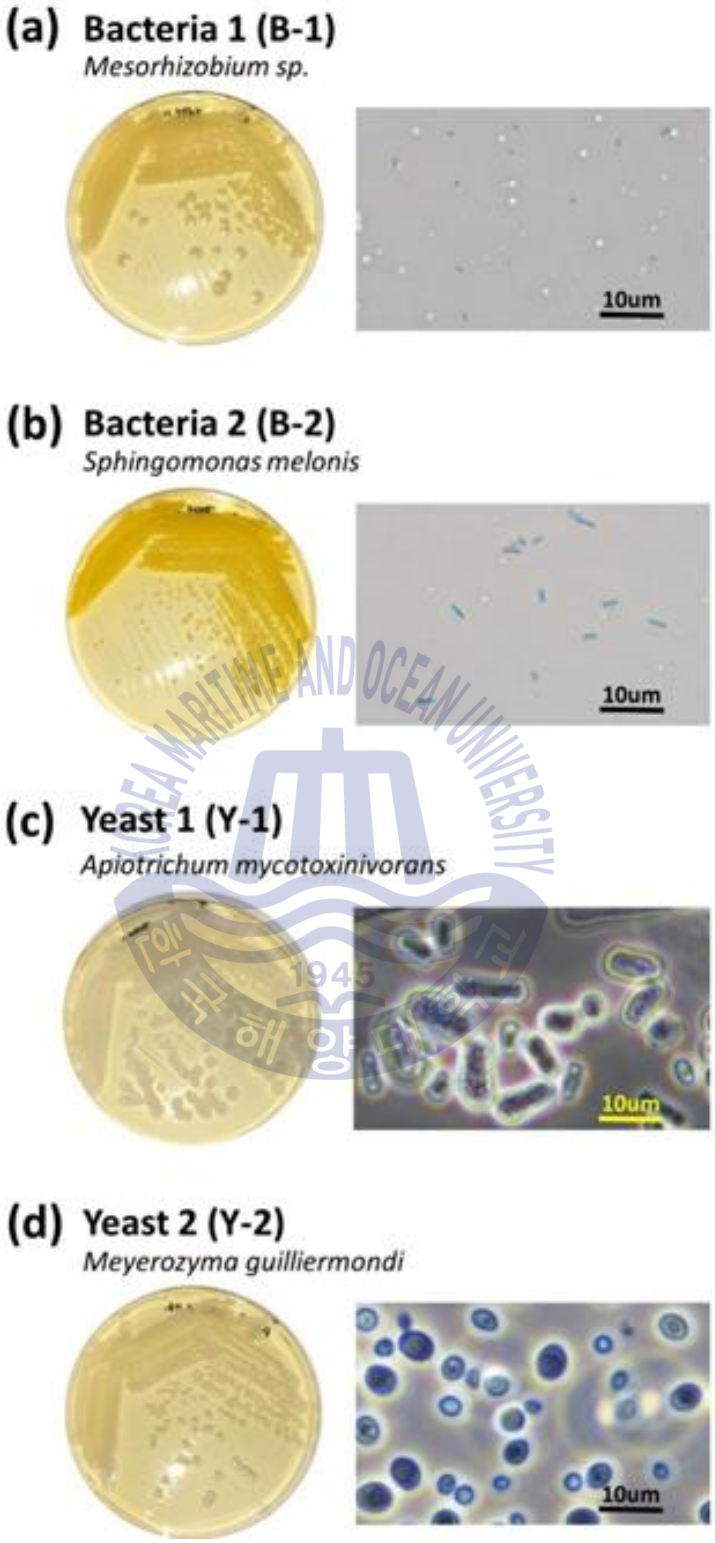

Figure 3.1 Colonial and cellular morphologies of the cultures isolated and identified in this study

3.2 Experimental culture setup for biodegradation of the azo dye Acid Blue 113

The Acid Blue 113 (CAS No. 3351-05-1) dye used in this study was purchased from Sigma Aldrich, South Korea. The cultures for biodegradation testing were set up according to the plan shown in Table 3.1; each culture carried 100 ml of MSM containing 0.03% (w/v) of glucose and 0.0006% (w/v) of yeast extract. The MSM had the following composition (g/L): Na_2HPO_4, 3.6; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 1.0; $Fe(NH_4)$ citrate, 0.01; $CaCl_2.2H_2O$, 0.1, and 10 mL of a trace element solution of the following composition (mg/L) was added to 1 L of the MSM: $ZnSO_4 \cdot 7H_2O$, 10.0; $MnCl_2 \cdot 4H_2O$, 3.0; $CoCl_2 \cdot 6H_2O$, 1.0; $NiCl_2 \cdot 6H_2O$, 2.0; $Na_2MoO_4 \cdot 2H_2O$, 3.0; H_3BO_3, 30.0 and $CuCl_2 \cdot 2H_2O$, 1.0 [5].

After autoclaving the MSM, the stock solution of glucose and yeast extract, which was filter-sterilized using Acrodisc syringe filters (0.2 μm; Pall Laboratory, Westborough, MA, USA), was added to the medium with AB113 dye at different dye concentrations (20, 50, and 100 mg/L). The medium of each culture was inoculated with an aliquot of each of the 24 h grown cultures (5%, v/v) and incubated at 27 ° C while shaking at 150 rpm. An appropriate amount of culture in each culture was withdrawn for spectrophotometric measurement of growth (at 600 nm) and azo dye biodegradation (at 560 nm).

Table 3.1 Setup of microcosms for biodegradation testing of the azo dye Acid Blue 113 using various cultures

Microcosms*	Species of cultures grown in microcosm	Inoculation amount (%)	Applied conc. of AB 113 (mg L-1)
B-1	*Mesorhizobium* sp.	5	20, 50, 100
B-2	*Sphingomonas melonis*	5	20, 50, 100
Y-1	*Apiotrichum mycotoxinivarans*	5	20, 50, 100
Y-2	*Meyerozyma guillermondi*	5	20, 50, 100
B-C [a]	*Mesorhizobium* sp. *Sphingomonas melonis*	2.5 2.5	20, 50, 100
Y-C [b]	*Apiotrichum mycotoxinivarans* *Meyerozyma guillermondi*	2.5 2.5	20, 50, 100
T-C [c]	*Mesorhizobium* sp. *Sphingomonas melonis* *Apiotrichum mycotoxinivarans* *Meyerozyma guillermondi*	1.25 1.25 1.25 1.25	20, 50, 100

* Each microcosm contains 100 mL of mineral salts medium (MSM) in an Erlenmeyer flask (250 mL) together with 0.03% glucose and 0.0006% yeast extract, incubated at 27 ℃ for 4 days in a shaking incubator (150 rpm).
[a] Bacteria consortium, [b] Yeast consortium, [c] Total microbial consortium.

3.3 UV-vis spectrophotometric analysis for cell growth and dye biodegradation

For cell growth monitoring, one milliliter of sample from each microcosm was regularly withdrawn and its growth monitored at λ_{600nm}.

For dye degradation monitoring, samples were withdrawn at regular intervals and centrifuged at 8000 rpm for 15 min. The absorbance of each supernatant was measured at maximum absorbance wavelength,

λ_{560nm}, using a UV visible spectrophotometer (Optizen POP, K Lab, South Korea).The decolorization efficiency was expressed as in the following equation, as previously described [66]:

$$\text{Decolorization rate (\%)} = \frac{A_{initial} - A_{final}}{A_{initial}} \times 100$$

where Ainitial and Afinal represent the initial and final absorbances at λ_{560nm}.

3.4 Monitoring of azo dye biodegradation based on FT-IR analysis

Samples were withdrawn at regular intervals and centrifuged at 8000 rpm for 15 min. The supernatants were then lyophilized without exposure to light. The lyophilized samples were examined in a Fourier transform infrared (FT-IR) spectrophotometer (Spectrum GX, PerkinElmer LST, South Korea) over the wavelength (4000–650 cm^{-1}) using the attenuated total reflection (ATR) method.

3.5 Monitoring of the fate of organic compounds (azo dye and glucose) based on COD analysis

It was necessary to monitor changes in concentrations of the only organic compounds (glucose as a reducing agent and azo dye AB113). This was because an absolute disappearance might be linked to the mineralization of these substrates through CO2generation.COD was measured using a commercial water analysis kit (HS-COD(Mn)-L, HUMAS, Daejon, South Korea). Samples were procured at regular intervals and analyzed according to the manufacturer's protocol.

3.6. RNA isolation and sequencing procedure for RNA-seq analysis for the dye degradation

In order to examine the different gene expression profiles and to perform gene annotation on set of useful genes based on gene ontology pathway information, whole transcriptome sequencing of Sphingomonas melonis was performed. The analysis compared the two groups of S. melonis growth depending on the experimental conditions the control (cultured in absence of azo dye Acid Blue 113 at 50mg/L) and the treatment (cultured in presence of azo dye Acid Blue 113 at 50mg/L) using RNA-sequencing. Total RNA from the control and treated samples were isolated and contaminated DNA was eliminated. The Ribo-Zero rRNA Removal Kit (Bacteria: Illumina, San Diego, CA, United States) was used to purify the RNA. The sequencing library was constructed using TruSeq Stranded Total RNA Sample Prep Kit (Illumina, San Diego, CA, United States) according to the manufacturer's protocol. These purified RNA molecules were randomly fragmented for short read sequencing and reverse transcribed into cDNA. Then, sequencing adapter is ligated onto both ends of the cDNA fragments. After amplifying fragments using PCR, fragments with insert sizes between 200-400 bp were selected and sequenced using Illumina's HiSeq 4000.

Chapter 4. Results and discussion

4.1 Dye decolorization by various cultures

The time courses for the decolorization rates (%) for all cultures at different concentrations of AB113 dye are shown in **Fig. 4.1**. Cultures B-1 and B-2 achieved maximum decolorization rates of approximately 67.8% and 75.4% in 50 mg/L dye within 72 h and 48 h, respectively (**Fig. 4.1a and b**).

The lower decolorization rate for B-1 seemed to be linked to its slow growth, and its maximum decolorization ability appeared after 60 h. In the consortium B-C, the slow growth of B-1 appeared to retard the faster growth of B-2 (50 and 100 mg/L dye), while B-1 did not interfere with the decolorization activity of B-2 at the lower dye concentration (20 mg/L).

As shown in **Fig. 4.1d and e**, Y-1 decolorized approximately 77.1% in 9 h while Y-2 achieved a decolorization rate of about 60% in 48 h (50 and 100 mg/L dye), indicating that Y-1 was a significantly faster degrader of the dye. However, when both of the yeast cultures were combined, not much of a synergistic effect was observed in decolorization, and even the speed was retarded. This might be because there was competition among these two cultures for glucose as the reducing agent required for decolorization.

Obviously, the Y-C decolorization rate was much higher than that of the B-C. Yeasts seemed to be less sensitive to the dye concentration gradient, while B-C decolorization was more negatively affected by the higher dye concentration. In general, however, decolorization rates

gradually increased over time. The total consortium carrying all four cultures (B-1, B-2, Y-1, and Y-2) achieved more than 80% decolorization after 24 h (50 and 100 mg/L dye) since the slow decolorization of B-C was overcome by the Y-C (**Fig. 4.1**).

Overall, T-C might be useful in bioremediation of azo dye materials and its degradative products since the consortium carrying all four members in an interaction would be more resilient to fluctuations in environmental conditions (e.g., kinds and amounts of target substrates, salinity, pH, temperature, ORP, etc.) than individual cultures [22,23].

The relatively lower decolorization activity of B-1 (20 mg/L dye) seemed to be due to a high concentration of carbon source compared to its initial dye concentration. Previous research reported that in biological decolorization of azo dyes, microorganisms require a carbon source as they are unable to use the azo compounds as a sole carbon source [67]. Nevertheless, excessive amounts of carbon can reduce the decolorization rate as microorganisms prefer to consume external carbon sources rather than dyes [68].

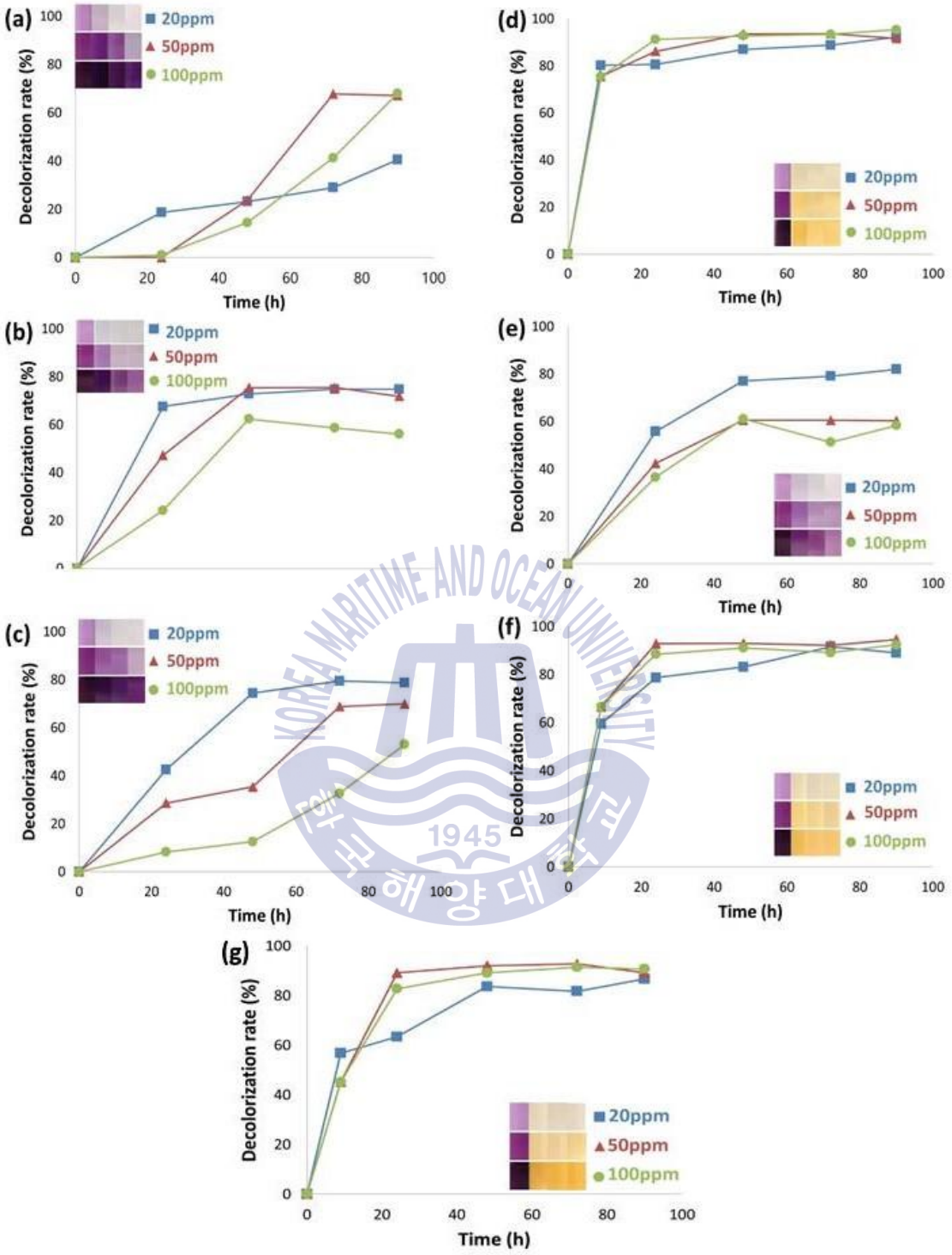

Figure 4.1 Time course for the azo dye decolorization rates for various pure cultures and consortia of bacteria and yeasts. (a) B-1, (b) B-2, (c) B-C, (d) Y-1, (e) Y-2, (f) Y-C, and (g) T-C

4.2 Monitoring of organics through COD analysis

The starting COD was in the range of 1314-1780 mg/L in the presence of glucose (300 mg/L) and the dye (50 mg/L); the control without inoculum showed the highest COD (Fig. 3). Higher COD removal rates of bacteria were observed in the order of B-2, B-1, and B-C, while those of yeasts were in the order of Y-1 (equal to Y-C) and Y-2, indicating there was little synergistic effect of COD removal for the B-C. The COD removal rate for B-C reached 97% in 72 h while Y-C and T-C achieved 98.0% and 97.5%, respectively, in 24 h. Therefore, the COD removal rates of Y-C (69.3 mg h^{-1}) and T-C (66.0 mg h^{-1}) were about 3-fold faster than that of B-C (22.2mgh-1). This appeared to be because the bacteria grew relatively slowly and took longer in COD removal, although the final COD removal rate was similar to other treatment. Also, the COD removal rate of T-C was slightly lower than that of Y-C, probably due to bacterial interference with the yeast cell metabolism of the organic substrates. In all, three different consortia showed similar COD removal rates after 3 days. It is worth mentioning that higher degrees of COD removal seemed to be related to higher decolorization rates regardless of whether pure cultures or consortia were used.

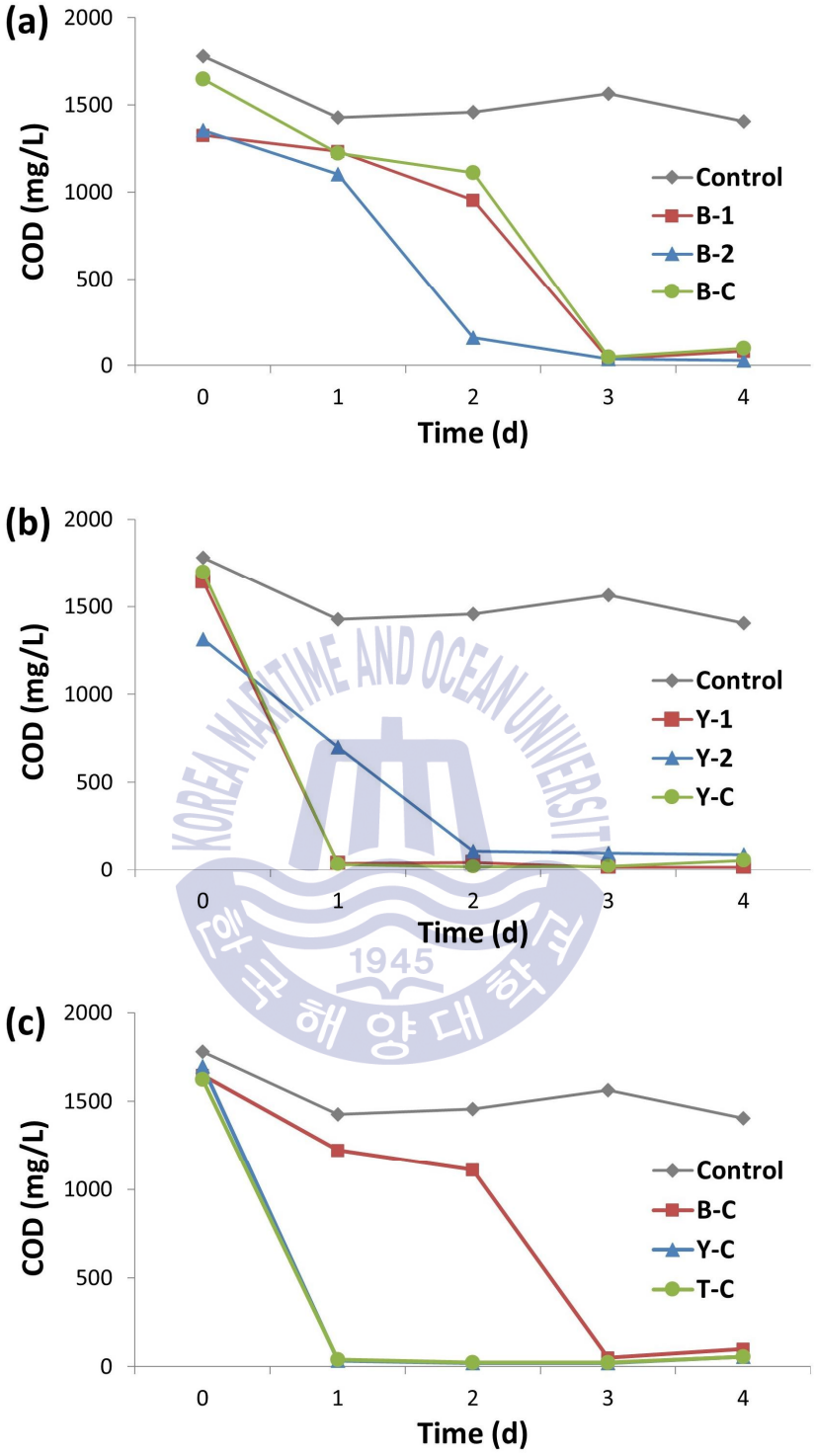

Figure 4.2 Time course for COD removal by various pure cultures and consortia of bacteria and yeasts during biodegradation of the azo dye Acid Blue 113 (50 mg/L). (a) bacteria, (b) yeasts, and (c) consortia

4.3 Comparison of cultural growth and concomitant decolorization activity in the microbial consortia

The cell population density (OD_{600}) and its concomitant decolorization activity at 560nm over time were comparatively analyzed (**Fig. 4.3**). At the beginning (Days 1 and 2), the growth of the B-C was much slower than that of the yeast. In the overall consortia, there was a strong negative correlation between OD_{600} and the λ_{560nm} because OD_{600} increased as the absorbance at 560nm decreased. The B-C had a longer lag phase (2days) while the Y-C seemed to have a shorter lag period. B-C reached its plateau growth after 3 days, while it took only a day for Y-C to reach its plateau.

There was a good positive relationship between the cell growth and the azo dye degradation rate. The OD_{600} for the Y-C decreased by 50% after significant decolorization due to the rapid depletion of glucose as a substrate for growth metabolism and the azo bond (-N=N-) reduction. However, T-C maintained its level (1.5 OD_{600}) due to the presence of B-C. This may indicate that the bacteria community could help the yeast community survive under adverse growth conditions.

The collaborative interaction between bacterial and yeast communities provides a great advantage for the bioremediation of azo dye compounds on a field scale as it has been shown that they can stably maintain a specific consortium that is more resilient to the target degradative compounds, their potentially toxic metabolites, and other nearby hazardous substrates as well as to varying environmental conditions.

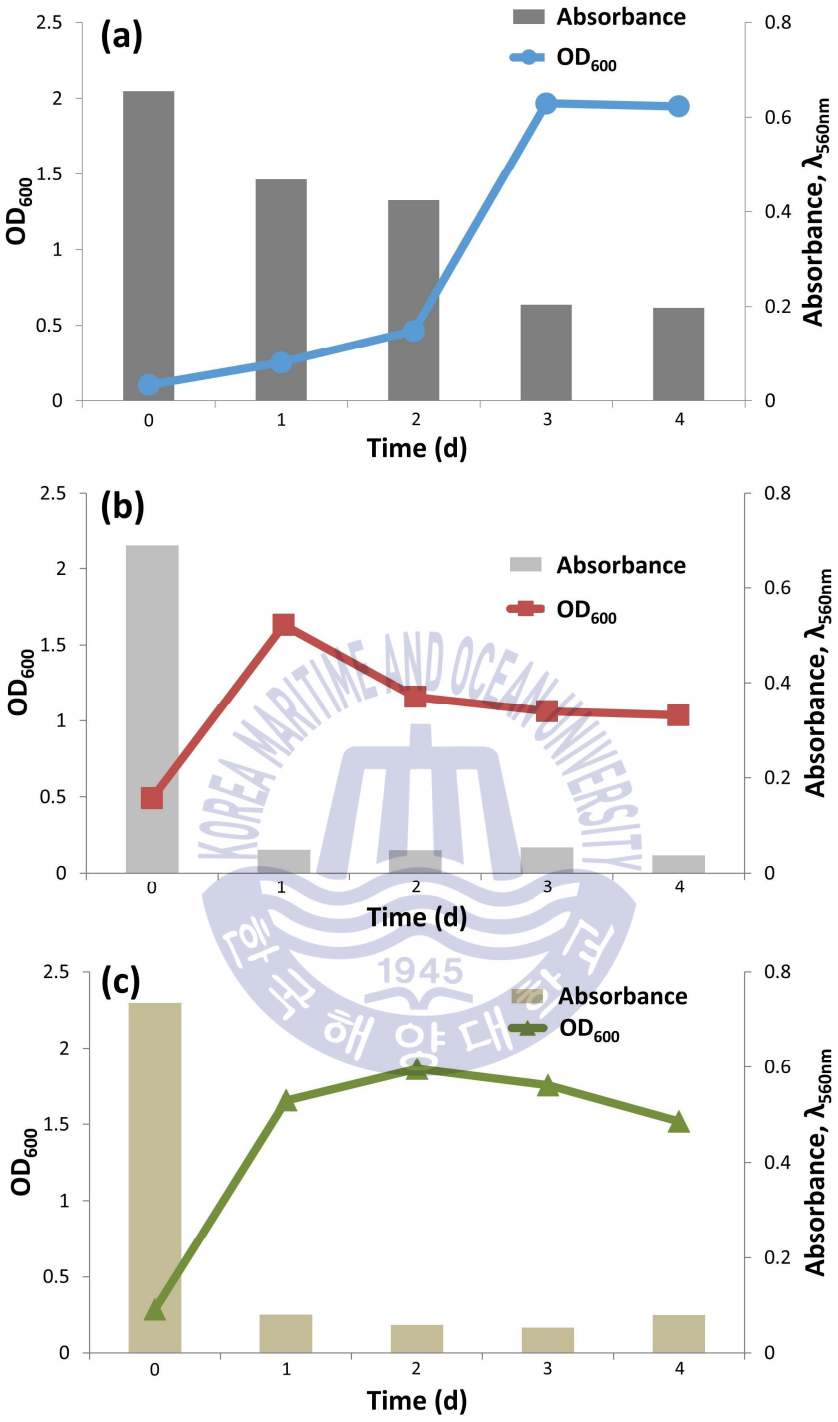

Figure 4.3 Comparative analysis of consortium cultural growth and concomitant decolorization of the azo dye Acid Blue 113 (50 mg/L) over time. (a) B-C, (b) Y-C, and (c) T-C

4.4 FT-IR and GC-MS analyses of the biodegradation process by microbial consortia for the azo dye AB113

The FT-IR profiles for the three different consortia during dye biodegradation are shown in **Fig. 4.4**. FT-IR spectra analysis enables the determination and understanding of the various functional groups involved in the process of biotransformation [69]. The peak at 1455 cm^{-1} indicates the presence of N=N stretching due to azo bonds [70]. The disappearance of this particular peak, therefore, means that decolorization has been achieved. As shown in **Fig. 4.4**, the arrows may indicate evidence of azo bond cleavage. All profiles showed decreasing intensity of peaks at the wavenumber 1455 cm^{-1}, in which the decreasing rate of yeast-containing consortia (Y-C and T-C) were much faster than that of B-C. These results corroborated with the previous decolorization results (**Fig. 4.1**) and both results confirm that significant decolorization was achieved after 72 h for B-C and 24 h for both Y-C and T-C. It was also observed that the number of OH or NH groups increased in all the consortia as the intensity of the 3210 cm^{-1} region increased as the dye degraded. The presence of OH group is expected to be related with the aromatic compounds that were detected by GC-MS (**Table 4.1**) Decanedioic acid and propanoic acid possess the OH group in their chemical structures. Again, the transmittance at 3210 cm^{-1} showed a similar trend to that of 1455 cm^{-1} for the azo bond. Moreover, the NH_2 groups were detected in the degraded samples at the 1300 cm^{-1} wavenumber. This is evidence of the biodegradation of AB113 [66, 71]. These NH_2 groups are also present in aniline which was detected as metabolites after decolorization. In addition, the size of the peak at 1100 cm^{-1} representing SO_2 decreased over time in B-C (**Fig. 4.4a**) due to bacterial biodegradation, as shown in a previous report [70]. In contrast,

the yeast group was not able to degrade this group, while gradual decrease of this peak was observed in T-C, which carried the B-C (**Fig. 4.4c**). In terms of secondary metabolites after decolorization, both the bacterial cultures showed their efficient degradative activity.

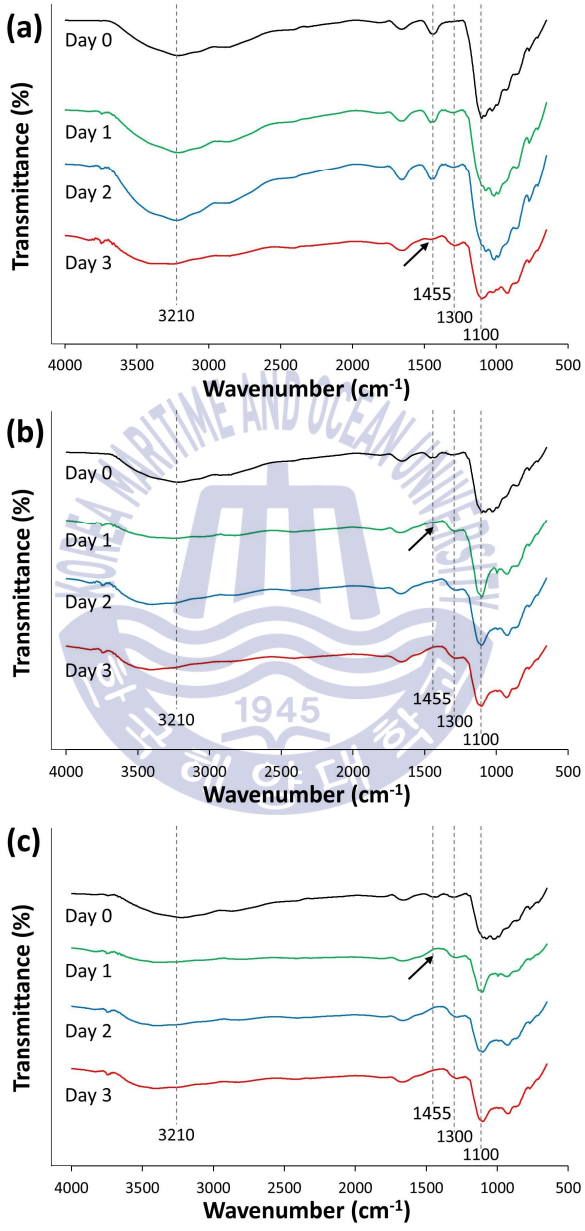

Figure 4.4 Time course for the FT-IR profiles of the three microbial consortia during biodegradation of the azo dye Acid Blue 113. (a) B-C, (b) Y-C, and (c) T-C

1-Butanol, propanoic acid, ethyl ester, 1,2-Benzenedicarboxylic acid (phthalic acid), mono(2-ethylhexyl) ester, decanedioic acid and bis(2-ethylhexyl) ester appeared to be background materials that might have been present as contaminants in Acid Blue 113. Butanoic acid (butyric acid) and aniline could be potential metabolites from the dye. There should be a further experiment to elucidate more metabolites in order to identify the degradation pathway of the dye.

Table 4.1 Metabolites from degradation of Acid Blue 113 detected using GC-MS

RT (min)	Metabolites	Control*	An-1	An-3	An-4	An-5	Mixed**
4.651	1-Butanol	+	+	+	+	ND	ND
4.904	Propanoic acid, ethyl ester	+	+	+	+	ND	+
5.296	Butanoic acid (butyric acid)	ND	ND	+	+	ND	ND
8.194	Aniline	ND	ND	ND	+	ND	ND
26.02	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	+	+	+	+	+	+
28.85	Decanedioic acid,bis(2-ethylhexyl) ester	+	+	+	+	+	+

4.5 Implications of the synergistic effects of inter genus co-culture in terms of bioremediation of azo dyes

Despite well-reported research on the biodegradation of azo dyes using a single culture, more successful degradation and complete mineralization can be achieved when a mixed culture is used [3, 24, 72]. In particular, bacteria are regarded as one of the most powerful means of bioremediation in terms of dye degradation. However, bacteria are sensitive to intermediate products such as carcinogenic aromatic amines, which can inhibit their large-scale activity [19, 73]. On the other hand, yeast can rapidly degrade these compounds using extracellular enzymes [20]. In this sense, the synergistic effect of a yeast-bacterial consortium may lead to enhanced degradation and detoxification of azo dyes, and thus provide a promising method for the efficient removal of azo dye contaminants [19, 74].

Therefore, a consortium of selected cultures could provide an effective approach for treating wastewater containing complex dye materials [72]. The physicochemical condition of wastewater is unstable, especially when the treatment system is exposed to the atmosphere. A combination of several cultures is able to easily adjust to the environment and effectively degrade the dyes [24]. Multi-strains of microcosm are able to attack the recalcitrant dye molecule at different stereo-specific positions or can collaboratively degrade the subsequencing metabolites further and may lead to eventual mineralization of the dyes with its ecological relevance [6,75].

4.6. Transcriptomic analysis of degradation of Acid Blue 113

The transcriptome represents the complete set of transcript in a cell under specific condition or stage [62]. For transcriptomic analysis, RNA sequencing is now actively applied in many research fields. **Figure 4.5** shows the brief mechanism and process of RNA sequencing that was applied in this study. The analysis compared the two groups of *S. melonis* growth depending on the experimental conditions the control (cultured in absence of azo dye Acid Blue 113 at 50mg/L) and the treatment (cultured in presence of azo dye Acid Blue 113 at 50mg/L) using RNA-sequencing. Total RNA was extracted for both groups and the cDNA was prepared through the reverse transcription. Then, all the genes were sequenced after several trimming process. Also, their quantity was identified so that it allowed the comparison of gene expression under the non-induction condition and induction condition using the dye. In the actual analysis for the study, the expressed values were normalized as shown in **Fig 4.6**.

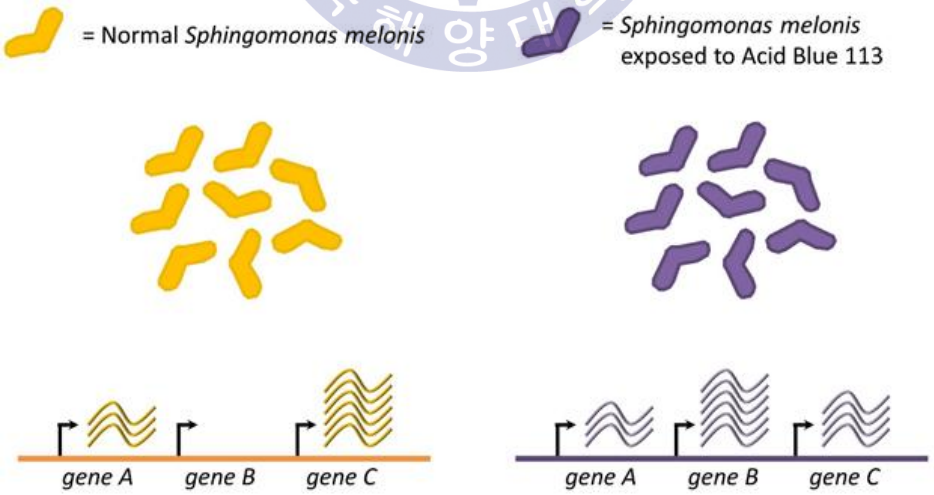

Figure 4.5 Scheme of RNA-seq analysis for the degradation of the azo dye Acid Blue 113 by *Sphingomonas melonis* under non-induction (left) and induction (right) conditions

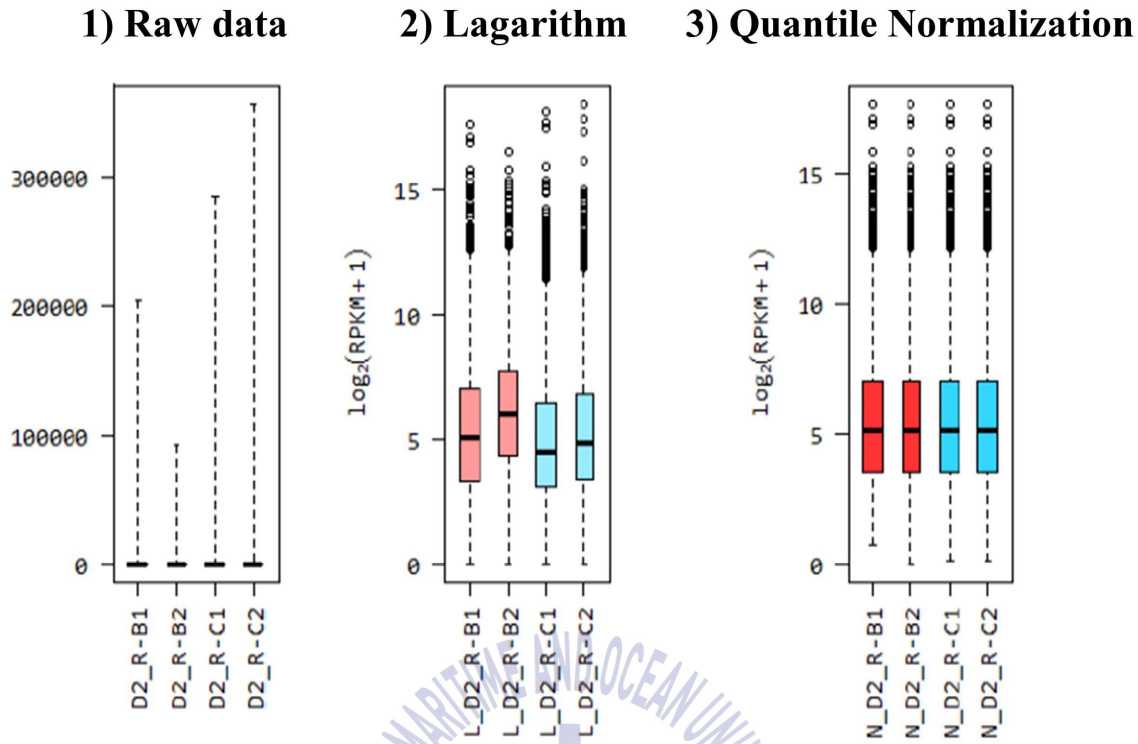

Figure 4.6 Box plots for normalization of the number of expressed genes during the degradation of the azo dye Acid Blue 113 by *Sphingomonas melonis* under non-induction ("C") and induction ("B") conditions

After RNA sequencing, trimming and normalization, 3830 differentially expressed genes (DEGs) were identified. Among them, significantly different DEGs were 272: 131 DEGs were up-regulated and 141 DEGs were down-regulated. This meant that 131 DEGs were expressed significantly higher in S. melonis exposed to Acid Blue 113 than that of the non-induction condition while 141 DEGs were expressed less than the control experiment.

Out of the 272 significantly expressed DEGs, top 25 genes which had the highest differences between the groups were shown in **Fig 4.7** as a

heatmap. In case of NAD(P)-dependent oxidoreductase, its expression was higher in treatment than control group. It is assumed that this oxidoreductase is responsible for azo group degradation, or hydroxyl acids and amino acids degradation [76]. Type 1 glutamine amidotransferase was also differentially expressed in the dye treatment. This enzyme is known to be involved in or responsible for aromatic compounds degradation pathways [75,78]. In addition, quinone oxidoreductase was highly expressed in treatment group (data not shown) and it was reported that it could enhance the azo dye decolorization [79].

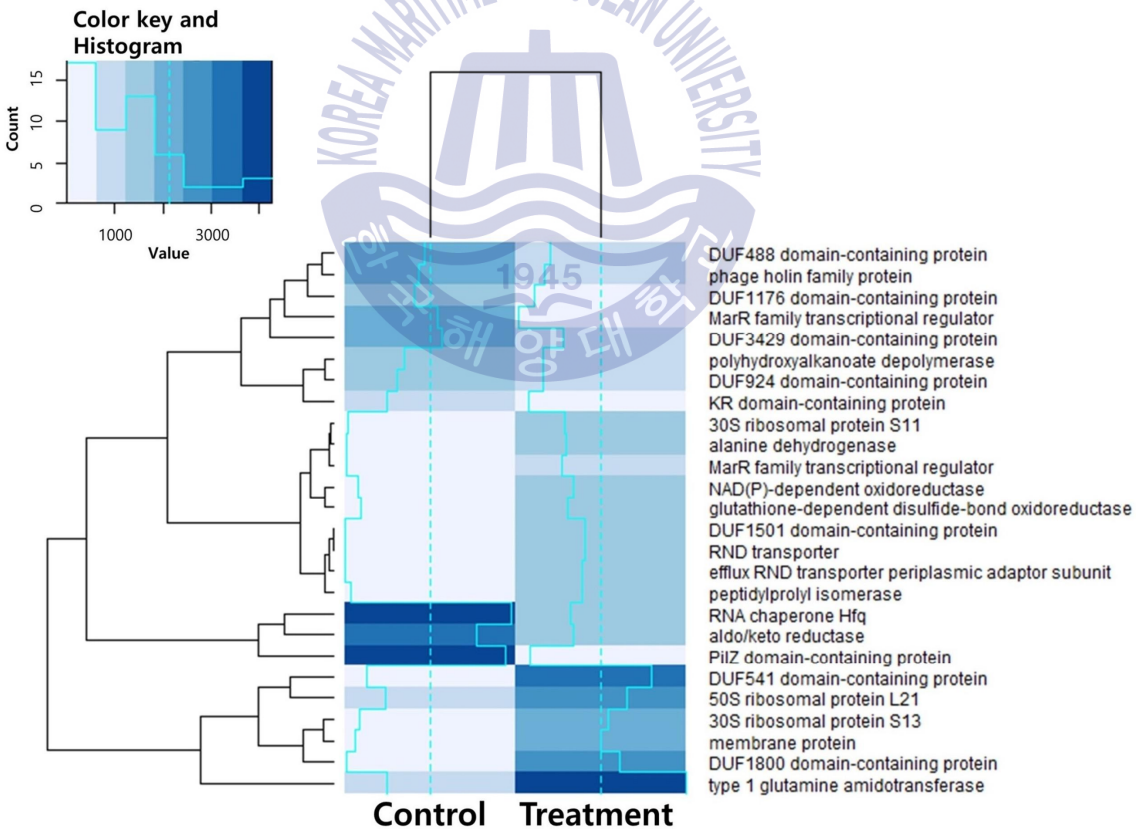

Figure 4.7 Heatmap for the top 26 genes differentially expressed under the azo dye Acid Blue 113 induction (treatment) condition

The 272 DEGs grouped according to functional categorization of gene ontology (**Fig 4.8**). They were mainly divided into three categories: biological process, cellular component and molecular function. The high number of genes was observed in cellular process and metabolic process in biological process and catalytic activity in molecular function. The genes that were mentioned were all included in metabolic process and catalytic activity. Furthermore, the genes were categorized according to species specific functional annotation as shown in **Table 4.2**. The eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) functional annotation of the genes produced the 16 different categories including 6 up-regulated categories and 10 down-regulated categories. Among the various categories, the carbohydrate transport and metabolism category was dominantly up-regulated and this might be due to the presence of carbon sources (i.e., glucose or dye) and their degradative products which can be utilized by the dye degrader.

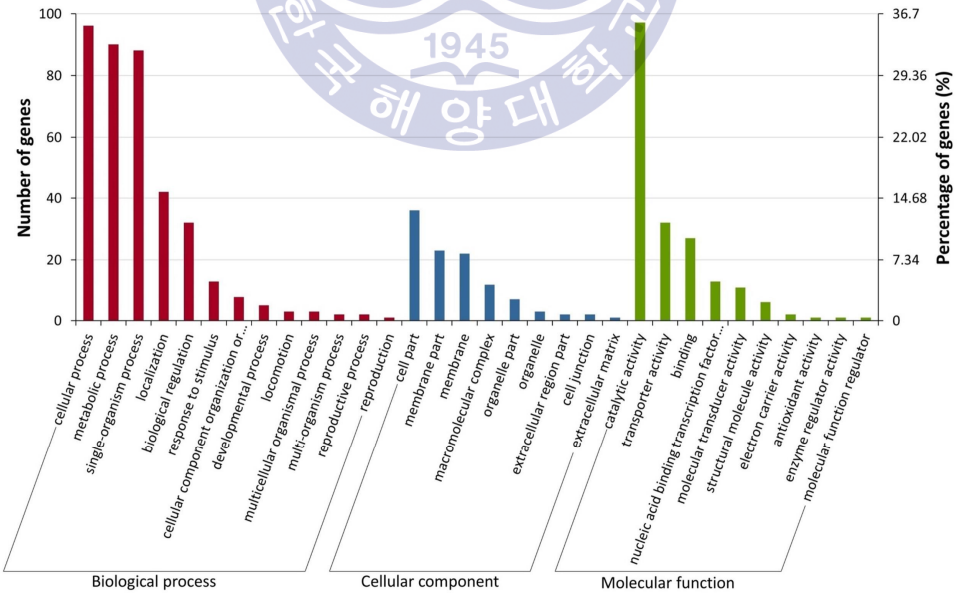

Figure 4.8 Functional categorization as gene ontology of genes differentially expressed under the azo dye Acid Blue 113 induction (treatment) condition

Table 4.2 eggNOG functional annotation and respective number of genes

eggNOG functional annotation	Number of genes	Up	Down
Amino acid transport and metabolism	12	12	0
Carbohydrate transport and metabolism	24	24	0
Cell motility, Intracellular trafficking, secretion, and vesicular transport	1	1	0
Cell wall/membrane/envelope biogenesis	6	6	0
Energy production and conversion	11	11	0
Function unknown	39	39	0
General function prediction only	9	0	9
Inorganic ion transport and metabolism	11	0	11
Lipid transport and metabolism	11	0	11
Nucleotide transport and metabolism	4	0	4
Post-translational modification, protein turnover, and chaperones	6	0	6
Replication, recombination, and repair	2	0	2
Secondary metabolites biosynthesis, transport, and catabolism	3	0	3
Signal transduction mechanisms	2	0	2
Transcription	12	0	12
Translation, Ribosomal structure, and biogenesis	12	0	12

Chapter 5. Conclusion

In this study, two bacterial cultures (*Mesorhizobium sp.* and *Sphingomonas melonis*) and two yeast cultures (*Apiotrichum mycotoxinivarans* and *Meyerozyma guilliermondi*) successfully decolorized the azo dye Acid Blue 113 and further showed the potential for an efficient biodegradation of secondary metabolites in the wastewater. More than 80% of decolorization within 24 h (50 and 100 mg/L dye) was achieved by the selected consortium composed of two bacterial cultures (*Mesorhizobium sp.* and *Sphingomonas melonis*) and two yeast cultures (*Apiotrichum mycotoxinivarans* and *Meyerozyma guilliermondi*). The yeast consortium and the total consortium (bacterial and yeast consortia combined) showed 98.0 % and 97.5 % of COD removal rates, respectively in 24 h, while the rate for the bacterial consortium reached 97% in 72 h, indicating a synergism between these inter-genus cultures and a potential mineralization of the azo dye Acid Blue 113. Moreover, there was a good positive relationship between the cell growth and the azo dye degradation rate which can also support the mineralization potential. It was concluded that all the data of decolorization, COD removal, cell growth, FT-IR spectra and GC-MS analysis results collectively provide a consistent evidence for the azo dye decolorization and a potential mineralization of the dye by the stably maintained bacterial and yeast cultures. Furthermore, transcriptomics data based on the RNA-sequencing showed that there was a significant difference in the gene expression of *Sphingomonas melonis* depending on the exposure to azo dye Acid Blue 113. Out of 3830 DEGs, the number of significantly different DEGs were 272: 131 DEGs were up-regulated and

141 were down-regulated. Especially, some of the highly up-regulated genes such as NAD(P)-dependent oxidoreductase from the treated group that was exposed to Acid Blue 113 appeared to be related to azoreductase which is a major enzyme responsible for cleavage of azo bond in the azo dye. In overall, these results may support that biological wastewater treatment using the bacterial-yeast consortium has a potential to be applied in many other related industrial wastewater treatments as well as the azo dye wastewater treatment in the future. Moreover, the transcriptomics technology based on RNA-sequencing to determine the unique gene expressions could enable the understanding of the azo dye biodegradation mechanisms of various microorganisms.

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Academic achievement

▶ **Peer-reviewed journal article**

1. Chaeyoung Rhee · Haham Kim · Aalfin Emmanuel · Hong-Gi Kim · Seonghun Won · Jinho Bae · Sungchul C. Bai · Sung-Cheol Koh, Microbial community analysis of eco-friendly recirculating aquaculture system for olive flounder (*Paralichthys olivaceus*) utilizing the complex microbial probiotics, *Korean Journal of Microbiology*, 54 (2018) 369-378

▶ **Conference presentations (International)**

1. Chaeyoung Rhee · Aalfin Emmanuel · Sung-Cheol Koh, Biodegradation of azo dye Acid Blue 113 by Sphingomonas melonis and its transcriptomic analysis of degradation pathway, *17th International Society of Microbial Ecology*, Leipzig, Germany, Aug 12 -17th, 2018

2. Aalfin Emmanuel · Chaeyoung Rhee · Woo-Jun Sul · Hoon-Je Seong · Sung-Cheol Koh, Metagenomic Analysis of Relationships between Carbon and Denitrification Metabolisms in Tannery Wastewater Treatment Plant Bioaugmented with Novel Microbial Consortium BMS-1, *9th International Conference on Environmental Science and Technology*, Houston, Texas, United States, Jun 25-29th, 2018

3. Chaeyoung Rhee · Aalfin Emmanuel · Hong-gi Kim · Sung-Cheol Koh, Efficient Biodegradation of Azo Dye Acid Blue 113 by Bacterial and Yeast Consortium and Molecular Analysis of Bacterial Biodegradative Pathway, *9th International Conference on Environmental Science and Technology*, Houston, Texas, United States, Jun 25-29th, 2018

4. Chaeyoung Rhee · Aalfin Emmanuel · Jae-Soo Chang · Sung-Cheol Koh, Effective and Eco-friendly Bioremediation of Acid Blue 113 Dye using a Microbial Bioaugmentation Technology, *International Environmental Engineering Conference*, Jeju, Korea, Nov 15-17th, 2017

5. Aalfin Emmanuel · Chaeyoung Rhee · Jae-Soo Chang · Sung-Cheol Koh, Metagenomic Analysis of Nitrogen Removal and Sludge Reduction Mechanisms in Tannery Wastewater Treatment Plant Bioaugmented with the Novel Microbial Consortium BMS-1, *International Environmental Engineering Conference*, Jeju, Korea, Nov 15-17th, 2017

▶ **Conference presentation (Domestic)**

1. 이채영 · Aalfin Emmanuel · 고성철, Sphingomonas melonis 를 활용한 azo dye의 생분해 및 전사체 분석을 통한 분해경로 이해, *2018 대한환경공학회*, 광주, 대한민국, Nov 14-16th, 2018

2. Chaeyoung Rhee · Aalfin Emmanuel · Hong-gi Kim · Sung-Cheol Koh, Azo Dye Biodegradation Using Bacterial and Yeast Bioaugmentation Technology, *2018 한국미생물학회*, 평창, 대한민국, Apr 25-27th, 2018

3. Woo-Jun Sul · Chaeyoung Rhee · Hoon-Je Seoung · Aalfin Emmanuel · Sung-Cheol Koh, Metagenomic Analysis of Relationships between Carbon and Nitrogen Metabolisms during Tannery Wastewater Treatment Undergoing a Microbial Bioaugmentation, *2017 한국미생물학회*, 부산, 대한민국, Apr 26-28th, 2017

▶ **Award**

Graduate Scientists' Forum Outstanding Presentation Awards, 2018 한국미생물학회, 평창, 대한민국, Apr 25-27th,2018