STUDY ON EFFECTS OF BORON AND ARSENIC IN YEAST CELLS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY
SEZİN ATICI

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CHEMISTRY

AUGUST 2016

Approval of the Thesis;

STUDY ON EFFECTS OF BORON AND ARSENIC IN YEAST CELLS

submitted by **SEZİN ATICI** in a partial fulfillment of the requirements for the degree of **Master of Science in Chemistry Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Cihangir Tanyeli
Head of Department, **Chemistry** _____

Assoc. Prof. Dr. Gülay Ertaş
Supervisor, **Chemistry Department** _____

Examining Committee Members:

Prof. Dr. Mürvet Volkan
Chemistry Department, METU _____

Assoc. Prof. Dr. Gülay Ertaş
Chemistry Department, METU _____

Assoc. Prof. Dr. Ali Çırpan
Chemistry Department, METU _____

Assist. Prof. Dr. Betül Sopacı
Chemistry Department, Ahi Evran University _____

Assist. Prof. Dr. Özgül Persil Çetinkol
Chemistry Department, METU _____

Date: 26.08.2016

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Sezin Atıcı

Signature:

ABSTRACT

STUDY ON EFFECTS OF BORON AND ARSENIC IN YEAST CELLS

Atıcı, Sezin

M.S., Department of Chemistry

Supervisor: Assoc. Prof. Dr. Gülay Ertaş

August 2016, 90 pages

Arsenic (As) has been the center of attention for researchers regarding its toxic effects in metabolism. Boron (B) can be both essential and toxic depending on its concentration level. The largest known boron deposits are in Turkey, the largest producer of boron minerals. Besides, there are some regions such as Balıkesir-Bigadiç and Kütahya-Emet having very high concentration of B and As in natural sources.

In this thesis, an analytical method based on growing yeast cells in the presence of B and/or As followed by determination using ICP-OES and ICP-MS was developed to follow the distribution of B(III) and As(III) in yeast cells and growth media separately. Yeast cell growth was followed by optical density measurements at 600 nm. Based on optical density measurements, 9^{th} hour was assigned as logarithmic phase and 24^{th} hour as stationary phase. Yeast cell growth decreased 30% in the presence of As compared to control group of yeast cells fed only with yeast extract peptone dextrose (YPD) solution. However, growth curves did not show differences between 0.25, 0.50 and 1.0 mM B and/or As supplemented groups and nonsupplemented groups. Results of elemental analysis showed that generally more than 90% of B and As remained in supernatant solution. However, arsenic concentration increased by almost 4 times as the spiked concentration level was increased up to 5.0 mM even though in all groups boron concentration was below detection limit with the exception of 5.0 mM. Genomic DNA of yeast cells was

followed by agarose gel electrophoresis for supplemented and control groups and no breakage was detected up to 5.0 mM As supplemented groups.

Keywords: Arsenic, boron, *Saccharomyces cerevisiae*, ICP-OES, ICP-MS, agarose gel electrophoresis.

ÖZ

MAYA HÜCRELERİNDE BOR VE ARSENİĞİN ETKİLERİNİN İNCELENMESİ

Atıcı, Sezin

Yüksek Lisans, Kimya Bölümü

Tez Yöneticisi: Doç. Dr. Gülay Ertaş

Ağustos 2016, 90 sayfa

Arsenik (As) metabolizmadaki toksik etkilerinden dolayı araştırmacıların ilgi odağı olmuştur. Bor (B) bulunduğu derişime bağlı olarak hem gerekli hem de toksik olabilmektedir. Bilinen en büyük bor rezervleri Türkiye'dedir ve Türkiye bor minerallerinin en büyük üreticisidir. Bununla birlikte, Balıkesir-Bigadiç ve Kütahya-Emet gibi bölgelerdeki doğal kaynaklarda yüksek derişimde B ve As bulunmaktadır.

Bu tez kapsamında B ve As varlığında büyüyen maya hücrelerinde ICP-OES ve ICP-MS kullanılarak B(III) ve As(III) dağılımını maya hücresinde ve hücre büyüme ortamında ayrı ayrı takip eden analitik bir metot geliştirilmiştir. Hücre büyümesi 600 nm'de optik geçirgenlik ölçümleriyle izlenmiştir. Optik geçirgenlik sonuçlarına göre 9. saat logaritmik faza karşılık gelirken, durgun faz 24. saattir. Yalnızca maya özütü pepton dekstroz (YPD) çözeltisiyle beslenmiş olan kontrol grubu hücreleriyle karşılaştırıldığında, maya hücrelerinin büyümesi 5.0 mM arsenik varlığında %30 azalmıştır. Ancak, büyüme eğrileri 0.25, 0.50 ve 1.0 mM B ve/veya arsenikle beslenen hücre grupları ve kontrol grubu arasında anlamlı bir fark bulunamamıştır. Arsenik ve borun genellikle %90'dan fazlasının üst fazda (süpernatant) kaldığı element analizi sonuçları ile gösterilmiştir. Hücre içi arsenik derişimi hücre besi ortamına eklenen As derişimi 0.25 mM'den 5.0 mM'a arttırıldığı zaman yaklaşık olarak 4 kat artmıştır. Ancak 5.0 mM olan örneğin dışındaki diğer bor örneklerinin hücre içi bor derişimleri tayin sınırının altındadır. Maya hücrelerinin genomik DNA'ları agaroz jel elektroforezi ile B ve/veya As ile beslenen ve beslenmeyen

kontrol grubunda takip edilmiştir ve 5.0 mM As ile beslenen grubun dışında herhangi bir hasar saptanmamıştır.

Anahtar kelimeler: Arsenik, bor, *Saccharomyces cerevisiae*, ICP-OES, ICP-MS, agaroz jel elekroforezi.

To Erhan Özdemir; Binnur Atıcı and Tüzel Atıcı

ACKNOWLEDGEMENTS

I would like to express my deep appreciation and respect to my supervisor Assoc. Prof. Dr. Gülay Ertaş for her endless guidance, support, encouragement, understanding, patience and suggestions throughout this study.

I am grateful to Prof. Dr. O. Yavuz Ataman not only for his guidance, valuable opinions and contributions during this thesis.

I would like to thank Prof. Dr. Mahinur Akkaya and Assist. Prof. Dr. Betül Sopacı for their ideas, comments and helps during this thesis.

My special thanks to my dearest friends Canan Höçük and Yeliz Akpınar for their endless help, moral support and friendship.

I would like to express my thanks to my teaching assistants Emrah Yıldırım, Pınar Mercan and Selin Bora for their help throughout the study.

I should thank to all my labmates, Başak Dügencili, Mehmet Bilgi Er, Anıl Ulukan, Ece Çiftcioğlu, and C-50 members, Zuhal Vanlı, Pakizan Tasman and Dilek Ünal and my friends Aylin Elçi, Merve Doğangün, Doruk Ergöçmen, Selin Yitkin and Hande Öktem.

I would like also thank members of Prof. Dr. Mahinur Akkaya research laboratory, Çağlar, Ayşe, İrem and Bayantes for their helps.

I am grateful to my special friends, Nazan Çapraz, Selen Temel, Elif Ceren Eren, Esra Gürlük and Damla Şenlik for their unique friendship.

Finally, my special thanks to my husband Erhan Özdemir and all of my family members for their patience, moral support and love. I know that they are always with me and never leave me alone.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

EPA	Environmental Protection Agency
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
ICPS	Integrated count per second
LD_{50}	Lethal dose 50%
LOD	Limit of detection
LOQ	Limit of quantification
MAPK	Mitogen activated protein kinase
N	Number of samples
n.d.	Not detected
NIST	National Institute of Standards and Technology
NRC	National Research Center of Canada
PE	Polyethylene
pK_a	Acid dissociation constant
PP	Polypropylene
PTFE	Polytetrafluoroethylene
RSD	Relative standard deviation
SRM	Standard reference material
UV	Ultraviolet
WHO	World Health Organization
YPD	Yeast extract peptone dextrose

CHAPTER 1

INTRODUCTION

1.1 Boron

1.1.1 Occurrence, Production and Use

Boron is classified as nonmetal in the Group IIIA with atomic molar mass of 10.81 g/mol [1]. Two stable isotopes of boron are ^{10}B and ^{11}B. Natural abundance of isotope of ^{11}B is 80.22% and isotope of ^{10}B is 19.78%. The electron configuration of boron is $[He]2s^2 2p^1$. There are three valence electrons in its outer shell forming three covalent bonds by electron sharing. Therefore, boron compounds are lack of octet and behave as Lewis acids [1]. Moreover, small covalent radius (80 pm) and high ionization energy of boron (1^{st} ionization energy: 799 kJ/mol) give rise to form covalent bonds rather than metallic bonds [2].

Boron cannot be found as its elemental form in the Earth. It has high affinity to oxygen, so it is found as borates which can be defined as boron-containing oxyanions in nature [3]. Approximately, 0.001% (w/w) corresponds to the concentration of the element on the Earth's crust in the form of borax ($Na_2B_4O_5(OH)_4.8H_2O$), kernite ($Na_2B_4O_6(OH)_2 \cdot 3H_2O$), tincalkonite ($Na_2B_4O_7 \cdot 5H_2O$), colemanite ($CaB_3O_4(OH)_3.H_2O$), tourmaline and sassolite [4]. The 'boron' term is used to indicate data as the equivalent boron content of a mineral independent from the form of the compound [5]. Boron is found in high amounts in sedimentary rocks, seawater, coals, and soils [6]. The average concentration in seawater around the world is estimated to be about 4.6 mg/L [6]. Release of boron into the atmosphere occurs via both naturally and commercially. Natural release from seawater, rock weathering, volcanos, coal combustion and forest

fires exists [7]. Production and applications of glass, ceramic, detergent, and fertilizer are responsible from the commercial sources [8].

Amorphous and crystalline boron are allotropes of the element. Amorphous boron is brown in powdered form, crystalline boron is black in crystal form, hard and refractory. At room temperature, boron is inert and finely divided form of it is attacked only by fluorine and concentrated nitric acid[1].

Boron was firstly isolated by Sir Humpry Davy, Joseph Louis Gay-Lussac and Louis Jacques Thenard in 1808 by the reaction of boric acid and potassium [4]. Jöns Jakob Berzelius classified boron as an element in 1824 [3].

For production of pure elemental boron, volatile boron halides are reduced in the presence of hydrogen at high temperatures using the following equation [1].

$$2BBr_3\,(g) + 3H_2\,(g) \rightarrow 2B\,(s) + 6HBr\,(g)$$

Boron compounds are used in different fields of industry including glass for making borosilicate glasses, cleaning for the preparation of laundry products, medicine for the preparation of antiseptics and drugs, semiconductor for changing conductivity properties of silicon and germanium for use in transistors, and as a fuel for rockets [9]. Moreover, elemental boron is used for hardening the steel. It is applicable for nonferrous metals as deoxidizer and degasifier [4].

1.1.2 Boron Compounds

72.5% of the world boron reserves are found in Turkey [10]. Production of borate compounds takes a biggest share in the world marketplace by exporting mineral concentrates of tincal, ulexite, colemanite in terms of unrefined boron and anhydrous borax, borax pentahydrate, borax decahydrate and boric acid in terms of refined boron [11].

Most common boron minerals, their chemical formula and location in Turkey are given in Table 1.1.

Table 1.1. Common unrefined boron minerals, their chemical formulas and locations.

Name	**Chemical Formula**	**Location**
Tincal (borax)	$Na_2B_4O_5(OH)_4.8H_2O$	Eskişehir-Kırka
Ulexite	$NaCaB_5O_6(OH)_6.5H_2O$	Balıkesir-Bigadiç, Kestelek-Bursa
Colemanite	$CaB_3O_4(OH)_3.H_2O$	Kütahya-Emet, Balıkesir-Bigadiç, Kestelek-Bursa
Probertite	$NaCaB_5O_7(OH)_4.3H_2O$	Kestelek-Bursa

Three classifications of boron minerals are unrefined boron, refined boron and end products. The most important consumption is refined compounds. The commercial boron products around the world are shown in the Table 1.2.

Table 1.2. Commercial boron products.

Product Name	**Chemical Formula**
Borax pentahydrate	$Na_2B_4O_7.5H_2O$
Borax decahydrate	$Na_2B_4O_7.10H_2O$
Unhydrated borax	$Na_2B_4O_7$
Boric acid	H_3BO_3
Unhydrated boric acid	B_2O_3
Sodium perborate	$NaBO_3.4H_2O$
Sodium metaborate	$Na_2B_2O_4.4H_2O$
Sodium octaborate	$Na_2B_8O_{13}.4H_2O$

End products of boron are obtained using both unrefined and refined boron. They contain boron nitride (BN), boron carbide (B_4C) and elemental boron [12]. Some physical characteristics of hexagonal BN and graphite are similar, but BN is a white and nonconducting solid [1]. However, cubic BN is as hard as diamond and maintain its properties up to 1320 °C.

The hardness of boron carbide is very close to that of diamond and the melting point is 2450 °C. Moreover, it is used as neutron absorber for neutron radiation forming in nuclear power plants and anti-personnel neutron bombs. Another nuclear application of boron carbide is usage as control rods in nuclear reactors to control rate of nuclear fission reactions [12].

1.1.3 Toxicity of Boron

Boric acid can show toxicity effect only if it is excessively consumed by humans and animals and excess boron is excreted primarily by urine within a few days [8]. Although 18 to 20 mg/kg of body weight boron for adults might be fatal [13], death from boron toxicity is rare [14].

Main exposure conditions of boron occur via ingestion or inhalation. Under these exposure period, the most critical irritants are boron, boric acid and boron oxide [14]. Toxic ingestions of them can result in diarrhea, nausea and vomiting. The color of feces may be blue-green, and renal injury, hypothermia, erythema, restlessness, and weariness may occur [13-14]. Loss of appetite and weight, and diminished seminal volume and sexual activity may be the results of chronic boron exposure [8].

Based on the current knowledge reflecting human poisoning events, data show that 3000-6000 mg boric acid is the level of acute poisoning for children, but this value is 15000-20000 mg for adults [15]. Dogs and rats are exposed to 1170-2000 ppm boron via their foods for 2 years and it was shown that stunted growth, less productive food use, skin breakdown, and gonadal disruption were observed [14].

In one study, six volunteers were ingested boric acid in an ointment or dissolved in water between 750 mg and 1500 mg levels. The urine samples were examined and 92 to 94% of boric acid was defecated at the end of 4 days [16]. An experiment conducted on rats, and between 0.5 and 2.1 mg boron as sodium pentaborate form was given to animals. Boron concentration in blood was followed with a function of time. It is reported that one half of the initial boron amount was removed from the body of the rat via urine in an hour [17]. Exposing a single dose of boron was not generally resulted in death, but vascular disorder and renal impairment conditions together with high boron exposure can lead to death [18].

1.1.4 Source of Exposure

Food and drinking water are the main sources of boron exposure in the environment [19]. Types of food affect the boron intake by people [20]. Boron concentration of some selected foods is listed in Table 1.3.

Table 1.3. Boron concentration in selected foods [8].

Type of Food	**(µg B /g of dry weight)**
Apple	42.5
Soy meal	28.0
Grape	27.2
Tomato	27.1
Celery	24.7
Almond	23.0
Broccoli	21.9
Banana	20.6
Wine	8.5
Honey	7.2

Since South Marmara Region of Turkey is rich in boron deposits, occupational exposure of the element is inevitable for workers and plants in mining field. As a result of geological activities, drinking water may be contaminated with boron. Guideline value of boron in drinking water was determined as 2.4 mg/L by WHO [21]. Boron concentration of drinking water in Bigadiç is reported between 8.5 and 29 mg/L [22]. Moreover, Korkmaz et al. reported the average boron intake of people

living in İskele-Balıkesir where the boron concentration in drinking water is higher than 2.0 mg/L [23]. In this study 66 men urine samples were analyzed and it was reported that 6.77 mg/day was the average boron intake of men living near the mining area, but the corresponding value of the control group living in Ankara was 1.26 mg/day [23].

Average boron intake changes with human metabolism and age in addition to food and location [24]. For example, it is 0.75 ± 0.14 mg/day for babies of 0-6 months, 1.34 ± 0.02 mg/day for middle-aged men and 1.39 ± 0.16 mg/day for nursing mothers [24].

Boron absorption in humans occurs by gastrointestinal and respiratory systems. When boron enters to the body tissues and fluids, it is absorbed mostly as boric acid and less amount as borate anion due to pH of human blood (pH=7.4) and weak acidity of boric acid (pK_a=9.25 at 25 ^oC) [11]. According to the pH-pC diagram, it is reported that 98.4% of boron is found as boric acid (H_3BO_3) while 1.6% of it is in borate form ($B(OH)_4^-$) in body tissues and body fluids [11].

According to WHO (World Health Organization), the expected results of daily boron intake are 0.44 µg /day by air, 0.2-0.6 mg/day by drinking water and 1.2 mg/day by diet [18]. According to the estimation of Anderson et al. [25], 10% of boron in air belongs to particulate boron and 90% of it corresponds to gaseous boron [25]. Therefore, the range is between 0.36 to 19.9 ng/m^3. Considering average adult air consumption (22 m^3/day) and using maximum value in the range (19.9 ng/m^3) result in exposure of 438 ng/day which is 0.44 µg /day.

Concentration of boron in drinking water is ranged from 0.01 to 15.0 mg/L in Chile, Germany, the United Kingdom and it is below 0.5 mg/L in USA [18]. According to these values 0.2-0.6 mg/day exposure comes from drinking water. Daily boron diet estimation can be made based on the consumed foods. While fruits, vegetables and legumes are rich in terms of boron, fish but meats are poor. According to the estimated values, consumption of 1.2 mg/day boron is required [18].

1.1.5 Importance of Boron for Plants, Animals and Humans

Boron is proved to be an essential element for plants growth in the early 1920s [26]. The plant scientists stated that "the essential role of boron in biological systems is stabilizing molecules with cis-diol groups, independently of their function" and "boron chemistry makes it a perfect candidate for atomic diester bridging" [27]. In the presence of boron, living media favor di-ester formation with boron instead of monoester ones, because only di esters could attain stabilities high enough to be of physiological relevance. Requirement of boron for best growth varies among plants. A list of the relative tolerance of some of the most common agronomic and horticultural crops is given in Table 1.4.

Table 1.4. Relative tolerance to B of some agronomic and horticultural crops [28-29].

Tolerant (4-15 mg/L)	**Moderately tolerant (2-4 mg/L)**	**Moderately sensitive (1-2 mg/L)**	**Sensitive (0.5-1 mg/L)**
Alfalfa	Oat	Broccoli	Avocado
Beet	Cabbage	Cucumber	Bean
Cotton	Celery	Carrot	Grape
Grain sorghum	Corn	Pea	Grapefruit
Parsley	Squash	Pepper	Lemon
Sugar beet	Sweet clover	Potato	Orange
Tomato	Turnip	Raddish	Wheat

Boron deficiency is an important problem for especially roots of vascular plants [26]. It is more common in acidic and light textured soils because of leaching of boron.

Cell membrane permeability may change and this also leads to boron deficiency. Membrane function may be affected as a result of oxidative free radicals deposit in cells. Therefore, boron deficiency occurs in root and leaf cells [30]. Although many studies are conducted on plants, exact mechanism of boron is still unclear. Cell walls are the place of boron gathering. As an instance, large proportion of boron in squash and tobacco plants were accumulated in cell wall structure together with pectin which was chemically extracted during cell wall preparation [31]. Cell walls were isolated from the plant cells and distributions of boron in both cells and cell walls were followed by ICP-OES. It was observed that majority of boron localized within cell walls. [31]. Cell membrane stabilization is provided by boron-pectin bonding [32]. These are structural roles of boron.

Relatively high amounts of boron are accumulated in cell membranes [26] where transport role of boron is activated. Boron induces H^+-pumping ATPase enzyme activity and intake of K^+ [33-34]. However, calcium and phosphate transport system activity decrease as a result of boron deficiency [35]. Boron is also beneficial in terms of defense system activity. Boron deficient plants suffer from damaged membranes [26]. Defense system of plants against pathogens occur via free and bound phenolic substances in the cell wall Moreover, boron has a role in DNA and RNA synthesis, hormone and carbohydrate metabolism [26]. Hormones affect flower and fruit improvement, and root extension in plants.

High concentrations of boron give rise to toxicity which is less likely to occur in nature compared to boron deficiency [36]. Symptoms of toxicity include cell death of some organs and death of entire plant according to boron exposure amount.

Although boron is essential for plant life, there is no evidence about its essentiality for animals and humans. However, it is considered to be ‘probably essential’ due to effects on mammalian life processes by the way of metabolism or exploitation of minerals and hormones [26].

One of the essentiality considerations of boron is related to bone metabolism. For example, chicks feeding with low B diet show gross bone abnormalities [37]. Moreover, addition boron supplement to low boron diet of rats (below 0.4 μg per g

body weight) increases absorption and retention of ions including calcium, magnesium and phosphorus [38]. Rats were fed with vitamin D deficient diets including 0.158 ppm or 2.72 ppm boron throughout 11 weeks. Ions apparent absorption were calculated on the next week. As a result, calcium and phosphorus values were higher for rats feeding with higher concentration (2.72 ppm) of boron [38]. The loss of bone weight in old people led to increased interest in the effects of boron on calcium uptake and metabolism in humans. Boron deficiency in post-menopausal women leads to low concentrations of ionized calcium and high total plasma calcium concentrations and calcium excretion [39].

In a recent study, NaBC1 was identified as a borate transporter in mammalians. It provides conduction of ions such as sodium, hydroxyl and hydronium when borate is absent. While boron is present, it functions as sodium-coupled borate transporter [40]. Low amounts of borate activate the MAPK pathway which provides convey of a signal from a receptor on cells to DNA. Borate was added in various concentrations ranging from 0.1 to 10 mM into HeLa cell cultures and they were incubated for 16 hours. As a result of cell counting, boron concentration for HeLa cell cultures was optimized and the optimum value is between 0.1 and 1.0 mM B but exposure to above 1.0 mM is toxic [41]. Human-plasma B levels are stated to be 13±3 μM which are lower than required boron concentration for optimal growth of HeLa cell cultures [42].

Dietary requirements of boron have been studied for variety of animals. For example, chick's requirement per day is about 0.4 ppm. [8]. In chicks, a daily boron supplement gives rise to beneficial change in mineral metabolism. When magnesium-deprived chicks were fed with supplemental boron, abnormalities due to insufficient magnesium intake were decreased. Moreover, boron addition results in enhancement of growth of the animals and increases in plasma magnesium and calcium levels [8]. Nielsen estimated that daily boron requirements of humans should be greater than this value based on the findings of such animal experiments including rats and chicks. Extrapolation of the value resulted in requirement of human daily diet at least 0.2 mg of boron. Therefore, Nielsen proposes that human's dietary requirement can be reached to 1 to 2 mg/day [43]. The Food and Nutrition Board of

the Institute of Medicine has put a Tolerable Upper Intake Level (UL) for boron of 20 mg/day for adults [8].

Boron results in a modification on calcium and bone metabolism via synthesis of male and female hormones [26]. For example, post-menopausal women were supplied with 3 mg B daily and as a result, rise in serum concentrations of testosterone was observed [39]. According to a study conducted on rats indicates that changes in plasma testosterone depend on concentration of boron. Testosterone levels increase when 2 mg boron per day are consumed, but decrease at daily 25 mg consumption [44]. Moreover, in one study it was shown that boric acid reduces in female ovulation, decreases and causes degeneration in sperm volumes for men in rats and mouse [3].

Boron functions as membrane integrity in the brain [26]. Mental and psychomotor functions of healthy elderly people are examined depending on boron concentration supplemented on diet. It was shown that when boron intake was lower (0.25 mg/day) as compared to other peoples' diet (3.25 mg/day), psychomotor and cognitive functions including manual dexterity, eye-hand coordination, attention, perception, short-term and long-term memories were reduced [45].

In addition to importance of boron in plants, animals and humans, boron is also critical for yeasts and bacteria [46-47]. Bennett et al. investigated the boron effects on yeast cells in terms of cell number growth [46]. Cells were grown in their feeding solution until reaching to early logarithmic phase (9^{th} hour). In this study, 18.5 mg/L B was added in the form of boric acid form into the working group, while an equal volume of deionized water was added to the control group. The number of cells of both groups was counted at 6^{th}, 9^{th}, 12^{th}, 15^{th}, and 24^{th} hours. It was reported that a significant increase in the number of cells was observed in the presence of boron [46].

1.2 ICP Techniques

1.2.1 ICP-OES

There are two parts in ICP-OES: ICP part corresponds to the source of ionization (temperature in the range of 6,000-10,000 K) and detection and quantification is provided by OES part [48]. Ionization begins with a spark from a Tesla coil. Interaction of ions and their associated electrons with magnetic field occur inside the coil [49]. Liberation of analyte elements as free atoms and collisional excitation of them in the plasma provide passage of free atoms from ground state to excited state. Conversion of atoms to ions and ions to excited states occur within the plasma. Relaxation of atomic and ionic excited states of elements results in emission of a photon. Since these photons have characteristic energies, the wavelength of them could be used for identification of elements [48]. Spontaneous emission of electromagnetic radiation from atoms and ions excited in plasma are followed by the separation of characteristic energies based upon the wavelengths of the analyte elements by means of a wavelength selector which is usually a monochromator. Finally, photodetector is used to convert the photon energy to electrical energy [48, 50].

Conversion of sample solution into aerosol occurs via nebulizer in the presence of argon gas. After that, aerosol particles passes through spray chamber in which larger aerosols trapped from the surface and poured into the waste container. Fine aerosol directed to plasma using argon as a carrier gas.

The plasma is produced by the interaction of magnetic field produced by radio frequency passing through a copper coil on a tangential flow of gas (argon) flowing through a quartz tube (torch). Torch is composed of 3 concentric quartz tubes which are outer, middle and inner. Argon flow in outer tube (10-15 L/min) called as coolant gas provides continuity of plasma. Moreover, it protects the outer quartz tube from melting. Inner argon flow (0.5-1.5 L/min) named as nebulizer gas carries the sample to the plasma. Annular shape of the plasma is sustained by the carrier gas [48].

Argon flow in intermediate tube (0-1.5 L/min) lifts the plasma softly if necessary and dilutes the carrier gas when organic solvents include in sample.

RF power in the range of 700-1500 Watt is supplied around the water-cooled, two- or three-turn copper coil for the formation of plasma [48]. Oscillation of magnetic field occurs inside the coil. The frequency used in ICP-OES instruments is either 27.12 or 40.68 MHz. While argon flows along the torch, free electrons are produced as a result of spark added from a Tesla coil. Ionization occurs by collisions of these electrons with atoms and molecules.

1.2.1 ICP-MS

ICP-MS is a popular technique due to its multi-element capability. Moreover, it has low limit of detections in variety of samples including environmental, biological and industrial applications [52].

ICP part is same as ICP-OES, but Mass Spectrometer (MS) part is based on the atomic mass to charge ratio (m/z) separation. Only ions can be analyzed with MS therefore ions formed in the plasma directed to the quadrupole or time of flight mass analyzer. Ions are transported to the mass analyzer along the two-stage ion extraction interface [52]. The first interface corresponds to sampler cone having small orifices around 1 mm. It is made of nickel or platinum because of high thermal conductivity and corrosion resistivity. When passage of ions through sampler cone is complete, skimmer cone is the second interface region. Its orifice (0.4-0.7 mm) is less than that of sampler cone [52]. Less than 1% total dissolved solids are the highest preferable value for best sensitivity values due to the possibility of clogging the orifices of the cones [9]. The region between these cones is held at a mild pressure of 1-2 Torr, but pressure behind the skimmer cone is highly lowered at the level of 10^{-6} Torr or less [51]. Turbomolecular pumps provide the vacuum maintenance [51]. After ions leave the interface region, focusing and transmission of them to mass analyzer provided by ion lenses. The interference from neutral species and photons are eliminated using these ion lenses which separate these interferences from the ions beam [53].

Quadrupole type mass analyzer is commonly used in ICP-MSs. It separates ions according to m/z ratio. It consists of four metallic rods in the same length with parallel to the ion beam [52]. It provides direct current in addition to alternating current. AC/DC ratio is adjusted so that only the selected m/z can reach to the detector. Then, ions are counted in the detector and they are converted to electrical signal [9].

1.3 Agarose Gel Electrophoresis

Agarose is a polysaccharide and it contains agarobiose (3,6-anhydro-L-galactopyranose and D- galactose) repeating units [54]. It is extracted from the seaweed genera Gelidium and Gracilaria. The melting temperature of it is approximately 90-95 °C while gelling temperature is around 34-38 °C. Agarose is a preferred matrix for separation of biomolecules such as DNA, RNA and proteins, because agarose is stable physically, chemically and thermally. Therefore, any interaction of agarose with biomolecules is less likely to occur. Moreover, its large pore-sized structure permits migration and separation of biomolecules [54].

Agarose gel electrophoresis is mostly used for separation, identification and purification of DNA fragments of varying sizes (0.2 to 60 kb) [55]. DNA samples are loaded into wells and a current is applied between electrodes. Migration happens through the positive electrode because of negative charge of phosphates in DNA. Since mass to charge ratio of DNA is constant (one negative charge per nucleotide) separation of molecules is based on the inverse proportionality with the logarithm of the molecular weight [56]. Plotting the relative mobility of DNA fragments versus logarithm of molecular weight of these fragments produce a straight line. As a result, smaller DNA fragments migrate faster than the large ones.

Important parameters affecting agarose gel electrophoresis quality include agarose concentration, applied voltage and electrophoresis buffer type which is either TBE (Tris Borate EDTA) or TAE (Tris Acetate EDTA). In general, the higher the

concentration of agarose, the smaller the pore size. Lower concentration of agarose (0.3 to 0.5% (w/v)) is suitable for separation of large fragments of DNA (20-60 kb), relatively high concentration of agarose (0.5 to 1.0% (w/v)) are used for small fragments of DNA (0.5-30 kb) separation. Concentrations larger than 1.0% (w/v) could separate small DNA fragments (0.2-0.5 kb) [55]. Moreover, low applied voltages (approximately 1 V/cm) are more appropriate for resolving large DNA fragments due to slowed down migration rate of large fragments through agarose pores. In addition, applying high voltage can increase the temperature and gel can be harmed. Buffer capacity is another important parameter in gel electrophoresis. TBE has a bigger buffer capacity than that of TAE and the former is more suitable for higher voltages [55].

After separation is complete, bands of DNA can be visualized under UV light after staining with an intercalating dye such as ethidium bromide. If bands are sharply visible, this means that DNA is unbroken. However, smeared DNA can show damage.

1.4 Arsenic

1.4.1 Occurrence, Production and Use

Arsenic is a metalloid belonging to group VA with atomic number of 33 and atomic mass of 74.92 g/mol. It is the 53rd most abundant element on the Earth's crust, 2 mg/kg in soil [57].

Arsenic can be found in the nature as a result of both natural and anthrophogenical sources. Arsenic occurs naturally in soils, some rocks and ores including lead and copper. It can pass to air and waters by means of dust particles transferred by air and leaking to underground water [58]. Mineral spring, volcanic rocks, inorganic/organic clays, metamorphic rocks, sea water, forest fires, volcanism and rock erosion are the natural sources of arsenic [59]. In addition to this, sulfide ores and iron oxide including arsenic are the other natural arsenic sources such as As_2S_3 (orpiment), AsS

(realgar), FeAsS (arsenopyrite), $FeAs_2$ (loellingite), NiAs (nickeline), CoAsS (cobaltite), $Cu_{12}As_4S_{13}$ (tennantite) and Cu_3AsS_4 (enargite) [60-61].

Anthrophogenical resources of arsenic are widely distributed in the industrial fields of wood preservatives, dye, cosmetics, medicine, herbicide, pesticide, leather, semiconductor materials, glass and cement. Moreover, copper, nickel, gold mining and usage of fossil fuels, and agricultural applications lead to arsenic pollution resulting from anthropogenic sources [62].

Two solid forms of arsenic are yellow arsenic and metallic arsenic. Both of them have tetrahedral As_4 structure [1]. Extraction of the metal is generally achieved from flue dust of copper and lead smelters. Moreover, heating the ores without oxygen environment is another extraction procedure of arsenic as shown in the following equation [1]:

$$FeAsS(s) \rightarrow FeS(s) + As(g)$$

In addition, elemental arsenic is produced by reduction of arsenic trioxide (As_2O_3) with charcoal in steel and iron casts as seen in the following equation [63]:

$$As_2O_3(s) + 3C(s) \rightarrow 2As(g) + 3CO(g)$$

Arsenic exits in four valence states (-3, 0, +3, +5) in their compounds in the nature. (-3) and (0) states of it is seen very rare in the nature [61]. That is, most common forms of it is trivalent (+3) and pentavalent (+5). Arsenic is found in the water as its dissolved forms such as arsenate (As(V)), arsenite (As(III)), methylarsonic acid (MMA) and dimethylarsinic acid (DMA). In water and sediments in the presence of oxygen, almost all arsenic is found in the As(V) oxidation state which is thermodynamically most stable specie of As [64]. The oxidation state of arsenic depends on pH and redox potential of water, presence of iron, calcium and sulfide and microbial activity in water [65].

In the periodic table, arsenic is below phosphorus (P) in the same group and their chemical and structural properties are very similar to each other. When arsenate (AsO_4^{3-}) enters to a cell, it may compete and replace phosphate (PO_4^{3-}) in biological

reactions due to physicochemical similarities between them. Phosphate has important biological functions including phosphorylation of proteins and energy transportation by ATP. Moreover, it is an essential component of nucleic acids and lipids. As a result, phosphate levels within cells are sufficiently high that arsenate cannot be directly responsible for arsenic-dependent toxicity [71].

1.4.2 Arsenic Compounds

Arsenic compounds could be classified as inorganic and organic forms. While organic arsenic compounds involve carbon and hydrogen, inorganic ones contain oxygen, sulfide or chlorine [67]. Inorganic arsenic is found in ground, surface and sea water, but organic arsenic is mostly present in natural gas and petroleum [62]. Naturally occurring organic and inorganic arsenic species are given in Table 1.5.

Table 1.5. Some organic and inorganic arsenic compounds [64].

Inorganic As compounds
Arsenic trioxide (As_2O_3)
Arsenic pentoxide (As_2O_5)
Arsenic trisulfide (As_2S_3)
Arsenic trichloride ($AsCl_3$)
Arsine (AsH_3)
Arsenious acid ($HAsO_2$)
Arsenic acid (H_3AsO_4)
Arsenenic acid ($HAsO_3$)
Organic As compounds
Arsenobetaine ($C_5H_{11}AsO_2$)
Dimethylarsinic acid ($C_2H_7AsO_2$)
Monomethylarsonic acid (CH_5AsO_3)
Arsanilic acid ($C_6H_8AsNO_3$)
Trimethylarsine oxide (C_3H_6AsO)

1.4.3 As Toxicity

In 2001, the Unites States European Protection Agency revised the drinking water standard 10 µg/L instead of 50 µg/L for better protection from arsenic exposure [68]. Moreover, International Agency for Research of Cancer classified the element as Group I human carcinogen [69]. In India and Bangladesh, a significant portion of the population was affected by arsenic in the drinking water [70].

Trivalent form of arsenic (arsenite, As(III)) is more toxic compared to pentavalent form (arsenate, As(V)) and is responsible for the toxicity of the element [71]. Its toxicity is mainly because of tendency to form very strong bonds with thiolates (RS^-) and imidazolium ($C_3H_5N_2^+$) nitrogen. Both of them plays critical role in structural and functional properties of proteins. As a result of nearly covalent bond formation between arsenic and these functional groups, inhibition of the enzymes active sites and receptors including thiolate and prevention of disulfide bond formation occur [69].

Toxicity of As(V) in cells is due to the fact that As(V) is a nonfunctional phosphate counterpart disturbing phosphate assimilation [71]. That means, $H_2AsO_4^-$ and $H_2PO_4^-$ ions are isoelectronic and in similar size. As a result, arsenate can compete for the same carriers of phosphate through the cell and it can pass into cells via phosphate transporters. As(V) enters into food chain by means of these transporters. When arsenate enters to the cell, it is reduced to arsenite by arsenate reductases which are thiol-based molecules [71].

A lethal dose of arsenic is considered to be 200-250 mg for humans [72]. For some laboratory animals, LD_{50} values were studied. LD_{50} can be defined as the dosage of a compound that is responsible for killing 50% of a population. For example, while LD_{50} value of mouse is 34.5 mg/kg in arsenic trioxide (As_2O_3) form [73], the corresponding value in rats is 15 mg/kg again in arsenic trioxide form [74]. LD_{50} value in mice is 8 mg/kg and 22 mg/kg in arsenite form and arsenate form, respectively [75].

When drinking water contains arsenic higher than the limit value, distortion in skin and internal organs were reported [66]. In south Calcutta, chronic arsenic poisoning occurs in 20 members of 17 families living near a factory producing copper acetoarsenite ($C_2H_3AsCuO_4$) [76]. According to the analysis, arsenic concentration was reported in well water as 5-58 mg/L [76]. This was higher than the limit value (10 μg/L) permitted by WHO. 20 affected patients among these members applying to hospitals were examined for clinical, hematological, neurological, liver and urine

tests. As a result, pigmentation disorders, weakness, muscle aches, cough were reported as the most critical signs of arsenic toxicity.

When As(III) enters to the cell, the change in methylation patterns of cellular DNA is observed. This kind of differentiation occurs usually in cancer cells [71]. Furthermore; liver, lung, skin, kidney and bladder cancers result from chronic exposure to high level of arsenic contamination [71]. Arsenic can be accumulated in nails, hair and skin by connecting with ceratine and sulfhydryl after a few weeks later from exposure [57]. On the other hand, acute exposure of arsenic results in vomiting, abdominal pain, and diarrhea. The symptoms proceed with numbness, muscle cramping and death in extreme situations [57].

Arsenic is one of the hazardous elements, but dose and source of exposure (air, foods or skin contact) determine the health effects [57].

1.4.4 Sources of Arsenic Exposure

Since arsenic is naturally occurring element, exposure to the element is inevitable [77]. The possible sources of contamination occurs from natural sources including volcanic activities, dissolution of minerals through the groundwater, air, foods, drinking water, and human activities like mining, smelting, fossil fuel combustion, producing pesticides and wood preservatives treatment [57]. Among them, ground and drinking water are the major sources of arsenic pollution and this is caused by natural geological sources rather than from mining, smelting and farming activities [60]. High concentration of arsenic in ground water becomes a major problem in countries including Bangladesh, Vietnam, China, Taiwan, Argentina and Canada. In Turkey, high concentration of arsenic are found mostly in Aegean region, Kütahya-Emet (448 µg/L) in drinking water, Balıkesir-Bigadiç (30-900 µg/L) and Uşak (50 µg/L) in ground water sources [57]. In all these regions, concentration of arsenic is above the WHO limit (10 µg/L).

Arsenic exposure via air is changing between 0.4 and 30 ng/m^3. According to the Environmental Protection Agency, daily arsenic inhalation of humans is in the interval of 40 to 90 ng. This value is approximately 50 ng or less for unpolluted places [77]. There is not any ambient air standard for arsenic [78]. Furthermore, tobacco plant includes arsenic, so smokers are exposed to arsenic. The concentration of arsenic in tobacco increases due to lead arsenate ($PbHAsO_4$) pesticide treatment [79]. Fish, shellfish, poultry, meat and cereals are main contributors of arsenic in daily diet. Fish and shellfish contain arsenic in organic forms such as arsenobetaine the toxicity of which is lower compared to inorganic arsenic forms [79].

1.5 Arsenic and Boron

Although studies in the literature is mainly focused on only boron or only arsenic exposure, there are few studies examining boron and arsenic effects together in living media.

Arsenic is a known carcinogen in the environment and leads to several cancer types in humans including lung, skin, kidney, bladder and liver [80]. Mechanism of its carcinogenicity has been still investigated such as induction of micronuclei (MN), DNA strand breakage, sister chromatid exchange (SCE), oxidative stress and inhibition of DNA repair [81].

Replication and transcription-independent DNA double strand breaks were observed in yeast cells in the presence of As [82]. This was indication of direct genotoxic role of arsenic in organisms rather than arsenic-induced oxidative DNA damage converted to replication-dependent strand breaks [82]. Investigation of arsenic-induced apoptosis which is programmed cell death were followed in yeast cells in another study [84]. Grown cells were fed with As(III) in the form of arsenite which was added to the cell culture in various concentrations (1, 3, 5 and 7 mM). Increasing concentration of arsenic resulted in decrease in the number of cells. Moreover, reactive oxygen species (singlet oxygen (O^*), superoxide anion (O_2^*), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH*) were produced as a result of arsenic

treatment [84]. Toxic levels of these species lead to oxidative stress which can be defined as balance disturbance between reactive oxygen species and antioxidant defenses [85]. Reactive oxygen species lead to protein oxidation, lipid peroxidation and DNA damage due to attack of cellular macromolecules [86]. Arsenic genotoxicity is mainly connected to the reactive oxygen species (ROS) formation during biotransformation of arsenic in the literature [87-88]. ROS elevation and glutathione (GSH) reduction were observed in leukemia cells exposed to low concentration of arsenic trioxide (1-2 μM) [89]. Cytotoxicity of sodium arsenite ($NaAsO_2$) was examined on PC12 cell lines at 5 and 20 μM concentrations [90]. Increase in ROS level and decrease in cell proliferation due to arsenic treatment were most critical results of cytotoxicity [90]. Proteins and enzymes are also affected by oxidative damage induced by arsenic because of its high affinity to sulfhydryl groups of proteins and enzymes [91]. Moreover, inactivation of many enzymes can be linked to oxidative damage [92]. Interference of As-induced oxidative damage with DNA repair mechanism is reported in many enzymes such as DNA β-polymerase [93].

Boron is essential for plants and nutritionally important for animals and humans [41]. It is critical in replication and growing for animal and human cells [41]. While relatively low concentrations of borate (0.1 mM to 1.0 mM) led to an increase in the number of HeLa cells, high concentrations (up to 10 mM) were toxic [41]. Furthermore, genotoxic effects were investigated in peripheral blood lymphocyte cells of boron exposed humans [94]. Lower levels of sister chromatid exchange (SCE) and micronuclei (MN) were observed for boron exposed people as compared to control ones. These lower results of genotoxicity assays could be an indication of less DNA damage as a comparison to controls. This interpretation could be linked to increase in glutathione (GSH) levels in cells [94]. As a result, boron may have a protective function against DNA damage, but more detailed studies are required to support this statement [94]. Effect of boron in yeast cell growth was investigated in the literature [46]. Yeast cultures were grown until reaching to early logarithmic phase of their growth. At this point, boric acid was added to liquid growth media of yeast to get final concentration of 0.185 mM B. Growth curve was followed over time by counting the cells on the microscope. Boron addition increased number of

cells when compared to control group in which no boron treatment occurred. According to ICP-MS boron results of cell and its supernatant at early logarithmic phase (9^{th} hour) and stationary phase (24^{th} hour), it was observed that boron concentration in cells were reduced at 24^{th} hour as a comparison to boron concentration at 9^{th} hour [46]. As a result, yeast cells lost their boron towards to their liquid growth media.

Effects of boron compounds (boric acid, borax, colemanite and ulexite) with or without heavy metals (arsenic, bismuth, cadmium, mercury or lead) were examined in human blood by Turkez et al. [95]. In this study, each boron compound was added to erythrocyte cells of blood samples in various concentrations (5, 10, 15, 20 mg/L). In addition, arsenic trioxide was also added to erythrocyte cells of blood at 3 and 5 mg/L concentrations. Boron and heavy metals added separately or together to the study group while none of these elements were added to the control group. According to the results of sister chromatid exchange (SCE), oxidative stress and micronuclei assays (MN), addition of boron compounds to erythrocyte cells of blood, genotoxic effects caused by arsenic (or other heavy metals) diminished. When a boron compound was added to erythrocyte cells, enzyme activities increased, and as a result antioxidant capacity also increased. However, heavy metal induced oxidative stress was greatest for arsenic among bismuth, cadmium, mercury, and lead. All of four boron compounds showed beneficial effect against heavy metals by decreasing effect of malondialdeyhe (MDA) level, MN and SCE formations when compared to only heavy metal added samples. It was shown that arsenic toxicity was decreased against especially in ulexite compound because of increasing glutathione (GSH) levels for antioxidant enzyme activities. In conclusion, Turkez et al. reported that boron may have a protective role against genetic disorders resulted in the presence of heavy metals [95].

Another study conducted to observe probable protective effect of boron against arsenic-induced oxidative stress in male and female rats [96]. While drinking water was given to control group, the second group was given *water including 100 mg/L* As_2O_3 and the last group was supplied with *water containing 100 mg/kg* H_3BO_3 *and 100 mg/L* As_2O_3. Hemoglobin, malondialdehyde (MDA) as a marker for lipid

peroxidation (LPO), glutathione (GSH) levels and antioxidant enzyme activities (SOD and CAT) were measured in blood, liver, kidney, hearth and brain tissues from rats fed with and without boron and/or arsenic. Hemoglobin levels of rats decreased as a result of arsenic exposure compared to control group and arsenic exposure in the presence of boron. Oxidative stress due to arsenic increases formation of free radicals. Level of free radicals in cells should be controlled to avoid any oxidative injury. This control is provided via nonenzymatic and enzymatic antioxidants such as SOD and CAT [97]. GSH, SOD and CAT activities decreased as a result of only arsenic treatment [95]. However, addition of boron increased levels of antioxidant enzymes and GSH. In conclusion, it is reported that boron can be protective to prevent oxidative stress caused by arsenic. Moreover, boron shows antioxidant characteristics against disorders due to arsenic exposure [95].

1.6 Aim of the Study

The scope of this thesis was to grow yeast (*Saccharomyces cerevisiae*) cells with and without B and/or As and to investigate synergic or antagonistic effect of boron and arsenic on DNA damage using gel electrophoresis and on the mobility of B and As in and outside the cell using ICP-OES and ICP-MS. Investigation of uptake of boron from natural sources is important since there are regional differences in boron concentration on the world depending on volcanic and hydrothermal activities. Especially west region of Turkey (Balıkesir-Bigadiç, Kütahya-Emet) is rich in boron reserves. Boron and arsenic levels were determined above the WHO`s limits in spring water of these regions [21, 57, 68]. Presence of boron in cell growth of organisms may change uptake of other elements such as arsenic into cell. In few studies, DNA damage was reported in the presence of arsenic species [95-96]. However, there are no studies examining the effect of boron and/or arsenic on DNA damage depending on the degree of uptake of these elements into yeast cells.

CHAPTER 2

EXPERIMENTAL

2.1 Chemicals and Reagents

1000 mg/L boron stock solution (High Purity Standards) was used from boric acid (H_3BO_3) in deionized water. For the distillation of analytical grade 65% (w/w) HNO_3 (Merck), the Berghof Acid Distillation System was used. Calibration standards were 0.05, 0.10, 0.25, 0.50, 1.0, 2.0 and 5.0 mg/L in 1% (v/v) HNO_3 prepared from 1000 mg/L boron stock solution. All of the dilutions were made by using 18.2 M Ω.cm deionized water from Elga, Purelab Option-Q Water Purification System.

1000 mg/L arsenic stock solution (High Purity Standards) was used from arsenic trioxide (As_2O_3) in 2% HCl solution. Calibration standards were 1, 5, 10, 25, 50, 100, 250 and 500 ng/mL in 1% (v/v) HNO_3 prepared from 1000 mg/L arsenic stock solution.

For cell growth; yeast from *Saccharomyces cerevisiae*, yeast extract peptone dextrose (YPD) agar and YPD broth were bought from Sigma-Aldrich. YPD broth can be defined as rich medium in the literature [98]. There is also minimal medium including yeast nitrogen bases without amino acids, ammonium sulfide and dextrose [98]. Growth of yeast cells can be affected depending on these minimal or rich media. In this study, only rich medium was used for the cell growth.

A special plant/fungi DNA isolation kit (Norgen, Biotek) was purchased for DNA extraction procedure. This kit included Lysis Buffer L and Binding Buffer I for preparation of lysate as a result of destructive process of lysis of yeast cells. 70% (v/v) ethanol was prepared from dilution of absolute ethanol (C_2H_5OH, 99.5% (v/v), Merck) in deionized water. It was used for washing lysates. Solution WN and Wash

Solution A were used to wash column. DNA elution provided by Elution Buffer B. 1.0% (w/v) agarose (Prona) was used in the electrophoresis experiments. 0.6 μg/mL ($C_{21}H_{20}BrN_3$, Sigma-Aldrich) of ethidium bromide was added to gel during the preparation of agarose gel to visualize DNA fragments. Intercalation of ethidium bromide with nucleotide bases provides the visualization of the dye under UV light [55]. Tris Acetate EDTA (TAE) buffer was prepared by addition of 48.4 grams of Trizma-base ($NH_2C(CH_2OH)_3$, Sigma), and 3.72 grams of ethylene diamine tetraacetic acid disodium salt dihydrate ($C_{10}H_{14}N_2Na_2O_8.2H_2O$, Fluka) in deionized water and they were stirred until complete dissolution. After that, 11.4 mL of glacial acetic acid (100% (v/v) CH_3COOH, Merck) were added to the solution and the final volume was adjusted to 1.0 L for 10X stock buffer solution. Working buffer solutions (1X) were prepared by diluting 10 times the stock solution with deionized water. DNA ladder (Biolabs, 50 base pair) and gel loading solution (Biolabs, 6X) were also used for electrophoresis to track DNA migration visually.

Boron concentration in 1573a Tomato Leaves (NIST) standard reference material (SRM) and arsenic concentration in DOLT-4 Dogfish Liver (NRCC, Ottawa, Canada) SRM were determined for the accuracy check of the method and the techniques used in this study. 38-40% (v/v) hydrofluoric acid (HF, Merck) and 65% (w/w) HNO_3 were used to digest 1573a Tomato Leaves SRM. On the other hand, 65% (w/w) HNO_3 and 30% (v/v) H_2O_2 were used to digest DOLT-4 Dogfish Liver.

2.2 Apparatus and Materials

100-1000 μL and 500-5000 μL range Eppendorf micropipettes were used to prepare working solutions. Polypropylene (PP) volumetric flasks were used instead of glass ones in order to avoid boron contamination from glass surfaces. Then, solutions were transferred to polyethylene (PE) flasks which were kept in fridge at 4 °C. Polytetrafluoroethylene (PTFE) digestion bombs were used for microwave-assisted digestion of samples.

Before using of all flasks and containers, they were immersed in 10% (v/v) HNO_3 solution for at least overnight. Then, they were washed with deionized water.

Plant/fungi DNA isolation kit included spin and filter columns used with their collection tubes and elution tubes to extract and elute purified DNA of yeast cells.

2.3 Instrumentation

2.3.1 Microwave Digestion System

Milestone Ethos PLUS microwave dissolution system was used for digestion of cell and supernatant parts, separately. Supernatant and cell samples were transferred into the PTFE digestion bombs and 2.0 mL of distilled concentrated HNO_3 and 1.0 mL of 30% (v/v) (H_2O_2, Merck) were added and then, digestion program shown in Table 2.1 was applied. After digestion, samples were diluted to 100.0 mL with deionized water.

Table 2.1. Microwave digestion program for cell and supernatant samples.

Temperature, °C	Ramp time, min	Hold time, min
100	3	5
150	3	5
180	3	5

2.3.2 ICP-OES

Leeman Labs Inc. DRE (Direct Reading Echelle) with an axial view configuration Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) was used for the determination of B in the samples using the continuous flow mode. Integration time for boron was 0.5 s.

Best signal to noise ratio was obtained with 249.773 nm emission line for boron. 0.1 mg/L B standard solution was used to optimize plasma and nebulizer parameters.

Standard addition method was used for analysis of boron for supernatant samples due to matrix interferences.

2.3.3 ICP-MS

Thermo X2 Series Inductively Coupled Plasma Mass Spectrometer with a concentric nebulizer and Peltier effect cooled spray chamber was used with continuous flow mode for the determination of arsenic in the samples. Optimization of instrumental parameters was performed with 10 µg/L ‘‘Tune A’’ mixed standard solution including Li^{+}, Co^{2+}, In^{3+}, Pb^{2+}, Bi^{5+} and U^{4+} in 0.5 mol/L HNO_3 solution. Moreover, 10 µg/L standard solution of As was used for best signal to noise ratio for ^{75}As isotope. Arsenic determination was done by following ^{75}As isotope with 100% natural abundance. Dwell time of As was 100 ms in standard resolution mode.

Spectral interference due to $^{75}ArCl^{+}$ was corrected by applying to software program of the instrument, PlasmaLab. Correction was made by applying the following equations:

$$^{82}Se^{+} = {}^{82}M^{+} - 1.001 \times {}^{83}Kr^{+}$$

$$^{77}ArCl^{+} = {}^{77}M^{+} - 0.860 \times {}^{82}Se^{+}$$

$$^{75}As^{+} = {}^{75}M^{+} - 3.12 \times {}^{77}ArCl^{+}$$

Standard addition method was used for analysis of arsenic in both cell and supernatant samples.

2.3.4 Centrifugation System

Liquid growth media (YPD broth) including yeast cell were centrifuged by a low-speed centrifuge (Nüve, NF200). Centrifugation of the media for 10 minutes at 5000 rpm required for separation of yeast cell suspension at the bottom and clear supernatant at the top.

High-speed centrifuge (up to 15300 rpm, Sigma) was used for extraction of DNA from yeast cells.

2.3.5 UV-vis Spectrometry

Optical density measurements at 600 nm were recorded by T80+ UV-vis spectrometer PG Instruments Ltd. These measurements were done for tracking yeast cell growth.

2.3.6 UV Transilluminator

The instrument (Kodak, Gel Logic 200 Imaging System) was used for viewing of separated DNA fragments by agarose gel electrophoresis.

2.4 Procedures

2.4.1 Growth of Yeast Cells

Yeast extract peptone dextrose (YPD) agar in deionized water was boiled to prepare agar plates. After that, this medium was put into the autoclave for sterilization of agar at 121 ^{o}C and at 0.3 MPa pressure for 15 minutes. While the agar medium was warm, it was poured in approximately equal volumes (25 mL) into the plates. The agar solidified and solid growth medium was ready for cell growth *(a).*

By applying the procedure conducted by Bennett et al. [46] dried yeast from *Saccharomyces cerevisiae* was activated in yeast peptone dextrose (YPD) broth which is the liquid growth medium. Liquid yeast cultures were spread onto agar plates (streaking process) by a flame sterilized platinum loop. After streaking procedure, plates were put into incubator at 30 ^{o}C, which is the optimal temperature for growth of yeast cells for two days. At the end of two days, around the streaks, individual colonies formed *(b).*

Once colonies formed, cells were inoculated in an autoclaved YPD broth media. Inoculation means picking a single colony and introducing it to the YPD broth media by mixing. After inoculation, the culture was incubated at 30 ^{o}C for overnight. Then, a portion of this overnight culture (2.0 mL) was transferred to the other sterile YPD broth medium (100 mL) until reaching to logarithmic phase (9th hour) *(c).* Various concentrations of boron and/or arsenic solutions were added to the liquid growth media including yeast cells at 9th hour (logarithmic phase) *(d).* After centrifugation *(e),* cell and supernatant samples were separated into different centrifuge tubes and stored in deep-freeze at -20 ^{o}C until analysis time.

2.4.2 Sample Preparation for ICP Analysis

When cells were reached to logarithmic phase (9^{th} hour), liquid growth media including yeast cells were divided in equal volumes (10 mL) and various concentrations of boron and/or arsenic (0.25 mM, 0.50 mM, 1.0 mM, or 5.0 mM) were added to each of these portions *(d)*. 5.0 mL aliquot from these solutions were taken into the centrifuge tubes. Cell and supernatant media parts were separated from each other by centrifuging at 5000 rpm for 10 minutes *(e)*. Remaining 5.0 mL portions of the liquid growth media were waited at 30 ºC incubator for another 15 hours to reach stationary phase (24^{th} hour). Same centrifugation process was applied as in the 24^{th} hour. By decantation, cell and supernatant phases were separated and put into centrifuge tubes and all these samples were waited in deep-freeze at -20 °C until analysis time.

To analyze cell and supernatant phases by ICP-OES and ICP-MS *(g)*, they were digested with an acid mixture (2.0 mL concentrated HNO_3 and 1.0 mL 30% (v/v) H_2O_2 for each sample) by microwave-assisted digestion system *(f)*. Schematic diagram of sample preparation for ICP analysis is given in Figure 2.1.

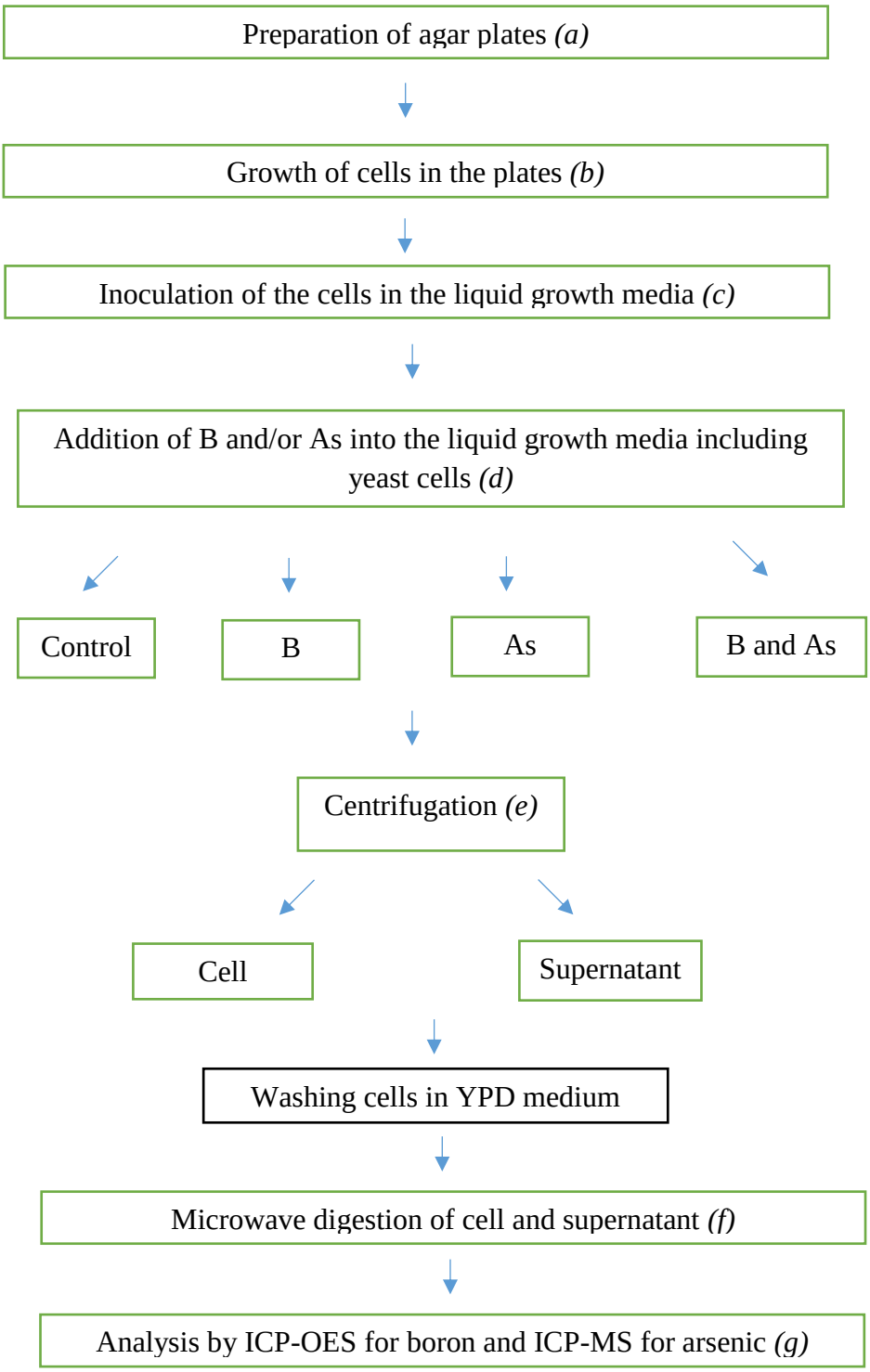


Figure 2.1. Schematic representation of cell and supernatant sample preparation for ICP analysis.

2.4.3 Cell Growth

Optical density value at 600 nm was measured at various time intervals using UV-vis spectrometer to follow cell growing. These measurements were done as two replicate cell samples. Optical density value is defined as logarithmic ratio of the incoming light intensity to transmitted light intensity through cell suspension. Number of cells is proportional to the measured optical density value.

Growth curves (OD_{600} vs. time) include four phases which are lag, logarithmic, stationary and death [99]. In lag phase, cells are acclimated to the growth medium and the number of cells starts to increase. In log phase, the number of cells increases dramatically due to active cell division which is growing and division of a parent yeast cell into daughter cells containing the necessary genetic material for cell replication. In stationary phase, cell division slows down and cell population remains almost constant. At the end, during death phase, the number of cell decreases as a result of depriving of nutrients in the liquid growing medium.

2.4.4 DNA Isolation from Yeast Cells

Genomic DNA was extracted from yeast cells via plant/fungi DNA isolation kit. Isolation procedure consisted of four steps including lysate preparation, binding to column, column wash and DNA elution [100].

a) *Lysate Preparation:* Firstly, yeast cells were put into a mortar and grinded into powder. After transferring of 100 mg grinded powder into Eppendorf tubes, 500 µL of Lysis Buffer L were added into the Eppendorf tubes. Incubation of them at 65 ^{o}C for 10 minutes was required for degradation of enzymes in cells. Then, addition of 100 µL of Binding Buffer I to the tubes was completed and followed by incubating of them in ice bath for 5 minutes. The lysates in the tubes were pipetted into filter columns and then, they were centrifuged for 2 minutes at 14000 rpm. Finally, clear supernatant (lysate)

were transferred into sterile Eppendorf tubes. 650 µL of 70% (v/v) ethanol were added to the lysates and vortexed for 30 seconds.

b) *Binding to Column:* 650 µL of lysates treated with ethanol were transferred to spin column and centrifuged for 1 minute at 10000 rpm. Flowthrough was discarded and proceeded to the next step.

c) *Column Wash:* 500 µL of solution WN were added to spin columns, and centrifuged at 10000 x g for 1 minute. After discarding the flowthrough, 500 µL of Wash Solution A were added to the column and centrifuged again at 10000 x g for 1 minute. Flowthrough was discarded and washing procedure was repeated once more. The columns were centrifuged at 14000 x g for drying the resin.

d) *DNA Elution:* The spin columns were placed into elution tubes. 100 µL of Elution Buffer B were added to the columns and incubation of them was performed at room temperature for 1 minute. Elution tubes were spinned for 1 minute at 10000 x g. Finally, purified genomic DNA was eluted. These DNA samples were stored at 2-8 ^{o}C.

2.4.5 Agarose Gel Electrophoresis

Firstly, Tris Acetate EDTA (TAE) buffer stock solution (10X) was prepared as explained in Section 2.1. Working buffer solution (1X) was used throughout electrophoresis. 200 mL of 1X TAE buffer solution including 1.0% (w/v) agarose were heated in microwave oven until obtaining a clear solution. After cooling of this solution around 60-65 ^{o}C, 12 µL of 10 mg/mL ethidium bromide were added into the mixture. Then, the solution was poured to gel tray and gelation occurred at 36 ^{o}C. After pouring working buffer solution to electrophoresis tank, 10 µL of extracted DNA samples with 2 µL of gel loading solution (6X) were loaded to wells. DNA samples were separated by applying 75 V for 90 minutes. At the end, gel images were observed under UV cabinet.

CHAPTER 3

RESULTS AND DISCUSSION

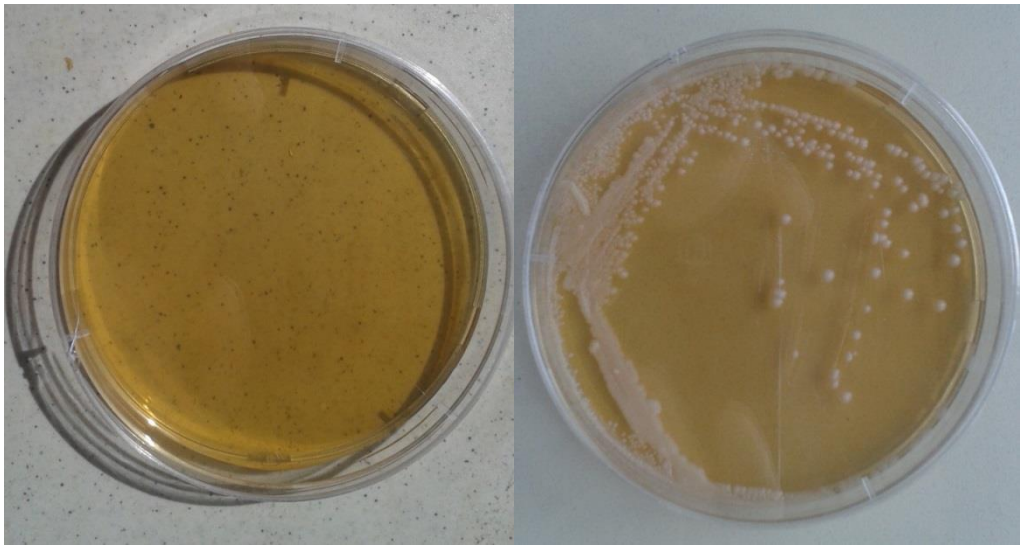
In the scope of this thesis, yeast (*Saccharomyces cerevisiae*) cells were grown to investigate effect of boron and arsenic in a living organism. After cells were grown up to logarithmic phase of growth (9^{th} hour), boron and/or arsenic (0.25, 0.50, 1.0 or 5.0 mM) were added to liquid growth media of yeast cells. Therefore, yeast cells were exposed to the elements of interest until stationary phase (24^{th} hour). Cell growth was tracked via optical density measurements at certain time intervals. Centrifugation was done to separate cell and supernatant phases in both logarithmic and stationary phases. After cell and supernatant solutions were digested separately, total boron and arsenic concentration were determined by ICP-OES and ICP-MS, respectively. As a result, mobility of B and As into cell from feed solution at certain time periods were aimed to be followed. Moreover, DNA damage due to arsenic and/or boron exposure was also followed by agarose gel electrophoresis to show possible DNA break.

3.1 Cell Choice

The reasons of choosing yeast (*Saccharomyces cerevisiae*) cells are as follows:

- It grows fast and simple. Cell doubling time duration is 90 minutes. After 48 hours of streaking, cell colonies in agar plate are as seen in Figure 3.1b. The number of cells are enough to use them in further studies.
- It is economical and commercially available. One package including 500 grams is approximately 100 €.
- It is simply conserved in laboratory conditions.

Picture of agar plate without and with yeast colonies on the plate were shown in Figure 3.1a and 3.1b, respectively.



(a) **(b)**

Figure 3.1. Agar plate for solid growth media of yeast cells (a), and yeast cell colonies appeared after 48 hours of streaking to the plate (b)

One of the critical parameters affecting yeast growth is temperature. Optimum temperature for yeast growth is 30 °C [98]. When temperature value is below than 30 °C, colonies do not appear in every streaked edge of the agar plate, so the growth is not as it should be. YPD (yeast extract peptone dextrose) is the required rich growth medium for yeast cells. YPD includes amino acids, vitamins, nucleotide precursors and essential metabolites in excess amounts [98, 101]. The agar medium should be prepared fresh otherwise growth of cell colonies becomes unsuccessful due to inadequate amount of nutrients as illustrated in Figure 3.2.

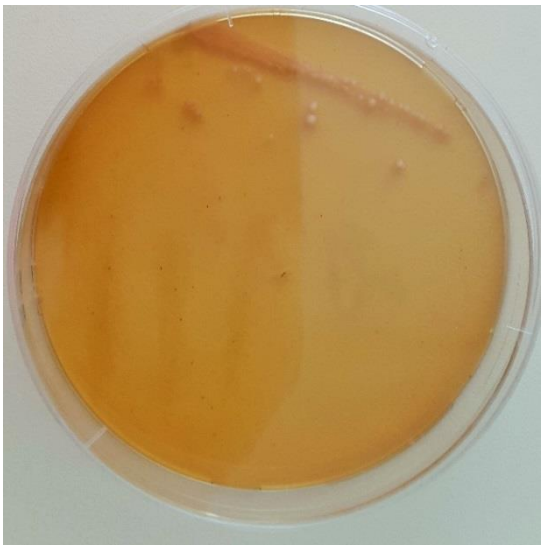


Figure 3.2. Yeast cell colonies in 2 weeks aged agar plate after 48 hours of streaking to the plate.

It is reported that YPD agar plates are stored in sealed parafilm at 4 °C up to three months [101], but in our study in such long-term storage, mold formation was frequently observed in addition to yeast cells as shown in Figure 3.3. YPD agar plates were not used in this study when mold formation was observed in long-term storage.

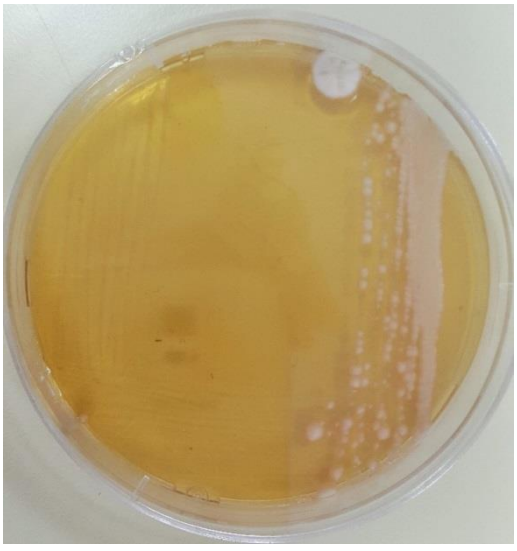


Figure 3.3. Yeast cell colonies in an agar plate after one month storage at 4 °C.

3.2 Yeast Cell Growth

3.2.1 Growth Curve of Yeast Cells in Various Concentrations of B and/or As

Optical density values of yeast cells at 600 nm (OD_{600}) were measured at certain time intervals with and without As and/or B in order to follow the effect of B and As on cell growth. A typical growth curve consisted of lag, log, stationary and death phases [100]. When cell number increase dramatically during logarithmic phase then, in stationary phase period cell population does not change drastically. In this study, logarithmic phase (time at which B added to the medium) and stationary phase were selected as 9^{th} and 24^{th} hour, respectively [46]. The time corresponding to these phases changes depending on the growth conditions (e.g. temperature, pH) and type of nutrients, so it may be different from one study to another study. For example, 9^{th} hour described as early logarithmic phase in Bennet and coworkers' study [46]; however, 9^{th} hour corresponded to logarithmic phase in this study. This is probably due to differences in type and amount of ingredients of growth media used. In Bennet et al. study, the media was composed of amino acids, salts, vitamins and carbon/nitrogen however, in this thesis commercially available growth media including peptone, yeast extract and glucose was used. Growth curves are shown in Figures 3.4, 3.5, 3.6 and 3.7 at various concentrations of As and/or B (0.25, 0.50, 1.0 and 5.0 mM). The initial OD_{600} value was adjusted to ca. 0.1 A by diluting 400 µL of cell culture 10 times.

Cells were grown as explained in the experimental section. Logarithmic phase is almost always the correct growth phase to harvest cells for experiments. Firstly, yeast cells were supplemented by spiking 0.25 mM B and/or As to liquid growth medium at logarithmic phase. Growth curves of feed solution including both cells and its liquid growth medium spiked with 0.25 mM B and/or As were compared with that of the control group as shown in Figure 3.4.

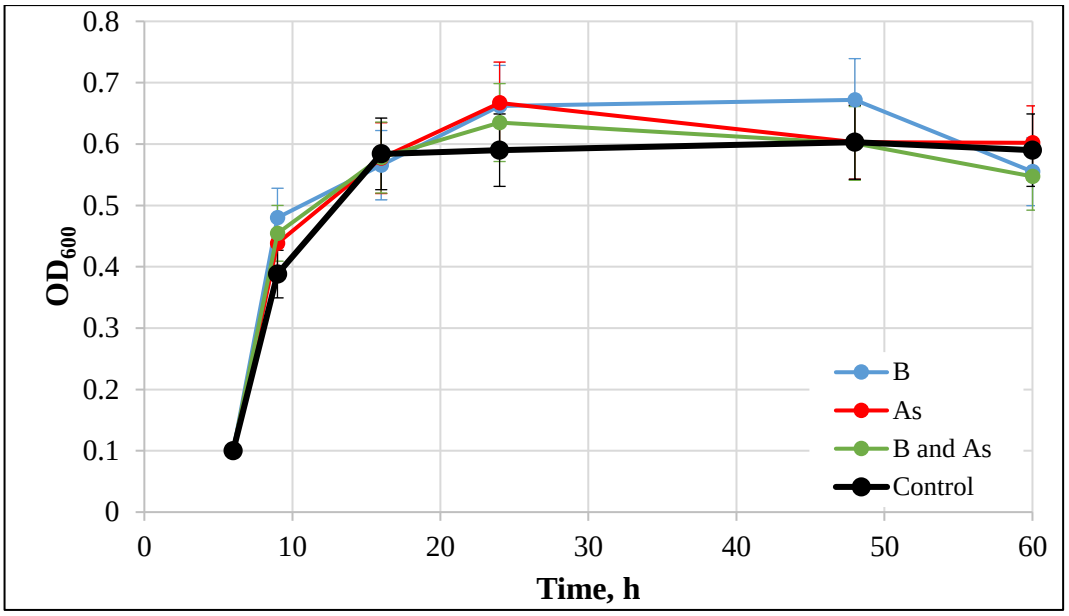


Figure 3.4. Growth curves of yeast cells fed with *0.25 mM* B and/or As (N=2). In control group yeast cells were waited only in growth medium without B and As.

As seen in Figure 3.4, there was a significant increase up to 9th hour (logarithmic phase) due to active cell division. Then, cell growth slowed down and cell population remained almost constant after reaching to 24th hour (stationary phase). Finally, death phase started with a decrease in optical density values after 48th hour. As shown in Figure 3.4, compared to control group cells fed with 0.25 mM arsenic only, boron only or boron and arsenic together, it was not observed a significant difference in the growing path of yeast cells. Additionally, any significant difference was not seen between cells fed with boron only, arsenic only or boron and arsenic together compared to control group at concentration level of 0.50 and 1.0 mM similar to that of 0.25 mM. The growth curves belong to feed solutions including cells and growth medium spiked with 0.50 and 1.0 mM B and/or As are shown in Figure 3.5 and 3.6, respectively.

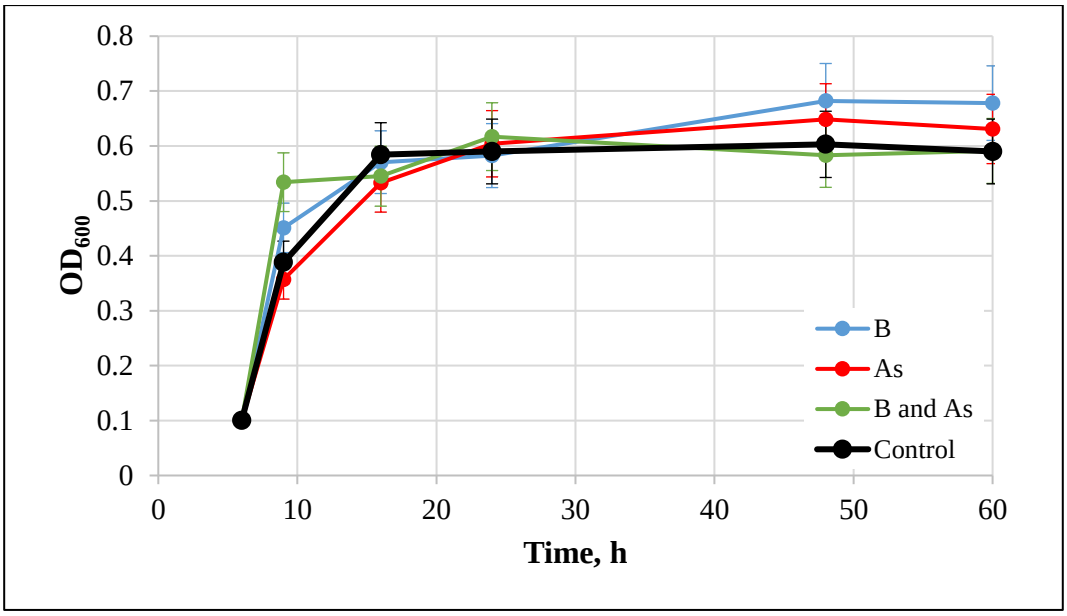


Figure 3.5. Growth curve of yeast cells fed with *0.50 mM* B and/or As (N=2).

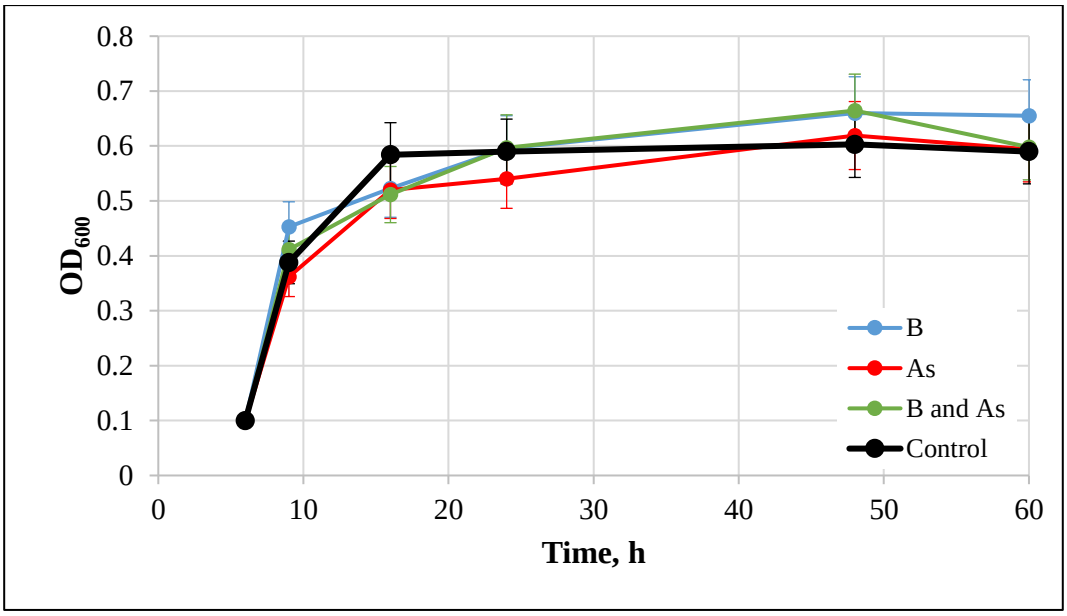


Figure 3.6. Growth curve of yeast cells fed with *1.0 mM* B and/or As (N=2).

Effect of highest concentration, 5.0 mM B or As, used in this study on yeast growth was shown in Figure 3.7. A dramatic decrease in OD_{600} value was observed with the exposure of 5.0 mM As only and 5.0 mM B and As together. Therefore, it can be concluded that the cell growth affected negatively as compared to control group at

5.0 mM concentration level of As exposure. However, 5.0 mM boron only spiking to the growth medium showed similar trend with the control group, so boron was not responsible for negative effect on the cell growth at 5.0 mM concentration level. In case of B and As together feeding, presence of B with As does not eliminate the decrease in the number of cells due to presence of As. Additionally, cell death began due to the presence of arsenic after 48th hour.

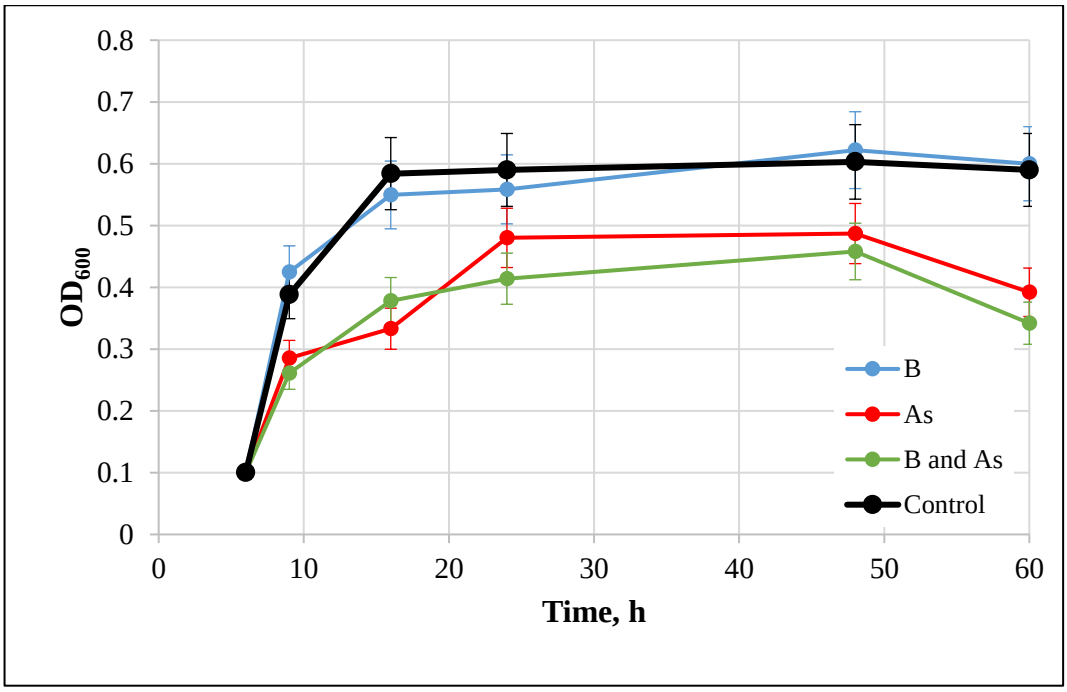


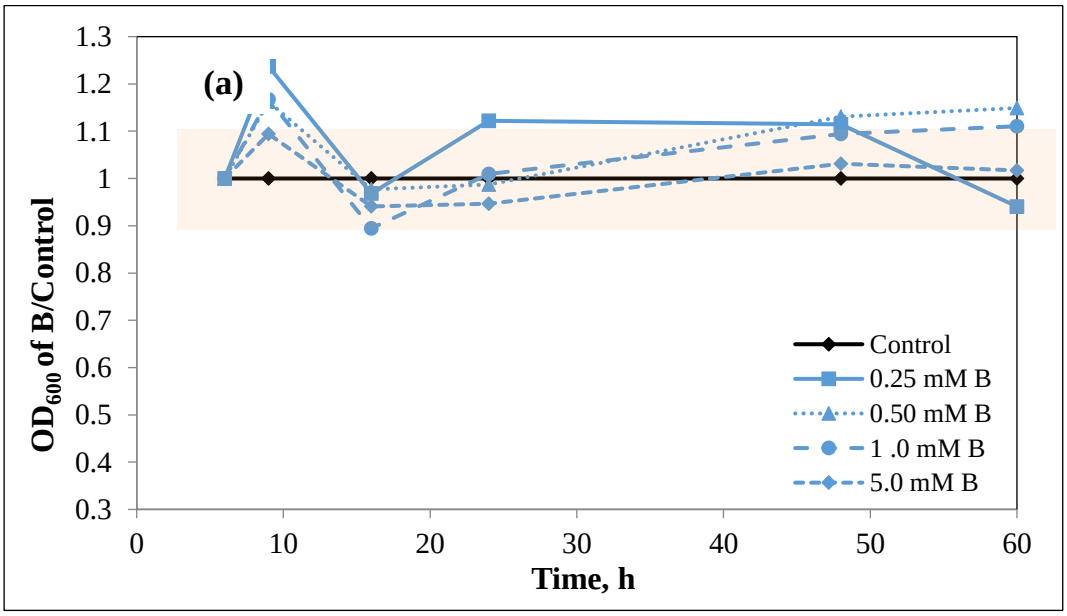
Figure 3.7. Growth curve of yeast cells fed with *5.0 mM* B and/or As (N=2)

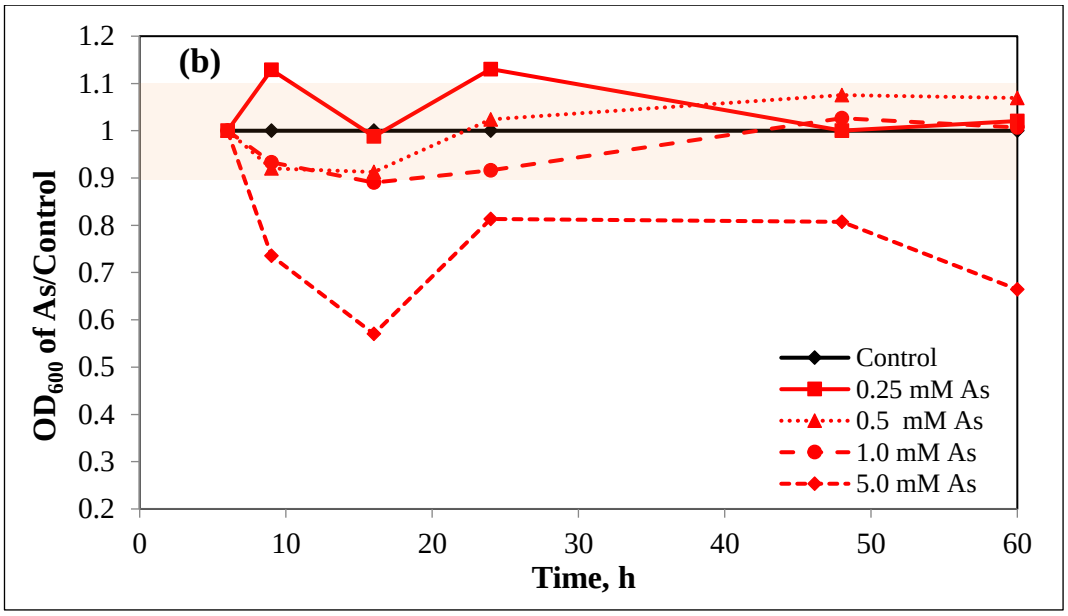
OD_{600} values with respect to time in Figures 3.4, 3.5, 3.6 and 3.7 were redrawn to show the effect of B and As concentration on the number of cell with time on the same graph. Ratio of OD_{600} *with supplemented cells* values to that of *control group* values vs time at 0.25 mM, 0.5 mM, 1.0 mM and 5.0 mM concentration level were calculated and these ratios vs. time graphs were shown in Figures 3.8a, 38.b and 3.9c. %RSD values were 10% in these measurements for 2 replicate measurements. Therefore, the ratio is equal to 1.0 for control group. If there is no effect of spiking

with B or As, then the ratio is equal to 1 or within the shaded area which shows the relative standard deviation in each measurement.

As shown in Figure 3.8a, the ratios with and without *boron* are not statistically different from each other, so positive or negative effect of boron on the number of yeast cells at none of log, stationary and death phases were not measured depending on increase in the concentration B as seen in Figure 3.8a. However, addition of 5.0 mM As to feeding medium of yeast cells decreases the number of cells by approximately 30% as shown in Figure 3.8b. Therefore, feeding yeast cells with As negatively affect the cell growth and the addition of B beside As does not influence the adverse effect of As on cell growth as illustrated in Figure 3.8c.

In Bennett et al. [46] study, yeast cells were fed with 0.185 mM B. They reported that presence of B stimulates yeast cell growth. In other words, growing in feeding medium supplemented with 0.185 mM B increased the number of yeast cells significantly compared to control group which were not fed with B [46]. However, in this thesis the use of B in growth media of yeast cell does not stimulate or negatively affect cell growth.





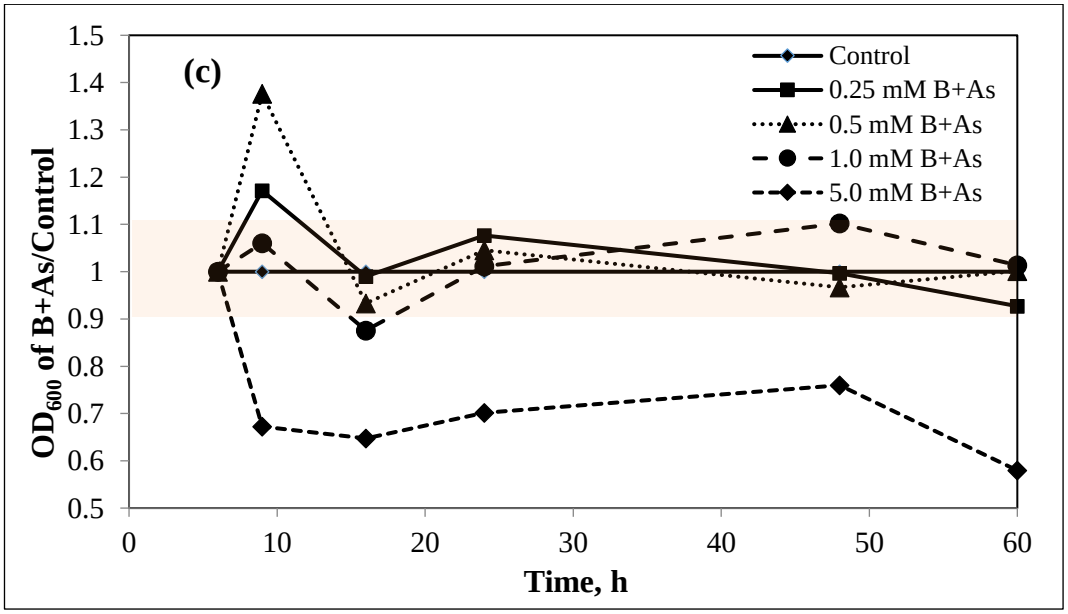


Figure 3.8. Ratio of OD_{600} values of yeast cells supplemented with boron only **(a)**, arsenic only **(b)** and arsenic and boron together **(c)** to that of control group. Shaded area shows the region corresponding to 10% RSD values.

3.3 Determination of Boron

In this study, sensitivity of ICP-OES instrument was better than that of ICP-MS for B, therefore two analysis technique, ICP-OES for B and ICP-MS for As, were used for the analysis of cell and supernatant samples.

3.3.1 Optimization of ICP-OES Parameters

Working plasma parameters were optimized to achieve highest sensitivity and precision for ICP-OES analysis. All these parameters affect significantly plasma stability and emission intensities. The optimum operating parameters for the instrument were given in Table 3.1.

Table 3.1. ICP-OES Operating Parameters

RF power, kW	1.3
Coolant gas flow rate, L/min	18
Auxiliary gas flow rate, L/min	0.5
Nebulizer pressure, psi	35
Sample flow rate, mL/min	1.0

Sample matrix contains high levels of dissolved solids due to the presence of yeast extract, peptone and dextrose in growth media. As a result, RF power was set at 1.3 kW, use of lower values resulted in instability of the plasma.

The nebulizer gas flow rate is required high enough for the penetration of aerosol to the plasma. On the other hand, the use of high flow rate may decrease the residence time of aerosols in the plasma and thus, the sensitivity decreases. The pressures of

the nebulizer was varied between 25 psi and 60 psi, and 35 psi was selected as the optimum value based on the maximum sensitivity.

Sustaining plasma was provided by coolant argon gas. Intensities were not affected from small changes in the flow rate of the plasma gas. The optimum flow was adjusted as 18 L/min to prevent torch overheating.

The function of auxiliary gas is to keep the plasma away from injector tube to avoid clogging of injector tube tip due to sample residues. Optimum value of auxiliary gas flow was selected as 0.5 L/min.

Pump rate was varied between 0.5 mL/min and 1.5 mL/min. Emission intensities increased up to 1.0 mL/min, but no significant change in signal intensities was observed beyond 1.0 mL/min. Therefore, 1.0 mL/min was chosen as the optimum pump rate value.

3.3.2 Calibration Plot of Boron

Calibration graph is drawn between 50 ng/mL to 5000 ng/mL concentration range as illustrated in Figure 3.9.

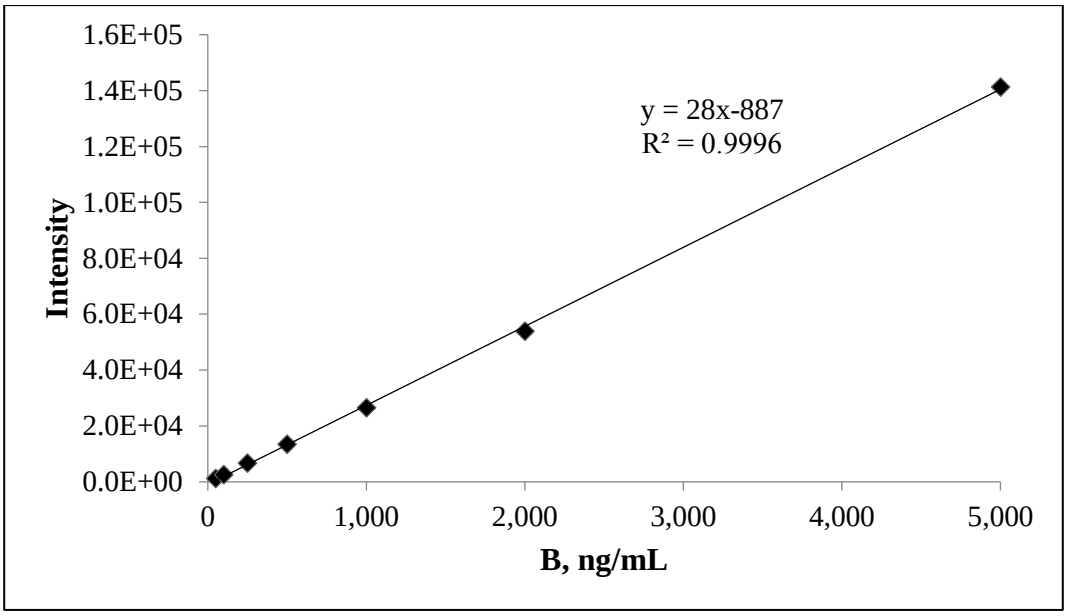


Figure 3.9. Direct calibration curve of B by ICP-OES.

Analytical figures of merit including Limit of Detection, LOD (3s/m), Limit of Quantitation, LOQ (10s/m) and working range values are listed in Table 3.2. LOD was calculated by dividing three times standard deviation (s) of seven replicate intensity readings (N=7) of 50 ng/mL B standard solution to the slope of the calibration plot (m).

Table 3.2. Analytical figures of merit for B

	B, ng/mL
LOD	11 (1.0 x 10^{-3} mM)
LOQ	35 (3.3 x 10^{-3} mM)
Range	50-5000

3.3.3 Accuracy Check of the Method

Accuracy of the method was checked using NIST 1573a Tomato Leaves Standard Reference Material (SRM). 9.0 mL of concentrated HNO_3 and 0.5 mL of concentrated HF were used to digest 0.5000 g of SRM. No precipitation was

observed at the end of acid digestion. Microwave digestion program was same with the program applied for digestion of cell and supernatant samples as shown in Table 2.1 in the experimental part. Direct calibration method was applied to determine the concentration of boron in SRM but the result was not in an agreement with the certified value. Therefore, standard addition method was used to match the matrix of standards and SRM. No significant difference was found between certified and experimental values when t-test was applied at 95% confidence level as shown in Table 3.3. Standard addition graph was shown in Figure 3.10.

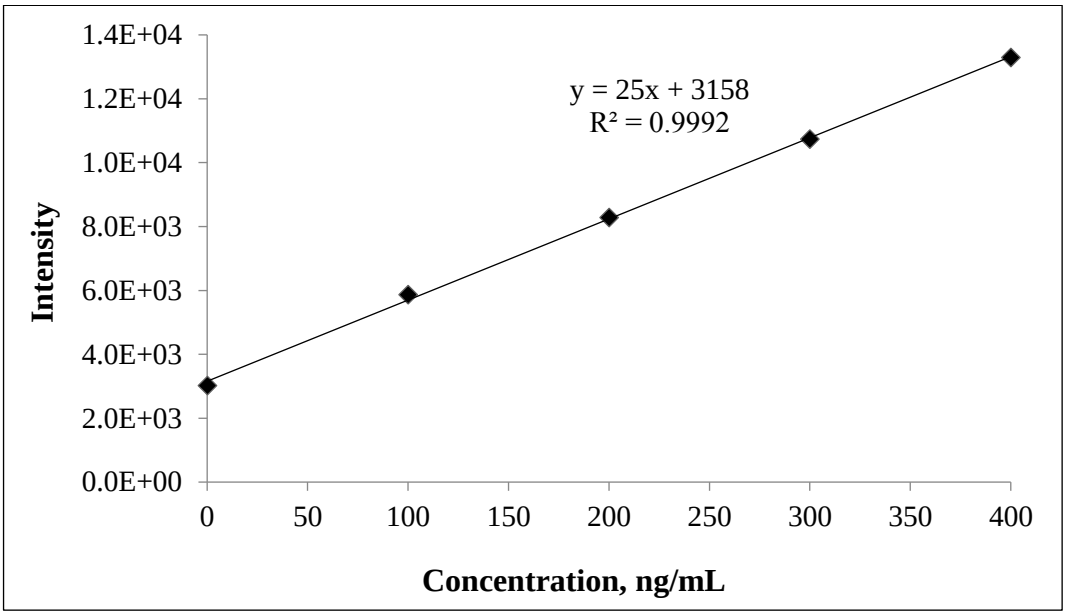


Figure 3.10. Plot of standard addition method for the determination of *B in SRM* by ICP-OES.

Table 3.3. Certified value and experimental result of SRM used for validation.

SRM	Certified Value*, mg/kg	This study*, mg/kg (N=3)
NIST 1573a Tomato Leaves	33.3 ± 0.7	31.1± 2.4

*Mean ± standard deviation

3.3.4 Boron Distribution in Yeast Cells and Supernatant

After addition of B at a certain concentration level to the feed solution including yeast cells and its liquid growth media at logarithmic phase, yeast cells and supernatant solution was separated from each other both at 9^{th} hour and 24^{th} hour by centrifugation. After applying acid digestion, boron concentration was determined in yeast cells and supernatant solution by ICP-OES.

Yeast cell and supernatant samples were digested separately. Acid combination of 4.0 mL of concentrated nitric acid and 2.0 mL of hydrogen peroxide was used firstly, but distorted signal shapes and high background intensities were measured from digested samples. As a result, acid content of the digestion procedure was reduced by half. Afterwards, both cell and supernatant samples were treated with 2.0 mL of concentrated nitric acid and 1.0 mL of hydrogen peroxide. Then, they were completed to 100.0 mL with deionized water. Better Gaussian signal shapes and lower background intensities were obtained by decreasing the acid content of digestion procedure.

Standard addition method was applied to determine boron in supernatant samples since matrix components of feed solution are complicated including such as glucose and amino acids. Standard addition graph was shown in the Figure 3.11. Quantification was done with ionic line of B at 249.773 nm.

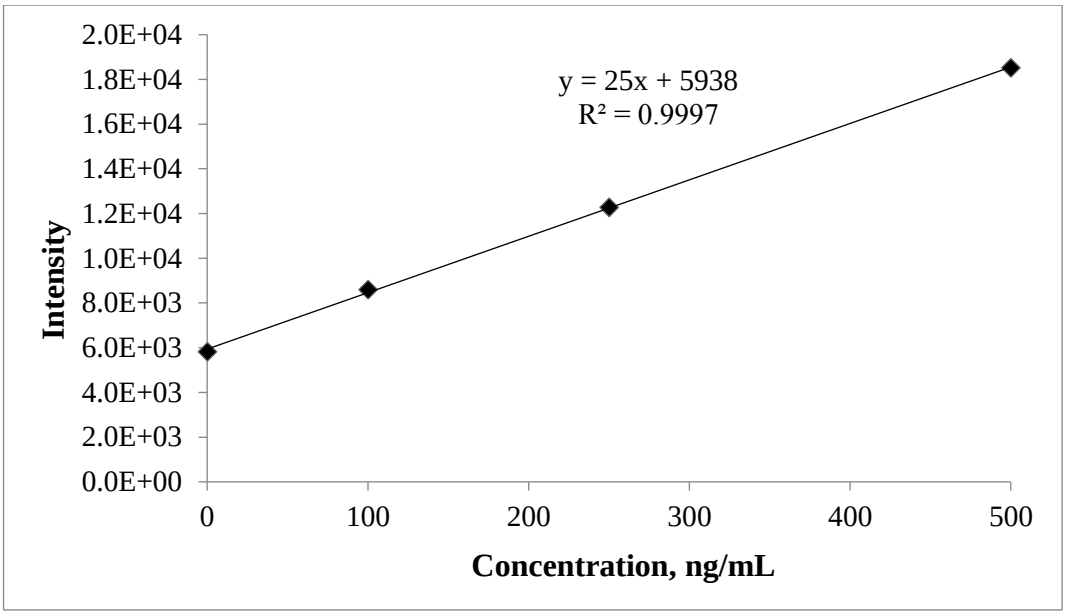


Figure 3.11. Standard addition graph of *B in supernatant* samples.

After measuring boron intensity of cell and supernatant samples, intensity of the sample was put into y variable in the best line equation of standard addition plot. The resulting x values are corresponded to concentration of the samples in ng/mL.

Control yeast cell results are not reported in the following sections since the concentrations of boron were below LOD values.

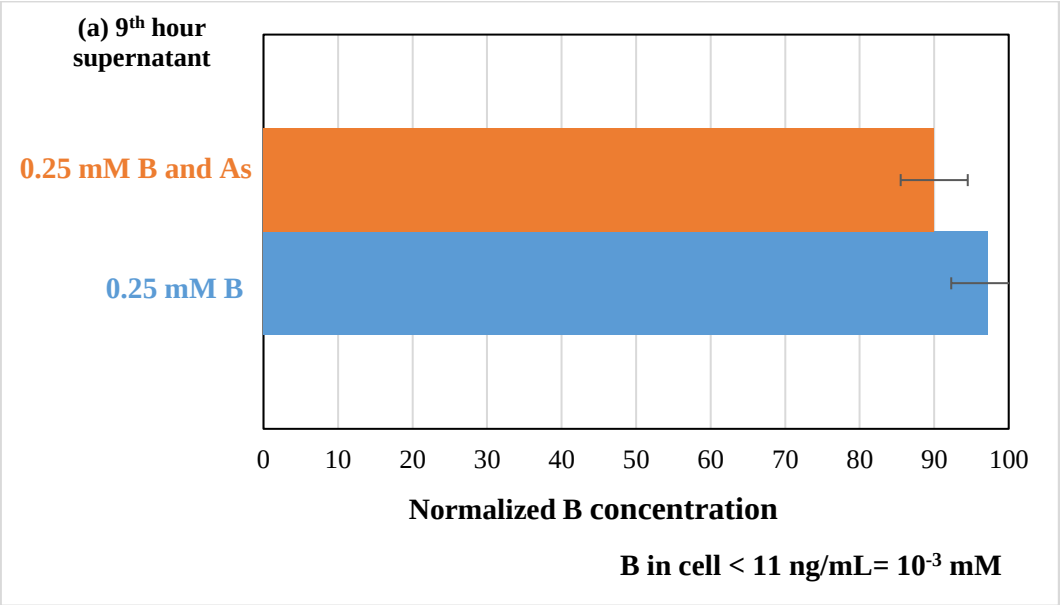
As mentioned above, feed solution contains cell and its supernatant which is in YPD media. After ICP-OES evaluation of digested cell and supernatant separately, B distribution graphs were obtained for 0.25 mM B in feed solution for both 9^{th} and 24^{th} hours as shown in Figure 3.12a and 3.12b, respectively. Three readings were taken for each sample and standard deviations corresponding to these replicate measurements were shown in the graphs.

Boron concentration was measured at 9^{th} and 24^{th} hours and boron distribution in supernatant solutions are given in Figures 3.12, 3.13 and 3.14 when yeast cells fed with, 0.25, 0.5 and 1.0 mM B, respectively. In these graphs, x-axis concentration values were normalized with respect to the spiked concentration values. In other

words, 0.25, 0.50, 1.0 and 5.0 mM concentrations were accepted as 100% and then, the measured values were normalized to 100% values for each graph. Only supernatant results are presented in these figures since boron in yeast cells at all concentration levels studied in this thesis except for 5.0 mM was measured below $1.0x10^{-3}$ mM, which is the detection limit of ICP-OES instrument.

Up to 5.0 mM B supplemented experiments, it was measured that more than 90% of B remained in supernatant part irrespective of yeast cells were fed with *B only* or *B and As together* in growth medium. Presence of As did not increase the diffusion of B inside yeast cells above the detection limit value of B.

When yeast cells were grown in the presence of 5.0 mM B, the concentration of B in the cell was measured above the detection limit. Therefore, distribution of B inside the cell and supernatant solution was shown in Figures 3.15a and 3.15b at 9^{th} and 24^{th} hours, respectively. There is not much change observed from 9^{th} to 24^{th} hour in the distribution of B in the cell and supernatant solution and the percentage of B in the cell was around 5% both only B supplemented yeast cells. Besides, addition of As increased the B inside the cell only few percent. From these results, it can be concluded that diffusion of B into yeast cells also did not influence the grown pattern of yeast cells if yeast cells were fed between 0.25 and 5.0 mM B only as explained in Section 3.2.



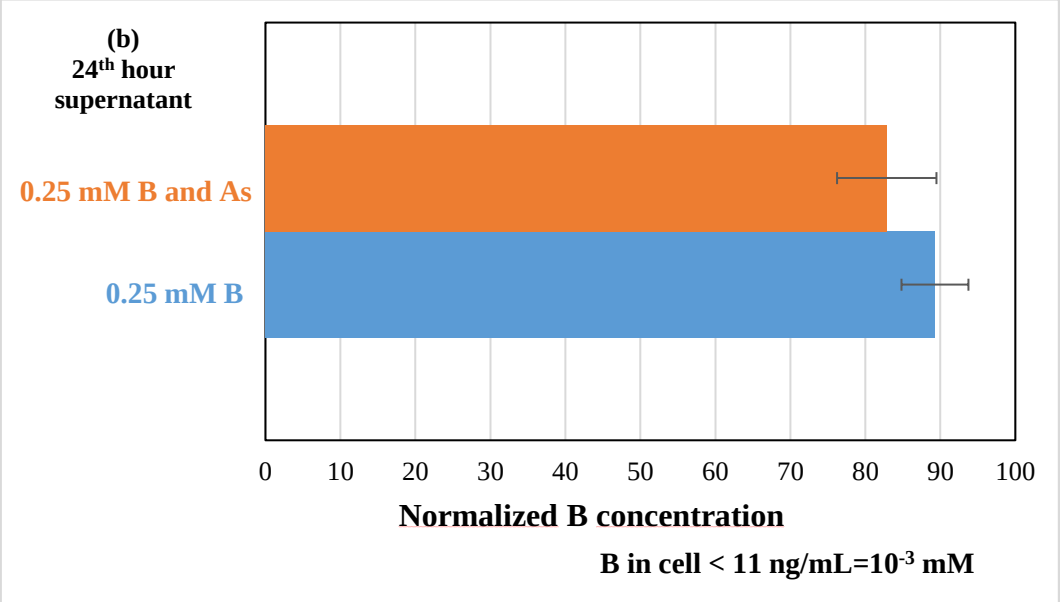
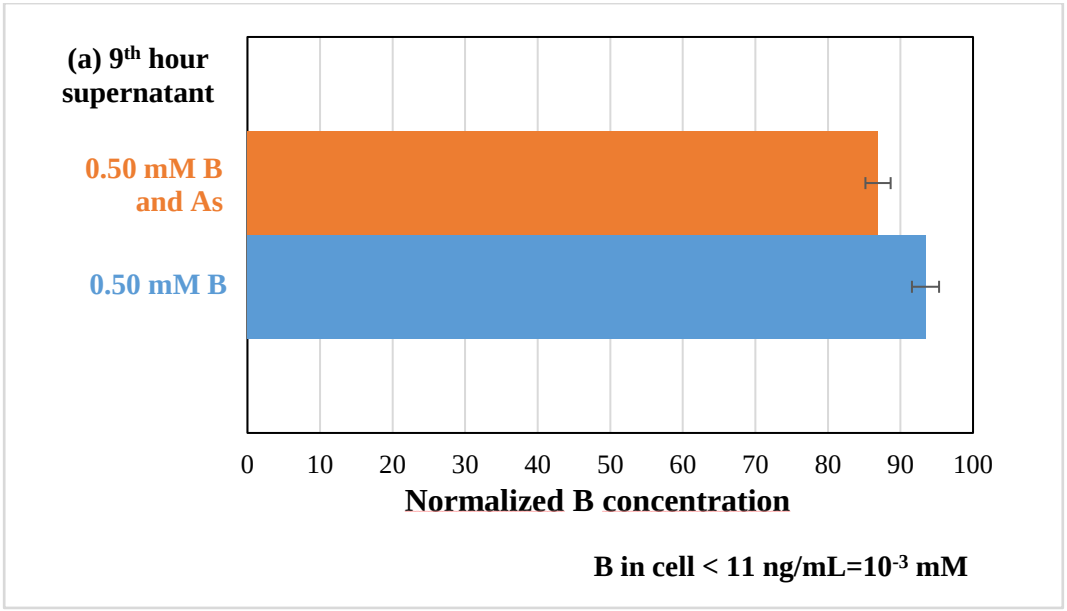


Figure 3.12. Distribution *B in supernatant solution* after supplementing the yeast cells with *0.25 mM B* or *0.25 mM B and 0.25 mM As spiked* growth medium at **(a)** *9th hour* (logarithmic phase) **(b)** 24th hour (stationary phase).



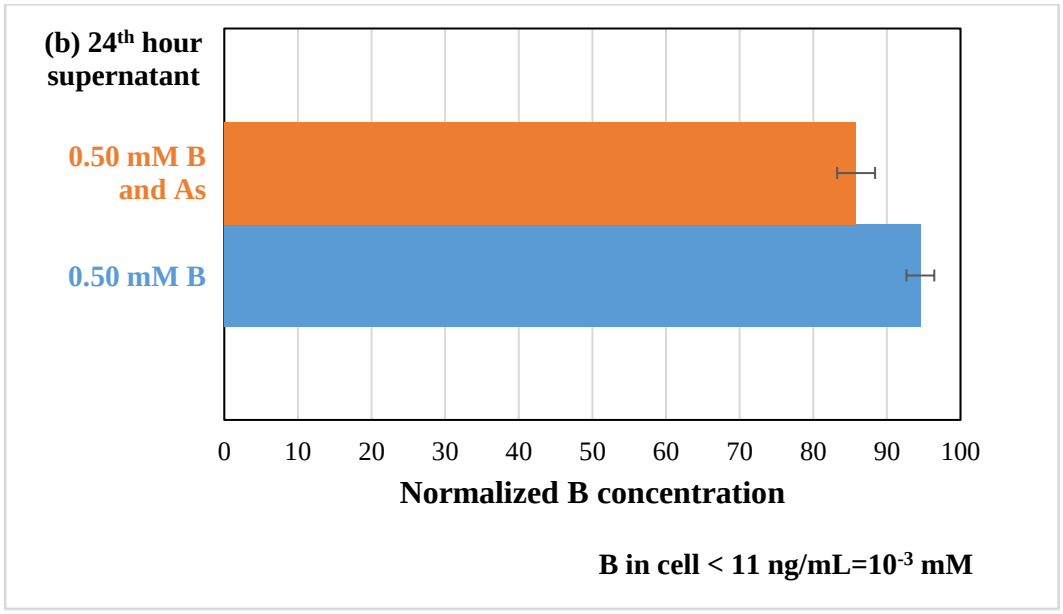
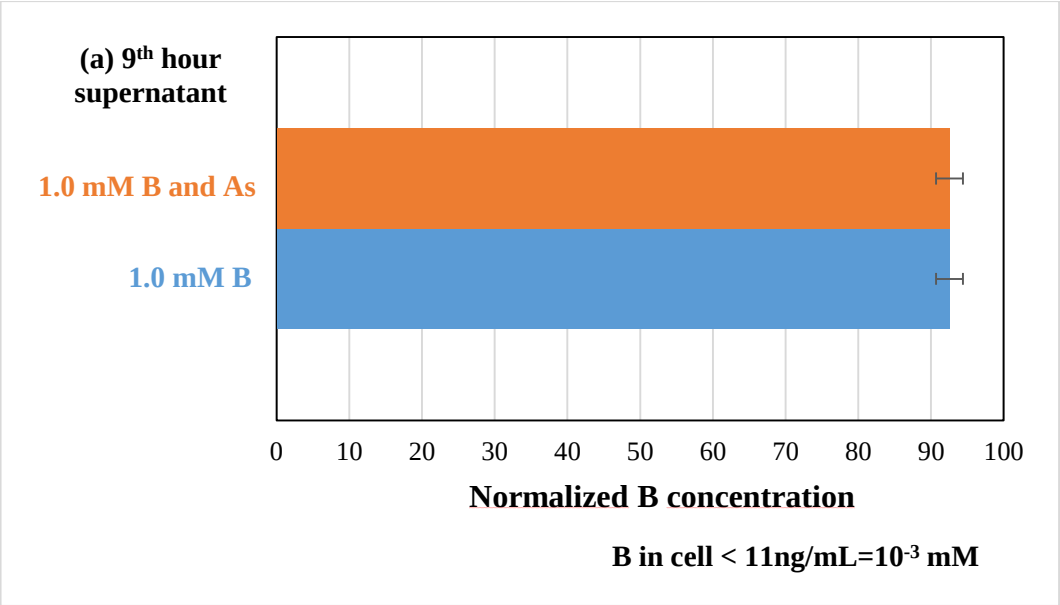


Figure 3.13. Distribution *B in supernatant solution* after supplementing the yeast cells with *0.50 mM B spiked* growth medium at **(a)** *9th hour* (logarithmic phase) **(b)** 24th hour (stationary phase).



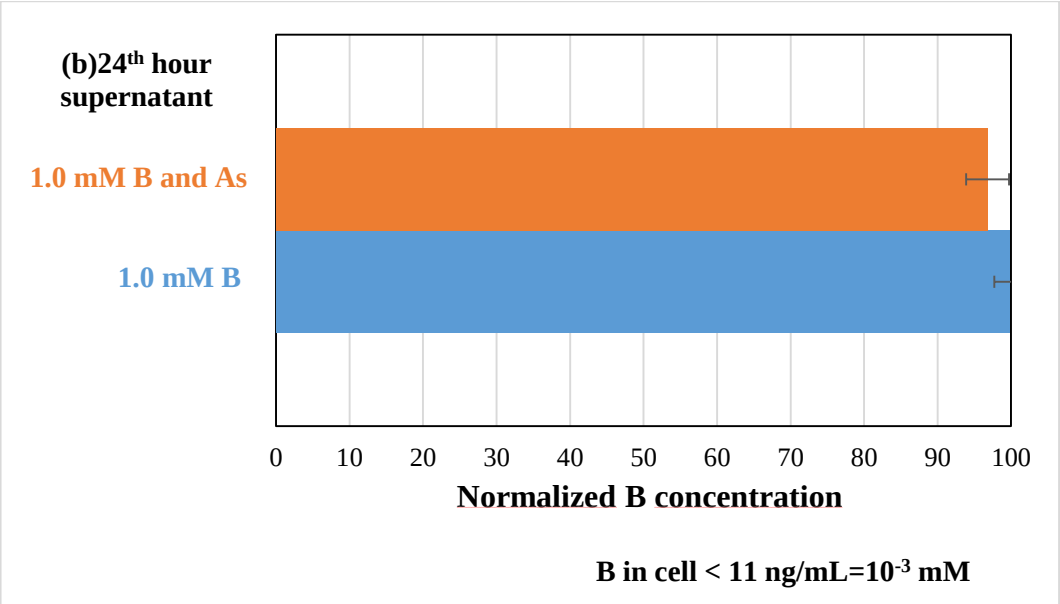
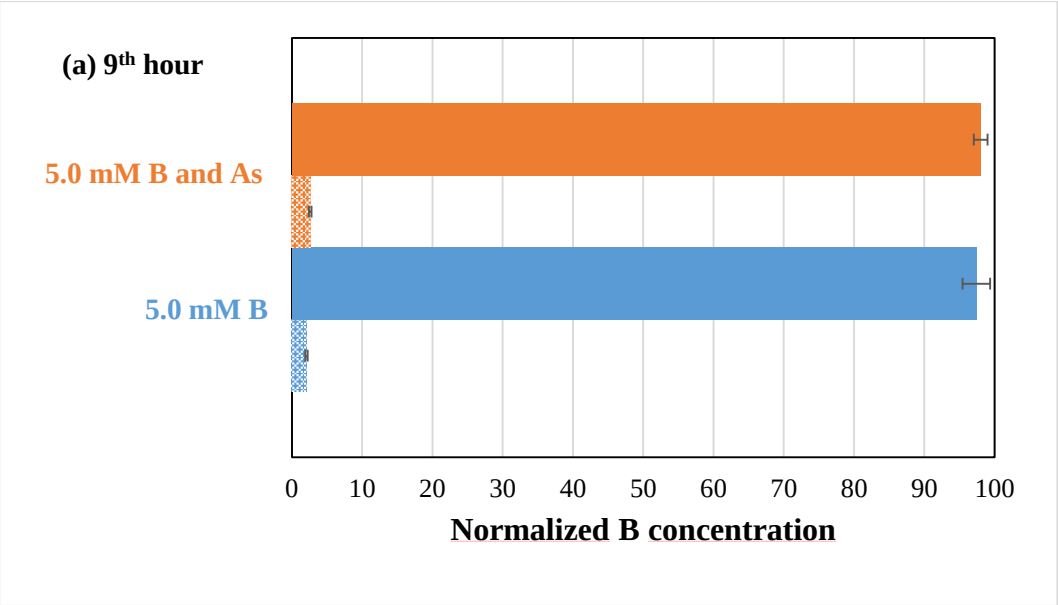


Figure 3.14. Distribution *B in supernatant solution* after supplementing the yeast cells with *1.0 mM B spiked* growth medium at **(a)** *9th hour* (logarithmic phase) **(b)** 24th hour (stationary phase).



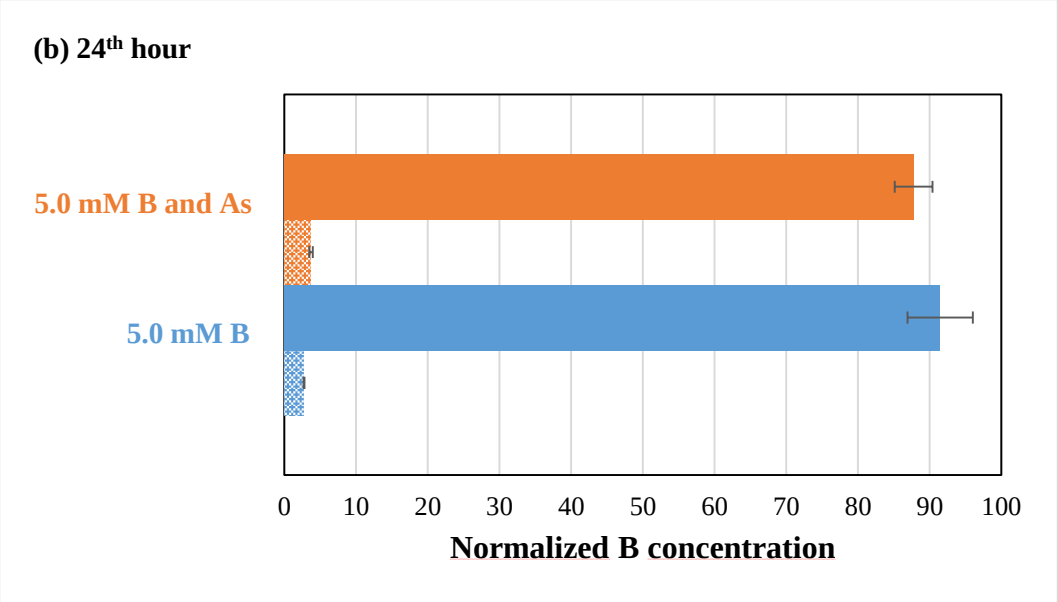


Figure 3.15. Distribution *B in yeast cells and supernatant solution* after supplementing the yeast cells with *5.0 mM B spiked* growth medium at **(a)** *9th hour* (logarithmic phase) **(b)** 24th hour (stationary phase). Patterned columns belong to cell samples, plain ones represent supernatant solutions results.

In Bennett and coworkers' study [46], it was reported that boron concentration decreased in both boron supplemented and non-supplemented yeast cells from early logarithmic phase (9^{th} hour) to stationary phase (24^{th} hour). In other words, boron inside the cell diffused to the liquid growth media by increasing the exposing period of cells to boron supplemented growth media. In our results, we do not observe the stimulation effect of B on yeast cells growth up to 5.0 mM B concentration unlike to Bennet et al. study, In their study, even though stimulation effect of boron on cell growth was connected to presence of boron, cellular boron concentrations of boron supplemented and non-supplemented cells were not significantly different from each other [46].

3.4 Determination of Arsenic in Samples

3.4.1 Optimization of ICP-MS Parameters

Working parameters of ICP-MS instrument should be optimized to obtain best sensitivities. These parameters are relevant to sample introduction, plasma conditions and ion sampling, and optic system together with detector parameters. Optimization of them was achieved by 10 µg/L ''Tune A'' solution including Li^{+}, Co^{2+}, In^{3+}, Pb^{2+}, Bi^{5+} and U^{4+} in 0.5 mol/L nitric acid solution. In addition, 10 µg/L As(III) solution were used for the optimization of ^{75}As isotope signal. The operating conditions for ICP-MS were given at the Table 3.4.

Table 3.4. ICP-MS Operating Parameters

Parameters	**Optimum Value**
Extraction Lens Voltage, V	-114
Lens 1 Voltage, V	-2.4
Lens 2 voltage, V	-45.5
Focus lens voltage, V	12.5
1. Diffraction aperture voltage, V	-40.0
2. Diffraction aperture voltage, V	-140
Quadrupole voltage, V	-2.5
Hexapole voltage, V	-4.0
Nebulizer pressure, psi	0.93
Lens 3 voltage, V	-197.6
Horizontal position of torch	104
Vertical position of torch	419
3. Diffraction aperture voltage, V	-29.8
Cooling gas, L/min	13.0
Auxiliary gas, L/min	0.80
Sampling depth**,** mm	152
Forward power, W	1400

3.4.2 Calibration Plot of Arsenic

Calibration standards with concentrations ranging from 1 to 500 ng/mL were used for plotting the direct calibration curve for arsenic as shown in Figure 3.16.

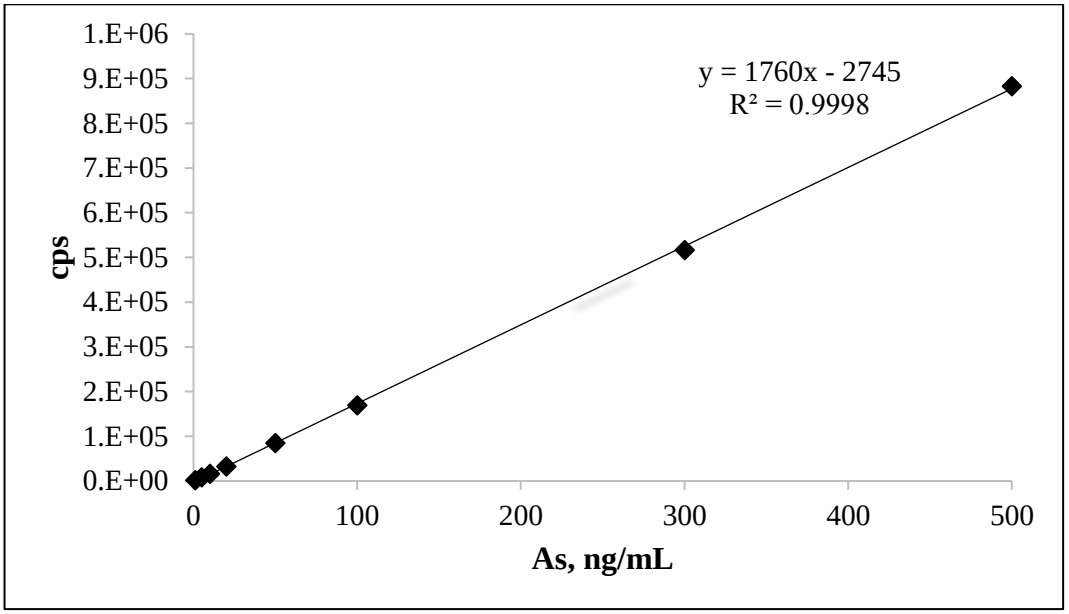


Figure 3.16. Calibration graph and best line equation for of ^{75}As ICP-MS.

For ICP-MS system, LOD (3s/m) and LOQ (10s/m) were calculated using 1.0 ng/mL As standard solution. Values of LOD, LOQ and range are listed in Table 3.5

Table 3.5. Analytical figures of merit of As.

	As, ng/mL
LOD	0.06 (8.0×10^{-7} mM)
LOQ	0.2 (3.0×10^{-6} mM)
Range	1-500

3.4.3 Accuracy Check of the Method

Accuracy of the system was checked by using DOLT-4 Fish Liver SRM. 0.1000 g SRM was weighed and digested with 3.0 mL of concentrated distilled HNO_3 and 3.0 mL of H_2O_2. SRM was digested using microwave-assisted digestion system. Applied microwave program was listed in Table 3.6.

Table 3.6. Microwave program for digestion of DOLT-4 Dogfish Liver

Temperature, °C	**Ramp time, min**	**Hold time, min**
100	5	10
150	5	10

Standard addition method was used to determine the concentration of As in SRM by ICP-MS as shown in Figure 3.17. No significant difference was found between the certified values and experimental values when t-test was applied at 95% confidence level. Results are given in Table 3.7.

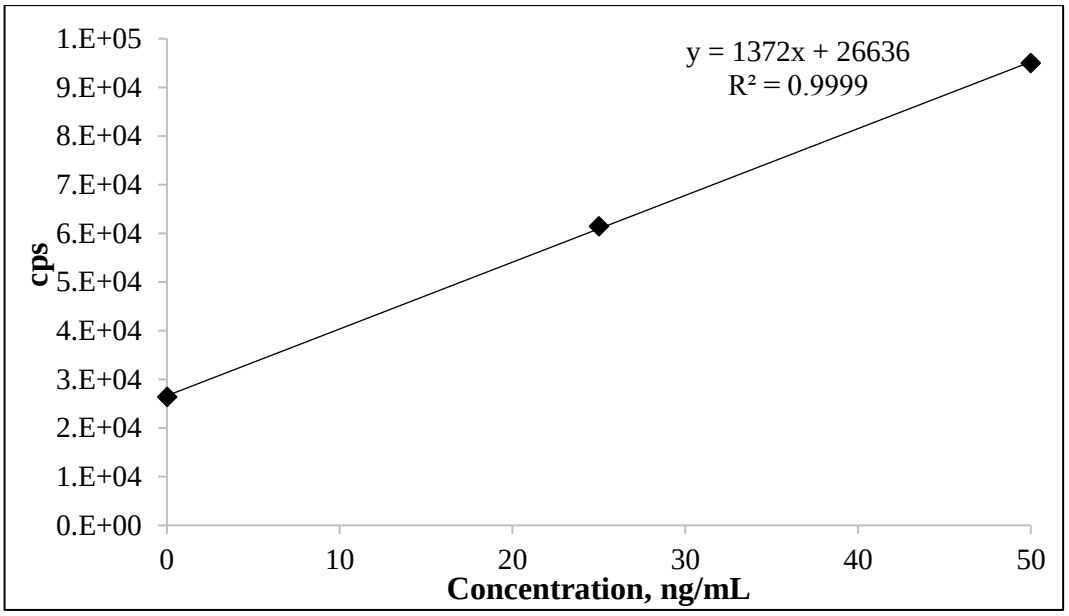


Figure 3.17. Plot of standard addition method for determination of *As in SRM* by ICP-MS.

Table 3.7. Certified value and experimental result of standard reference material used for validation.

SRM	Certified*, mg/kg	This study*, mg/kg (N=5)
DOLT-4 Dogfish Liver	9.66 ±0.62	9.16 ±0.45

*Mean ± standard deviation

3.4.4 Arsenic Distribution in Yeast and Supernatant Samples

Total As concentration in cell and supernatant solutions was determined using ICP-MS. 50 fold dilution was done to eliminate the effect of matrix interferences due to complex matrix of liquid growth media of yeast cells. However, slopes of direct calibration and standard addition plots correspond to cell and supernatant samples are significantly different from each other and this difference is a strong evidence for matrix interference. Therefore, standard addition method was used for quantitative

analysis of 50-fold diluted supernatant samples and 5-fold diluted cell samples. The standard addition plots are shown in Figures 3.18 and 3.19 for supernatant and cell samples, respectively.

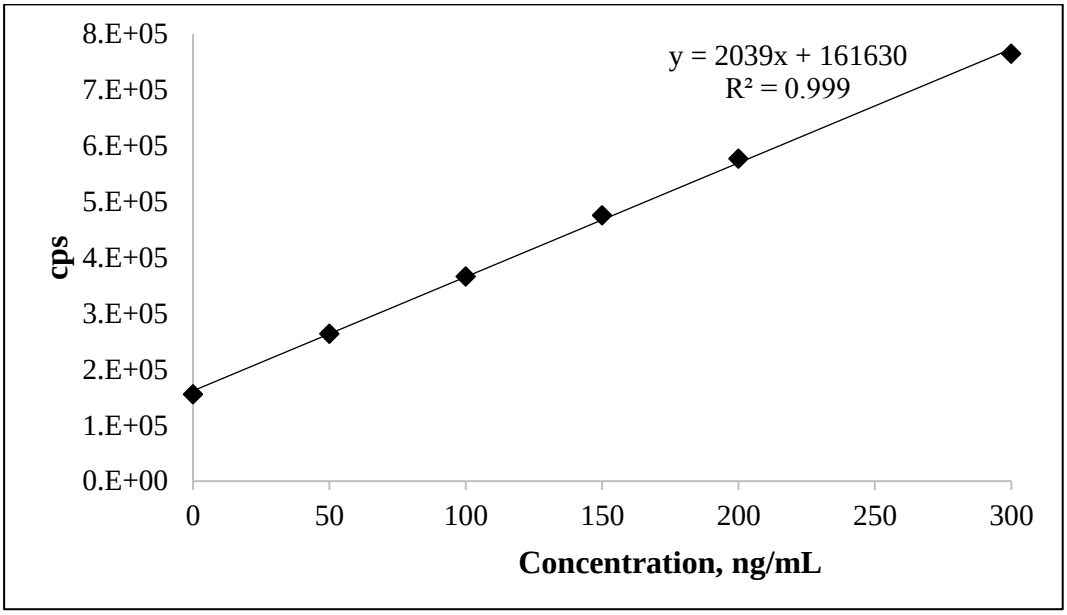


Figure 3.18. Standard addition graph of *As in supernatant* samples.

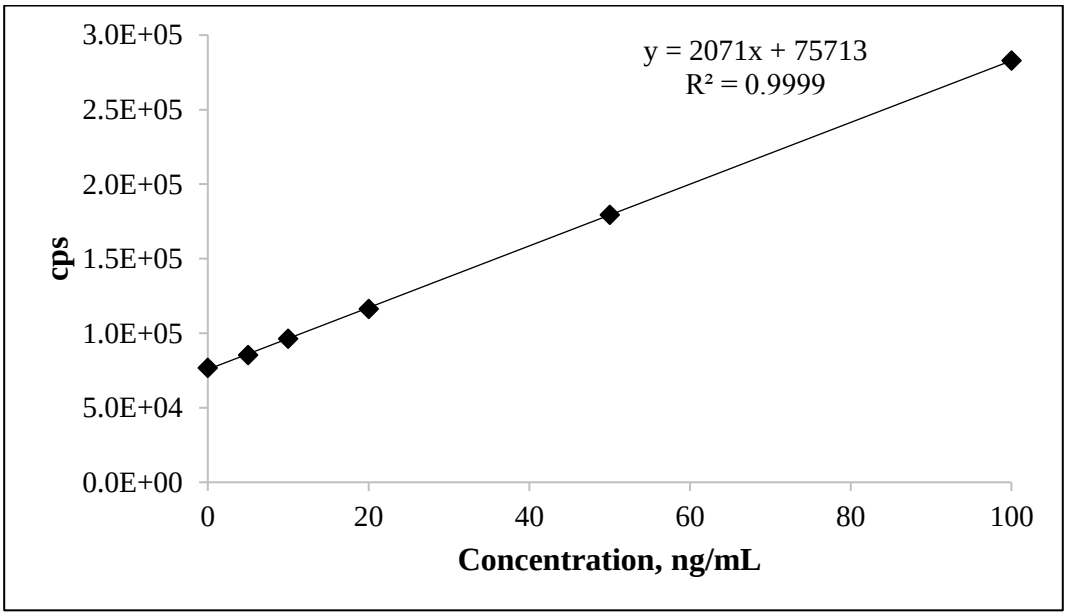


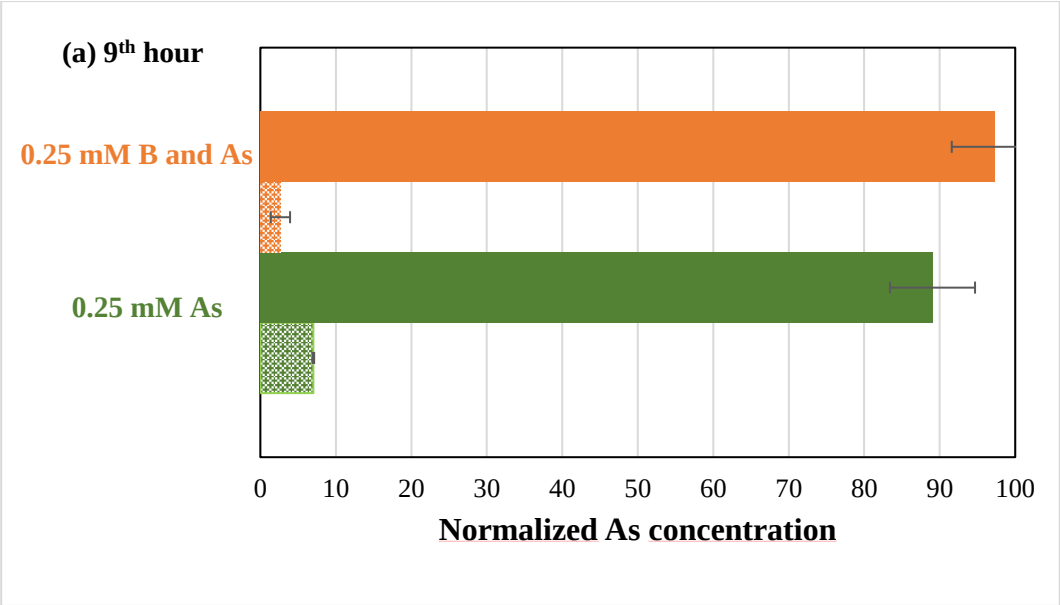
Figure 3.19. Standard addition graph of *As in cell* samples.

The mobility of boron between cell and growth medium were followed in the literature [31, 46]. However, to the best of our knowledge there is no study tracking

the distribution of arsenic or B and As together within yeast cells using elemental techniques. Studies of antioxidant effect of boron against the toxicity of arsenic in the literature [94, 95] were the motivation of this study to follow the diffusion of B and As to inside and outside of yeast cells to develop a possible synergistic or antagonistic effect of boron on arsenic.

Arsenic distribution between cell and supernatant solutions was shown in Figures 3.20, 3.21, 3.22 and 3.23 at 0.25, 0.5, 1.0 and 5.0 mM As with and without B, respectively. In these graphs, x-axis As concentration values were normalized with respect to the spiked concentration values. In contrast to boron, arsenic concentration in the cell was above detection limit, so in these distribution graphs, both cell and supernatant As distribution was shown. The results belong to two replicate samples and their standard deviations were also shown in these figures.

Up to 5.0 mM B supplemented experiments, it was measured that more than 80% of As remained in supernatant part irrespective of yeast cells were fed with *As only* or As *and B together* in growth medium. Presence of B did not increase the diffusion of As inside yeast cells. There is no significant difference observed from 9^{th} to 24^{th} hour in the distribution of As in the cell and supernatant solution and the percentage of As in the cell was below 10% As supplemented yeast cells with or without B.



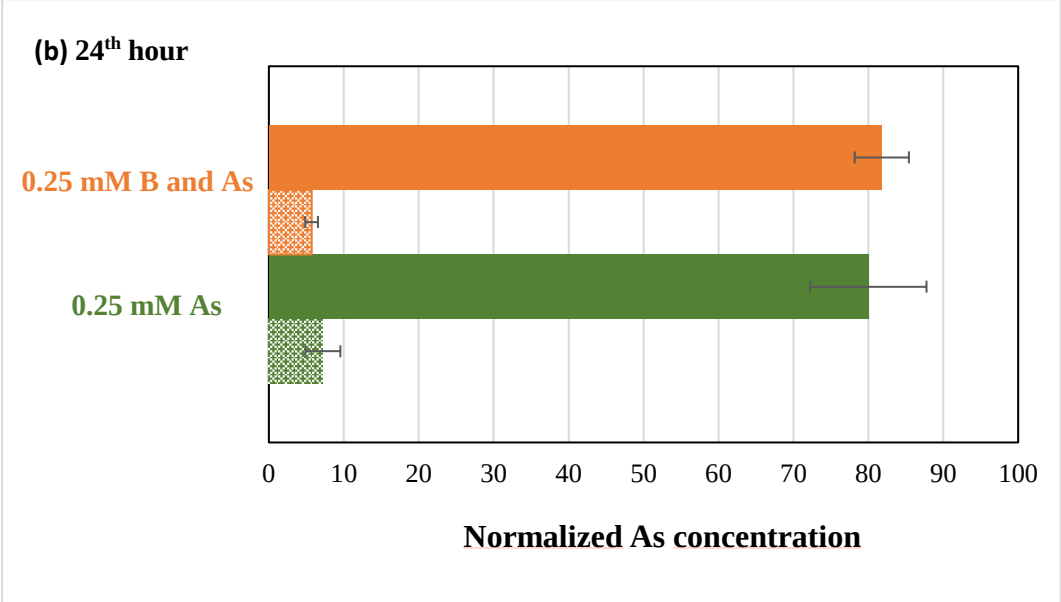
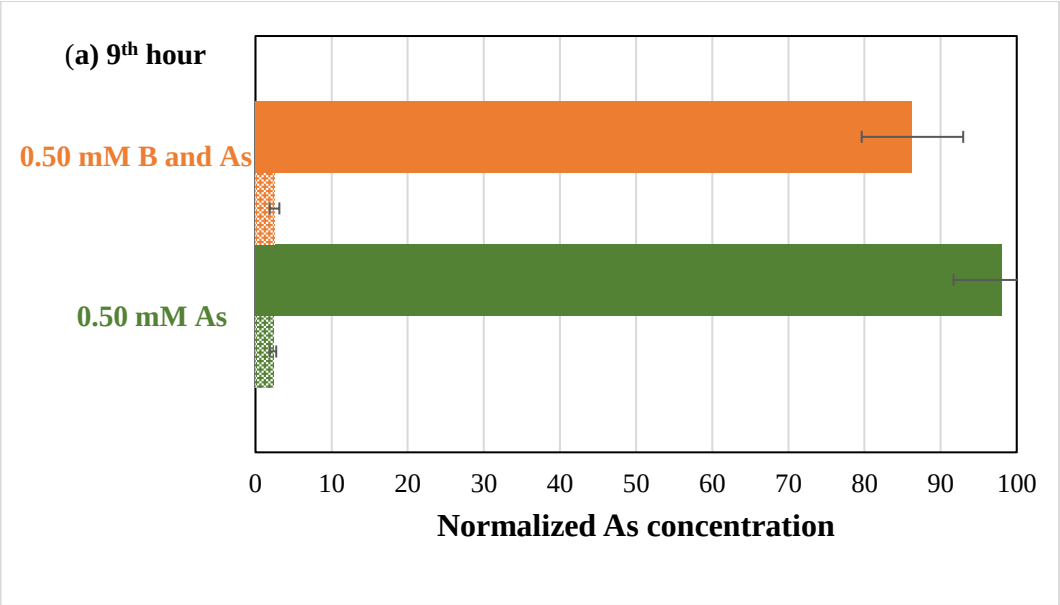


Figure 3.20. Distribution *As in supernatant and cell solution* after supplementing the yeast cells with *0.25 mM As* or *0.25 mM B and 0.25 mM As spiked* growth medium at **(a)** *9th hour* (logarithmic phase) **(b)** *24th hour* (stationary phase).

Patterned columns belong to cell samples, plain ones represent supernatant solutions results.



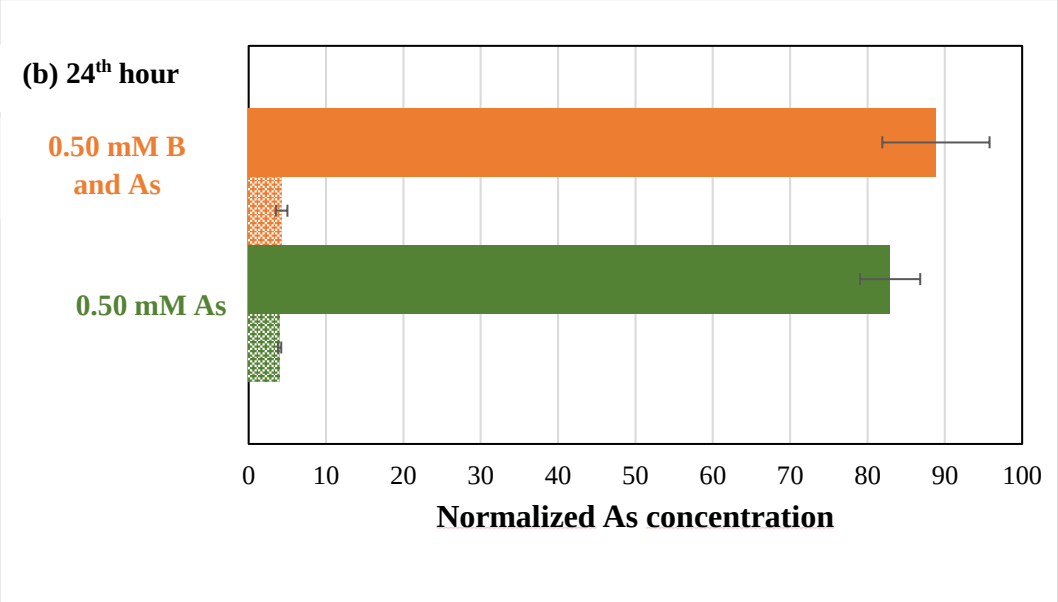
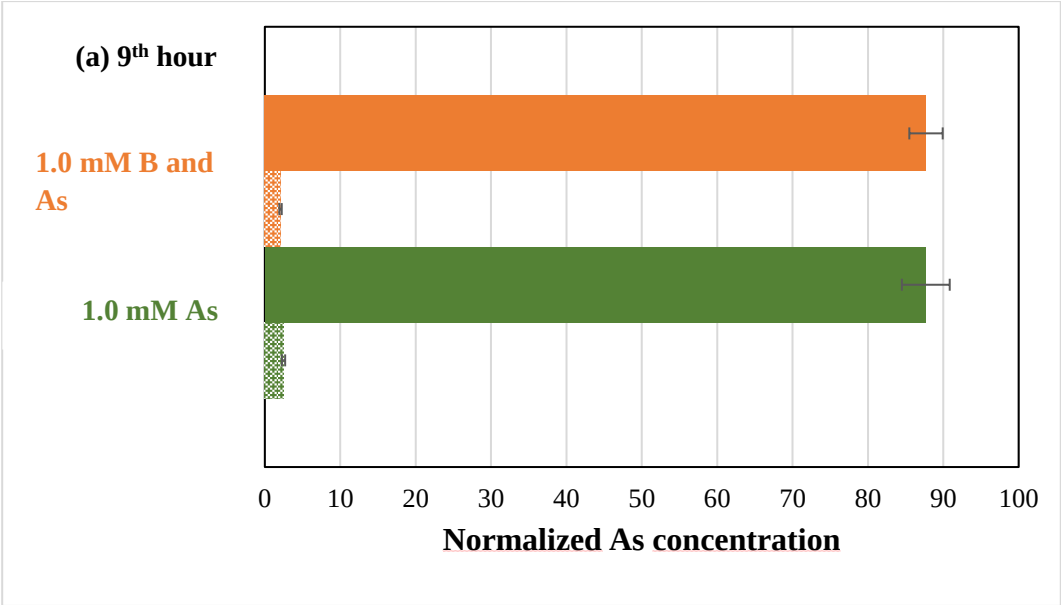


Figure 3.21. Distribution *As in supernatant and cell solution* after supplementing the yeast cells with *0.50 mM As* or *0.50 mM B and 0.50 mM As spiked* growth medium at **(a)** *9th hour* (logarithmic phase) **(b)** *24th hour* (stationary phase).



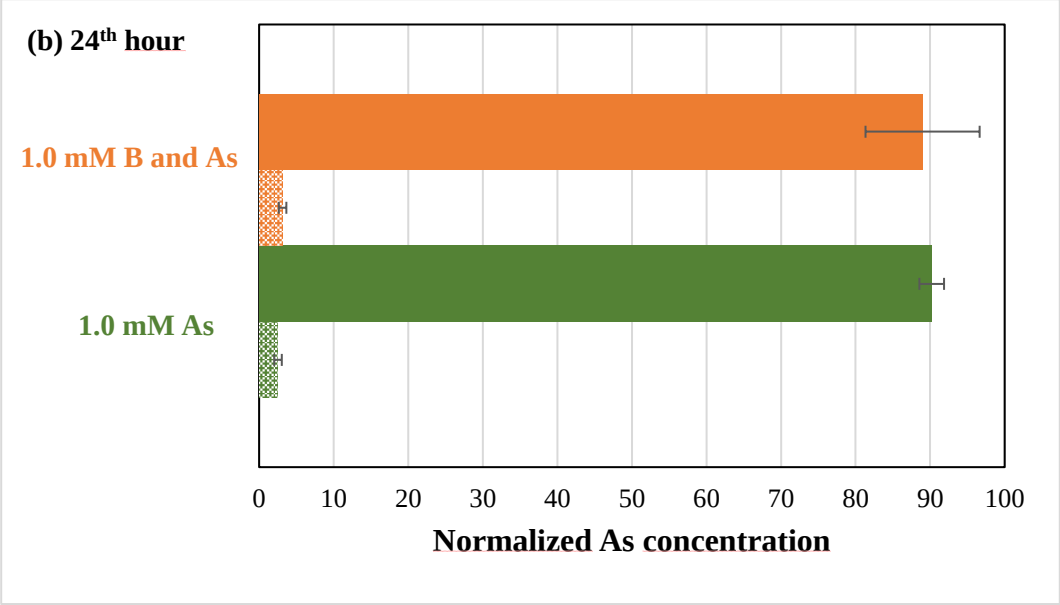
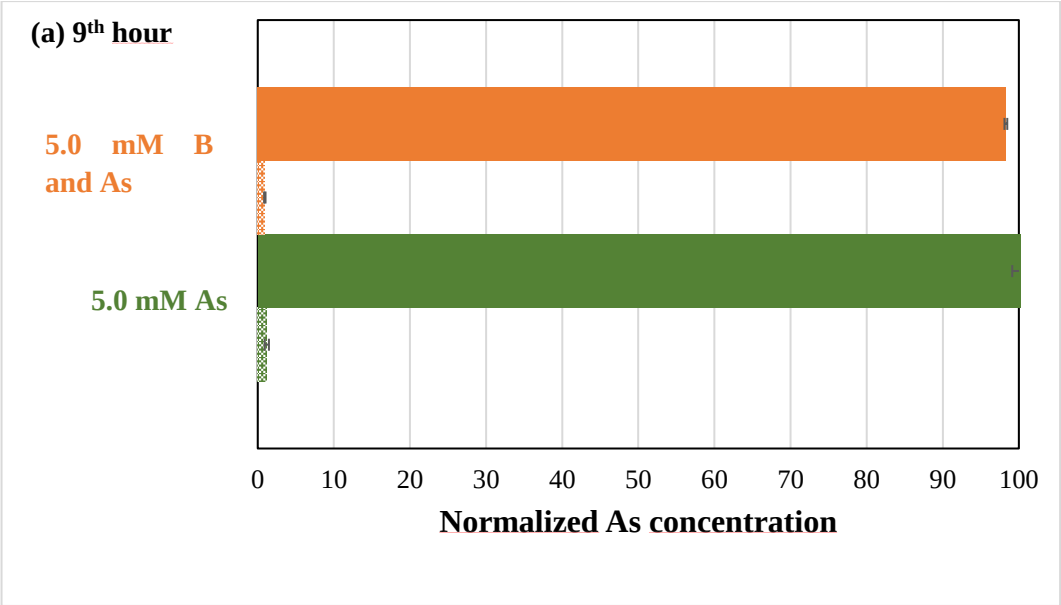


Figure 3.22. Distribution *As in supernatant and cell solution* after supplementing the yeast cells with *1.0 mM As* or *1.0 mM B and 1.0 mM As spiked* growth medium at **(a)** *9th hour* (logarithmic phase) **(b)** 24th hour *(*stationary phase).



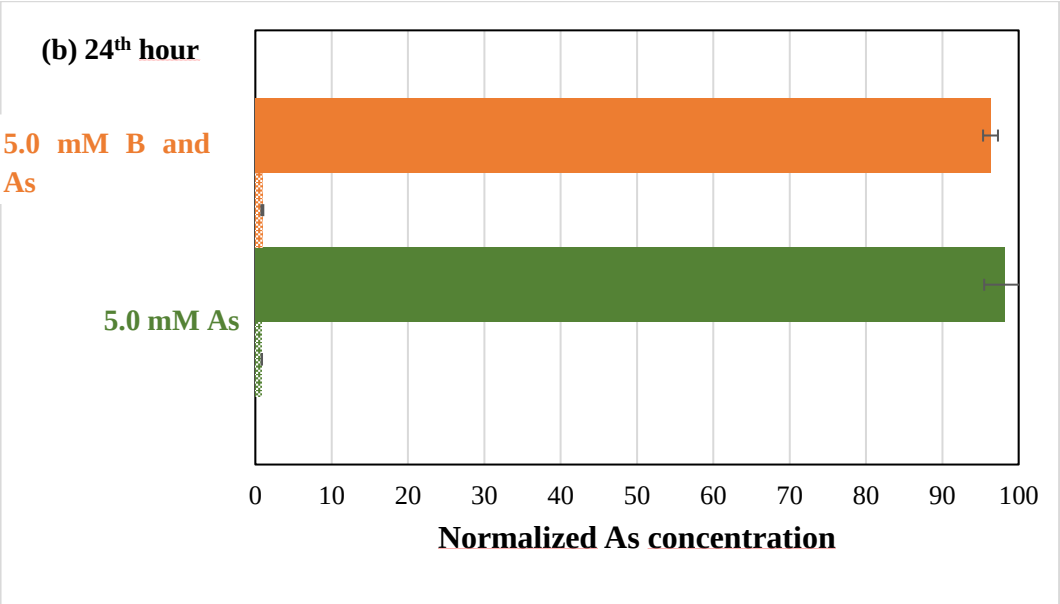


Figure 3.23. Distribution *As in supernatant and cell solution* after supplementing the yeast cells with *5.0 mM As* or *5.0 mM B and 5.0 mM As spiked* growth medium at **(a)** *9th hour* (logarithmic phase) **(b)** *24th hour* (stationary phase).

Measured As concentration in cell samples were shown in Figures 3.24 and 3.25 at 9th and 24th hours, respectively when the growth medium of yeast cells were spiked with As with and without B. As can be seen in these figures when the exposure

concentration of As increases from 0.25 to 5.0 mM, the diffusion of As into the cell increases linearly and the difference between these two concentration is almost 4 times. However, the presence of B besides As does not cause any significant effect on the diffusion of As into the cell. Additionally, arsenic inside the cell did not diffuse into the liquid growth media by increasing the exposing period of cells to boron supplemented growth media from 9 to 24 hour.

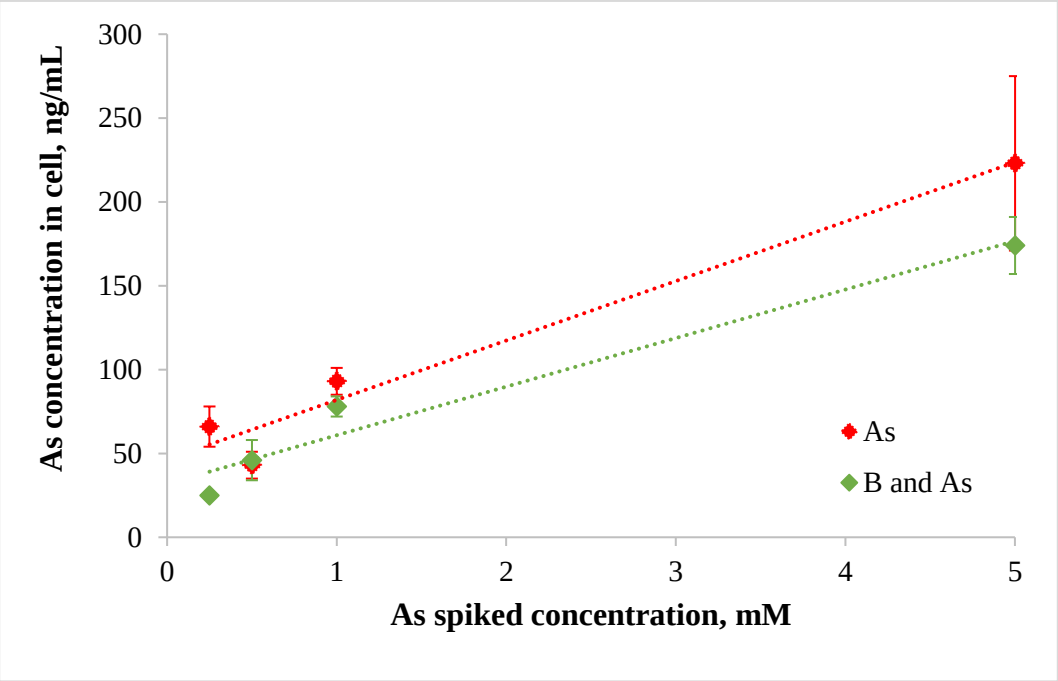


Figure 3.24. Arsenic concentration in the cell when yeast cells were exposed to As or As and B together at *9th hour*.

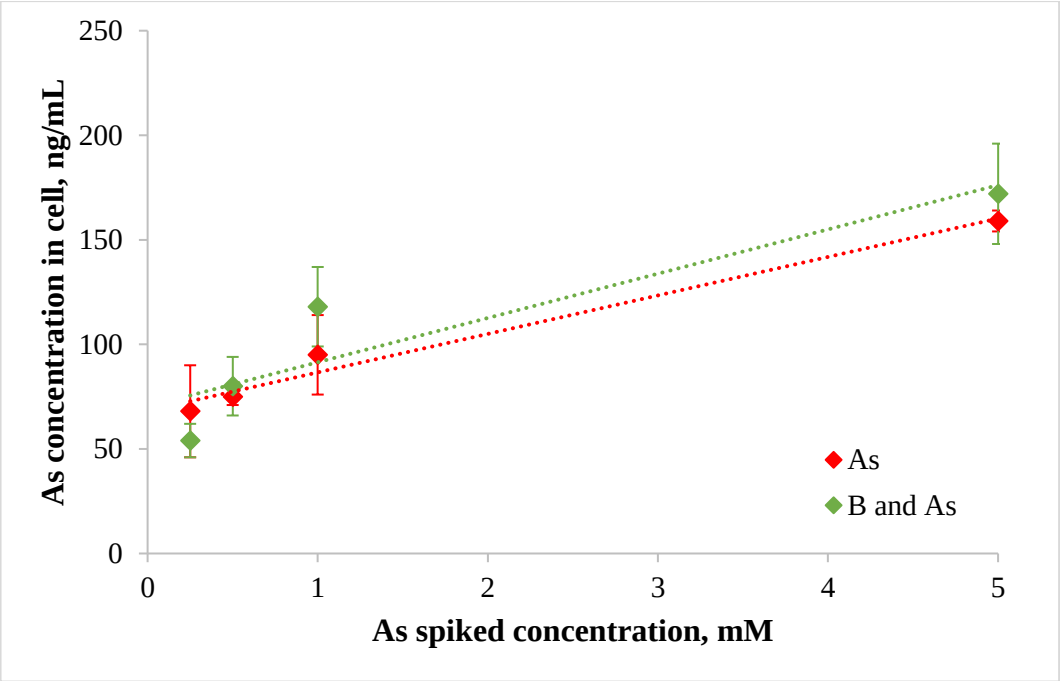


Figure 3.25. Arsenic concentration in the cell when yeast cells were exposed to As or As and B together at 24th *hour*.

If results of growth curves and elemental analysis are evaluated together, it can be concluded that the diffusion of As into the cell effect negatively on growth of yeast cells only at 5.0 mM concentration level due to the increase of As concentration in the cell. In conclusion, the negative effect of As on the growth of yeast cells starts if the concentration of As reaches to the level of 200 ng/mL which is corresponding to the 5.0 mM As exposure.

3.5 Agarose Gel Electrophoresis

Antioxidant and protective properties of boron against arsenic in human erythrocyte cells and rats was studied in the literature by measuring antioxidant enzyme activities [95-96]. Protective role of boric acid against other toxic elements such as lead and cadmium was also shown in the literature [102]. In this study, DNA strand breaks

were shown in lung fibroblast cells of hamster by means of comet assay. The assay encompasses inclusion of cells in a low melting agarose and electrophoresis was applied to show possible DNA breaks. While damaged DNA forms a tail, undamaged ones remain intact. DNA strand breaks were higher in case of Cd or Pb only treated cells as compared to Cd or Pb in the presence of boric acid by measuring tail intensities of DNAs [102].

Possible DNA breakage induced by arsenic and/or boron was aimed to investigate toxic or possible protective characteristics of As and B. In this study elemental analysis results showed the diffusion of boron and arsenic into cells but at very low amounts when yeast cells were exposed to B and/or As. Growth curve experiments showed that the effects of B and/or As depends on the exposure concentration of B and As. None of these experiments provides information about the biological effects of B and/or As induced on yeast cells. Therefore, agarose gel electrophoresis was applied to show the possible effects of these elements on DNA damage.

Agarose percentage is one of the important parameters in visualization of gel images, because migration rate of negatively charged DNA through anode depends on agarose percentage. Increases in agarose percentage decreases the pore size of agarose and as a results movement of DNA fragments slows down [55]. Genomic DNA can be defined as the DNA holding all genetic data of an organism. Genomic DNA of yeast cells were extracted by applying the protocol provided from the manufacturer as explained in the experimental part. In the first step, 1.5% (w/v) agarose in 1X TAE (Tris Acetate EDTA) buffer was used for genomic DNA samples of the control group. The gel electrophoresis image is illustrated in Figure 3.26. Movement of large DNA molecules was not enough in this percentage of agarose gel since the pore size of agarose was not large enough to visualize high molecular weight genomic DNA.

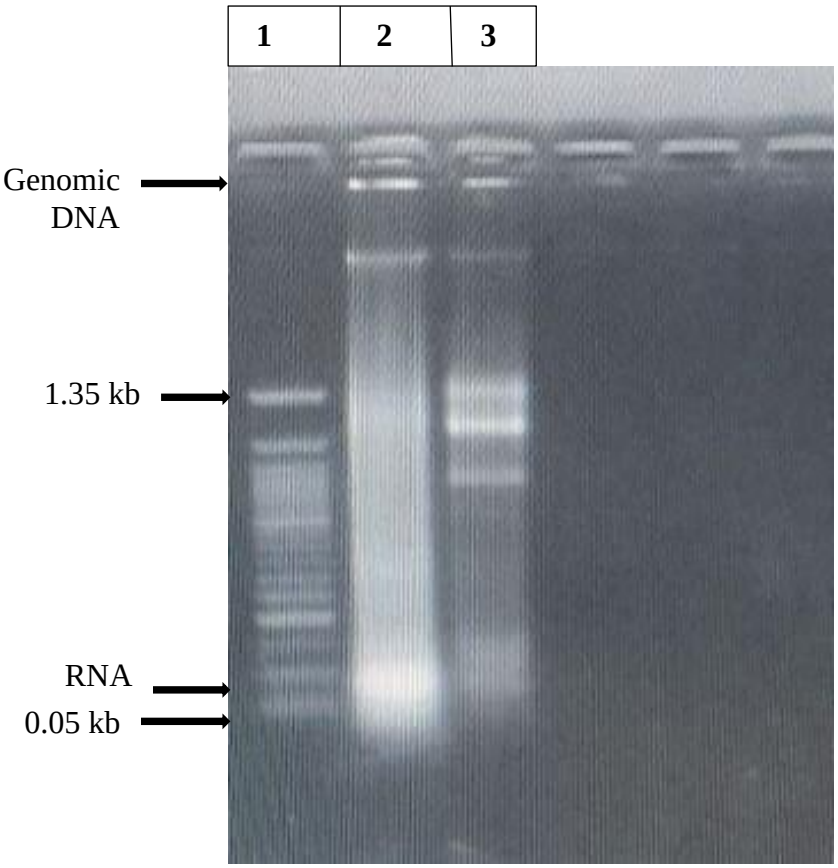


Figure 3.26. 1.5% TAE-agarose gel image of extracted genomic DNA of *control* group (in the absence of As and B) samples: Lane 1: 50 base-pair (0.05 kb) DNA ladder; lane 2 and lane 3: two replicates of *control* group DNA of yeast cells.

Afterwards, agarose percentage was decreased to 1.0% (w/v) to increase resolution of DNA fragments for the following agarose gel electrophoresis experiments.

Any genomic DNA breakage could not be observed up to 5.0 mM due to B and/or As exposure since any low molecular weight smear could not be seen from the images as shown in Figures 3.27, 3.28 and 3.29 belonged to 0.25 mM, 0.50 mM and 1.0 mM B and As, respectively. RNA of yeast cells was seen at the bottom side of gel images [103], since RNAse enzyme was not added to all DNA samples.

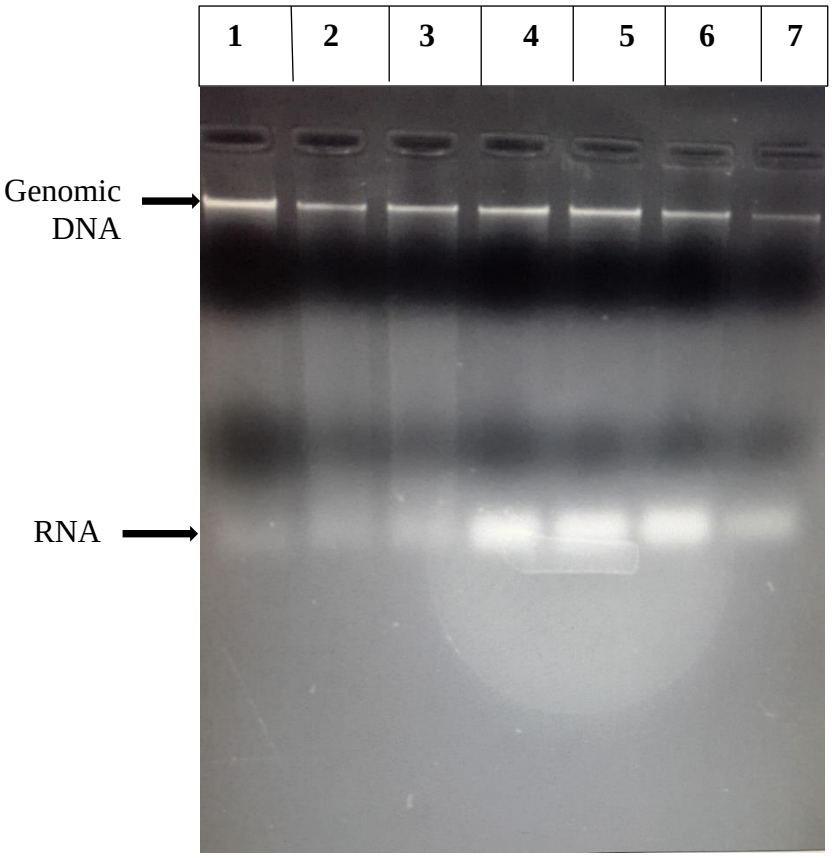


Figure 3.27. 1.0% TAE-agarose gel image of extracted genomic DNA of *control* group and *0.25 mM* B and/or As supplemented yeast cells: Lane 1: *control* group DNA of yeast cells; lane 2 and lane 3: *0.25 mM B* supplemented yeast cells' genomic DNA; lane 4 and lane 5: *0.25 mM As* supplemented yeast cells' genomic DNA; lane 6 and lane 7: *0.25 mM B and* As supplemented yeast cells' genomic DNA.

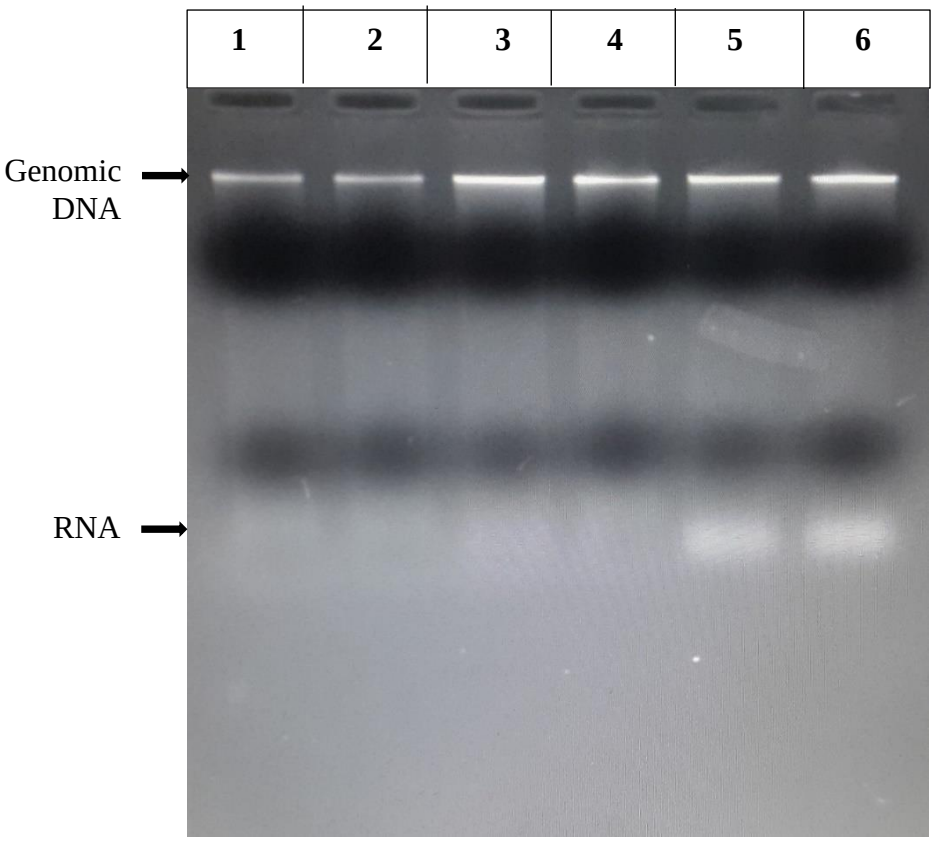


Figure 3.28. 1.0% TAE-agarose gel image of extracted genomic DNA of *0.50 mM B and/or As* supplemented yeast cells: Lane 1 and lane 2: *0.50 mM B* supplemented yeast cells' genomic DNA; lane 3 and lane 4: *0.50 mM As* supplemented yeast cells' genomic DNA; lane 5 and lane 6: *0.50 mM B and As* supplemented yeast cells' genomic DNA.

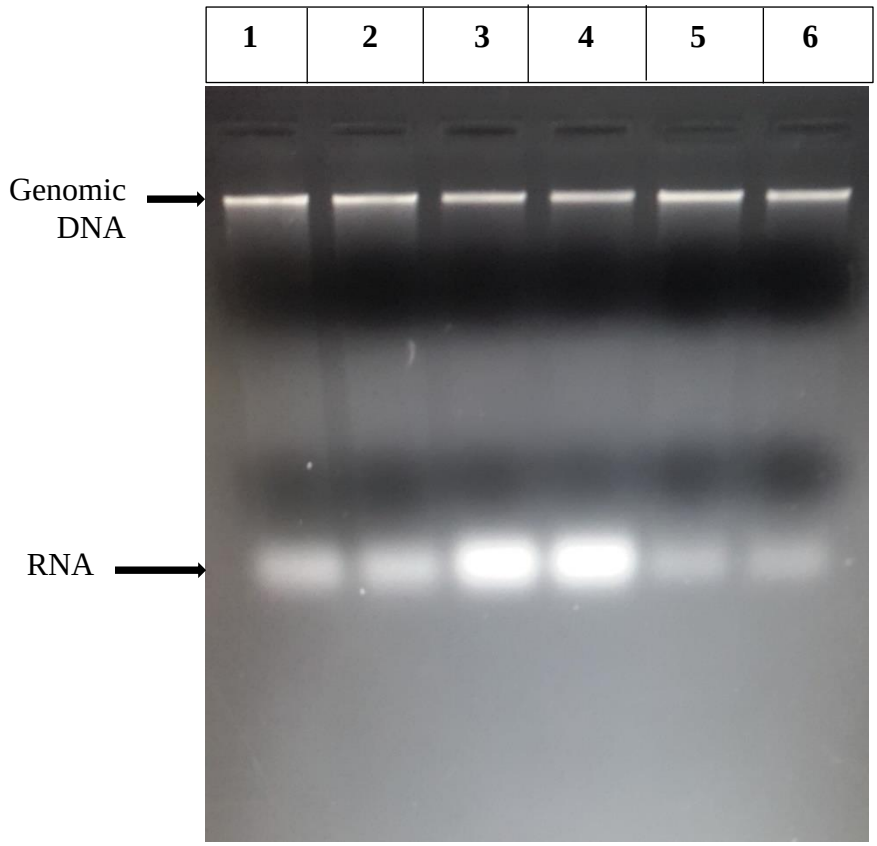


Figure 3.29. 1.0% TAE-agarose gel image of extracted genomic DNA of *1.0 mM B and/or As* supplemented yeast cells: Lane 1 and lane 2: *1.0 mM B* supplemented yeast cells' genomic DNA; lane 3 and lane 4: *1.0 mM As* supplemented yeast cells' genomic DNA; lane 5 and lane 6: *1.0 mM B and As* supplemented yeast cells' genomic DNA.

When yeast cells fed with 5.0 mM boron, DNA fragments was seen as shown in Figure 3.30, but genomic DNA could not be isolated with addition of 5.0 mM As to B or As only used to fed yeast cells in YPD medium as illustrated in Figure 3.30 lane 3, 4, 5 and 6. The extraction procedure was repeated three times as independent experimental experiments for 5.0 mM As only and 5.0 mM B and As. However, no genomic DNA was observed in the images even though RNA could be separated from cells and detected at the bottom of the gel images. It might be due to failure of DNA extraction kit when such high concentration of As was used. In these experiments presence of RNA of cells in the gel images eliminates the possibility of degradation of DNA due to high toxicity of arsenic.

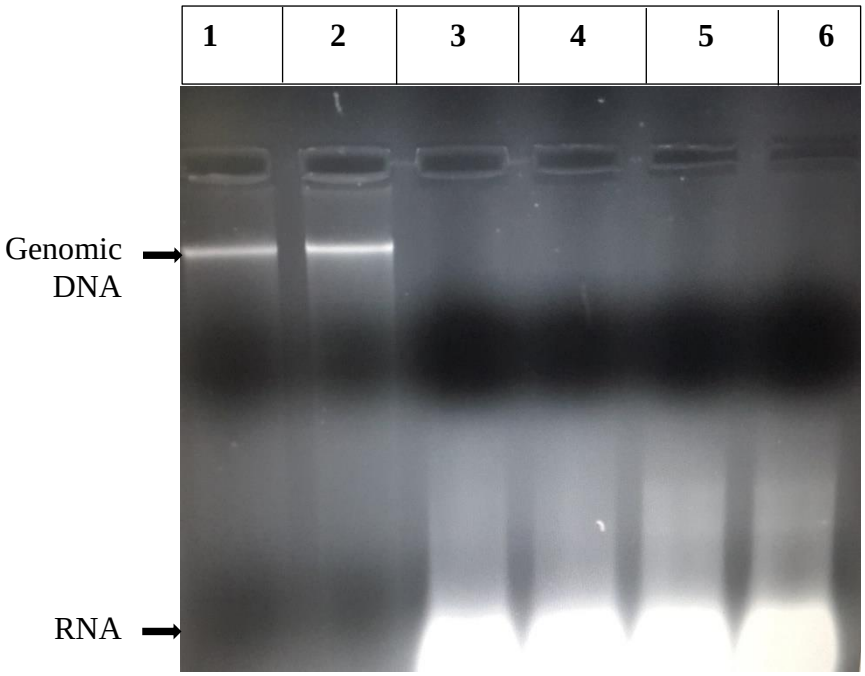


Figure 3.30. 1.0% TAE-agarose gel image of extracted genomic DNA of *5.0 mM B and/or As* supplemented yeast cells: Lane 1 and lane 2: *5.0 mM B* supplemented yeast cells' genomic DNA; lane 3 and lane 4: *5.0 mM As* supplemented yeast cells' genomic DNA; lane 5 and lane 6: *5.0 mM B and As* supplemented yeast cells' genomic DNA.

Another As concentration between 1.0 and 5.0 mM was also used to fed yeast cells in YPD medium. Agarose gel image of 2.0 mM B and/or As spiked DNA samples was shown in Figure 3.31.

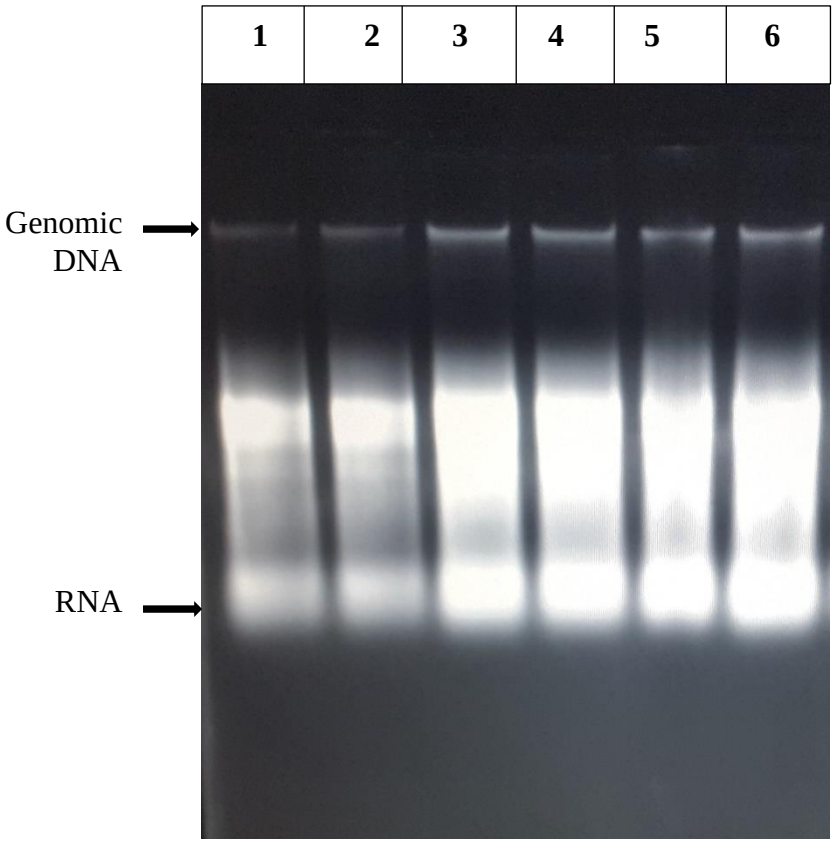


Figure 3.31. 1.0% TAE-agarose gel image of extracted genomic DNA of *2.0 mM B and/or As* supplemented yeast cells: Lane 1 and lane 2: *2.0 mM B* supplemented yeast cells' genomic DNA; lane 3 and lane 4: *2.0 mM As* supplemented yeast cells' genomic DNA; lane 5 and lane 6: *2.0 mM B and As* supplemented yeast cells' genomic DNA.

Similar to 5.0 mM As gel images, it was not observed any smear belonged to genomic DNAs from 2.0 mM B and/or As treated yeast cells. However, the number of fragments was more compared to that of 5.0 mM As fed yeast cells as seen in Figure 3.31. These additional fragments may belong to degraded RNA of yeast cells due to smeared bands through the bottom side of the gel.

Double strand DNA breaks of chromosomes isolated from yeast cells treated with As(III) up to 25.0 mM were observed using pulsed-field gel electrophoresis (PFGE) in Litwin et al. study [82]. If distinct chromosome bands were absent and low molecular weight smear was accumulated through the bottom of the gel, it was interpreted as the presence of DNA double strand breaks [82]. DNA breaks were not

be detected up to 1.0 mM As (III) treated logarithmically grown yeast cells by PFGE [82-83], but the breaks induced by such relatively low levels of As(III) could be measured by single cell gel electrophoresis (comet assay) [82]. On the other hand, application of PFGE to 20 mM As(III) treated yeast cells would form DNA fragmentation and degradation of chromosomes of the cells became apparent at the concentration of 25 mM [82].

Although DNA breaks using yeast comet assay was revealed with 1.0 mM As(III) treatment, PFGE results would not show them. According to Litwin and coworkers, this could be possible by assumption of forming of DNA breaks indeed, but these breaks are not resected in yeast cells at this growth stage of yeast cells (logarithmically growing cells exposing As(III) for 6 hours) and signal of DNA damage is not transduced [82]. Same possible explanation might be valid for this study of As and/or B exposed-cells for 15 hours. However, using different type of genomic DNA extraction kit and using agarose gel electrophoresis instead of pulsed-field gel electrophoresis could affect the images of DNAs on the gel. Moreover, fragments of DNA could be seen in more detail by PFGE as compared to agarose gel electrophoresis which was used in this study.

CHAPTER 4

CONCLUSIONS

The effect of B(III) and As(III) on the grown yeast (*Saccharomyces cerevisiae*) cells by tracking mobility of B and/or As inside and outside the cells with ICP-OES and ICP-MS was studied in this thesis. Additionally, biological damage possibly induced by As and/or B was followed by agarose gel electrophoresis.

Firstly, yeast cells were grown in both yeast extract peptone dextrose (YPD) agar and YPD broth media until reaching to logarithmic phase. After logarithmic phase was reached, As and/or B was added to growth medium, so yeast cells continued to grow in the presence of 0.25, 0.50, 1.0 or 5.0 mM As and/or B.

Results of the growth curves suggested that presence of boron had no positive or negative effect on cell growth when compared to control group of cells irrespective of the concentration of boron used in this thesis. Moreover, there were not any significant difference between B and/or As treated yeast cells from the control group up to 1.0 mM concentration level. However when 5.0 mM As and B and As together spiked in feed solution the number of yeast cells decreased by 30% compared to control group probably due to toxic effect of As. Besides, presence of 5.0 mM B with 5.0 mM As did not show synergistic or antagonistic effect on the cell growth.

Total B and As concentrations in cell and supernatant samples was determined by ICP-OES and ICP-MS after digesting samples by microwave assisted acid digestion using 2.0 mL of concentrated nitric acid and 1.0 mL of 30% (v/v) hydrogen peroxide solution. After dilution of the samples, standard addition method was applied on both cell and supernatant samples because of matrix interferences caused by complex matrix of YPD growth medium.

Total B in cell and supernatant samples was determined by ICP-OES while total As was determined using ICP-MS since detection limit of ICP-OES is better than that of ICP-MS for B Elemental analysis of yeast cells and growth medium separately showed that more than 90% of spiked boron remained in supernatant solution at all concentrations irrespective of yeast cells fed with B or B and As together in growth medium. Cellular B concentration was below detection limit value of ICP-OES up to 5.0 mM. At 5.0 mM concentration level, B uptake by cells did not increase with the addition of arsenic.

Similar to boron, arsenic also remained more than 90% in supernatant solution. Any significant difference was not observed from 9^{th} to 24^{th} hour in the distribution of As in yeast cell and supernatant solutions at all of the studied concentrations of As and B and As together. Moreover, diffusion of As into yeast cells from the As spiked growth medium was not increased or decreased with the addition of B. Negative effect of arsenic on the cell growth at 5.0 mM concentration could be attributed to the increase of As concentration inside yeast cells up to the level of 200 ng/mL.

Minor uptake of As and B into cells may not have impact on biological damage. Agarose gel electrophoresis was applied for investigating DNA damage possibly induced by As and/or B. No detectable breakage of extracted genomic DNAs was seen in the presence of As and/or B up to 2.0 mM. Genomic DNAs at 5.0 mM As only and B and As together could not be detected even though their RNAs seen from the gel image. Therefore, interpretation about degradation of DNA was not done because of observable RNA fragments. The most probable reason can be due to failure of DNA extraction kit at such high concentration of As.

The results are applicable only for *Saccharomyces cerevisiae* cells grown in the conditions given in the experimental chapter. Results can change depending on cell type and experimental conditions.

Although effect of boron on yeast cell growth [46] and DNA damage induced by arsenic in yeast cells [82] was investigated in the literature, effect of boron and/or arsenic on yeast cells was not studied in terms of biological damage upon degree of uptake of these elements inside cells till now. Total element distribution was

examined in both cell and supernatant parts together with DNA damage analysis by agarose gel electrophoresis in this study. However, any significant antagonistic or synergistic relation between boron and arsenic could not be found during this study.

Total element determination in cells and supernatants will be studied to follow whether changes in uptake of elements into and out of cells or not beyond the stationary phase, i.e. 48^{th} hour, as a future study.

REFERENCES

[1] P. Atkins, “Shriver and Atkins’ Inorganic Chemistry”, OUP, Oxford, 2010, pp. 325–335.

[2] F. S. Kot, “Boron sources, speciation and its potential impact on health”, *Rev. Environ. Sci. Biotechnol.*, vol. 8, pp. 3–28, 2009.

[3] S. Bakırdere, S. Örenay, and M. Korkmaz, “Effect of boron on human health”, *Open Miner. Process. J.*, vol. 3, pp. 54–59, 2010.

[4] R. F. Moseman, “Chemical disposition of boron in animals and humans”, *Env. Heal. Perspect*, vol. 102, pp. 113–117, 1994.

[5] C. Çöl, M., Çöl, “Environmental boron contamination in waters of Hisarcik area in the Kutahya Province of Turkey”, *Food Chem. Toxicol.*, vol. 41, pp. 1417–1420, 2003.

[6] S. Samman, M. R. Naghii, L. W. P.M., and A. P. Verus, “The nutritional and metabolic effects of boron in humans and animals”, *Biol. Tr. Elem. Res.*, vol. 66, pp. 227–235, 1998.

[7] P. D. Howe, “A review of boron effects in the environment”, *Biol. Tr. Elem. Res.*, vol. 66, pp. 153–166, 1998.

[8] A. D. Tara and L. V. Stella, “The physiological effects of dietary boron”, *Crit. Rev. Food Sci. Nutr.*, vol. 43, pp. 219–231, 2003.

[9] S. Bora, “Boron determination in body fluids by inductively coupled plasma optical emission spectrometry and inductively coupled plasma mass spectrometry”, MS Thesis, 2010.

[10] Boren, “Reserves”, 2012. [Online]. Available: http://www.boren.gov.tr/en/boron/reserves. [Accessed: 17-Jul-2016].

[11] W. G. Woods, “An introduction to boron: history, sources, uses, and chemistry”, *Env. Heal. Perspect*, vol. 102, pp. 5–11, 1994.

[12] N. Acarkan, “Boron products and their uses”, in *Proceedings of the 1st*

international boron symposium, 2002.

[13] C. D. Hunt, "Boron," *Encyclopedia of food science, food technology and nutrition*. London: Academic Press, pp. 440–447, 1993.

[14] R. Von Burg, "Toxicology update", *J. Appl. Tox.*, vol. 12, pp. 149–152, 1992.

[15] Agency for Toxic Substances and Disease Registry (ATSDR), "Toxicological profile for boron." [Online]. Available: https://www.atsdr.cdc.gov/toxprofiles/tp26-c2.pdf. [Accessed: 21-Jul-2016].

[16] J. S. Schou, J. A. Jansen, and B. Aggerbeck, "Human pharmacokinetics and safety of boric acid", *Arch. Toxicol. Suppl.*, vol. 7, pp. 232–235, 1984.

[17] L. E. Farr and T. Konikowksi, "The renal clearance of sodium pentaborate in mice and men", *Clin. Chem.*, vol. 9, pp. 717–726, 1963.

[18] WHO, "Boron", *Environ. Heal. Criteria 204*, pp. 1–125, 1998.

[19] C. J. Rainey, L. A. Nyquist, R. E. Christensen, P. L. Strong, B. D. Culver, and J. R. Coughlin, "Daily boron intake from the American diet", *J. Am. Diet Assoc.*, pp. 335–340, 1999.

[20] (EVGM) The Expert Group on Vitamins and Minerals, "Revised review of boron", pp. 2–5, 2002.

[21] WHO, "Guidelines for drinking-water quality", 4th ed., World Health Organization, 2011, , p. 178.

[22] Y. Duydu, N. Basaran, A. Ustundag, S. Aydin, U. Undeger, O. Y. Ataman, K. Aydos, Y. Duker, K. Ickstadt, B. S. Waltrup, K. Golka, and H. M. Bolt, "Reproductive toxicity parameters and biological monitoring in occupationally and environmentally boron-exposed persons in Bandırma, Turkey", *Arch. Toxicol. Suppl.*, vol. 85, pp. 589–600, 2011.

[23] M. Korkmaz, U. Sayli, B. S. Sayli, S. Bakirdere, S. Titretir, O. Y. Ataman, and S. Keskin, "Estimation of human daily boron exposure in a boron-rich area", *Br. J. Nutr.*, vol. 98, pp. 571–575, 2007.

[24] Food and Nutrition Board: Institute of Medicine, “Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc”, Washington, D.C: National Academies Press, 2001, pp. 510–521.

[25] D. L. Anderson, W. C. Cunnigham, and T. R. Lindstrom, “Concentrations and intakes of H, B, S, K, Na, Cl and NaCl in foods”, *J. Food Compos. Anal.*, vol. 7, pp. 59–82, 1994.

[26] M. Benderdour, T. Buivan, A. Dicko, and F. Bellevilp, “In vivo and in vitro effects of boron and boronated compounds”, *Trace Elem. Med. BioI.*, vol. 12, pp. 2–5, 1998.

[27] H. E. Goldbach and M. A. Wimmer, “Boron in plants and animals: Is there a role beyond cell-wall structure?”, *J. Plant Nutr. Soil Sci.*, vol. 170, pp. 39–48, 2007.

[28] E. V. Maas, “Salt tolerance of plants” in *Handbook of Plant Science in Agriculture*, CRC Press., lB.R. Christie, Ed. 1987, p. 57.

[29] Food and Agriculture Organization of the United Nations, “Water quality evaluations” [Online]. Available: http://www.fao.org/docrep/003/T0234E/T0234E05.htm. [Accessed: 17-Jul-2016].

[30] I. Cakmak and V. Römheld, “Boron deficiency-induced impairments of cellular functions in plants”, *Plant Soil*, vol. 193, pp. 71–83, 1997.

[31] H. Hu and P. H. Brown, “Localization of boron in cell walls of squash and tobacco and its association with pectin”, *Plant Physiol.*, vol. 105, pp. 681–689, 1994.

[32] T. Matoh, K. I. Ishigaki, O. Kaori, and .T.I. Azuma, “Isolation and characterization of a boron polysaccharide complex from radish roots”, *Plant Cell Physiol.*, vol. 34, pp. 639–642, 1993.

[33] T. Tanada, “Localisation of B in membranes”, *J. Plant Nutr.*, vol. 6, pp. 743–749, 1983.

[34] J. Blaser-grill, D. Knoppik, A. Ambergre, and H. Goldbach, “Influence of

boron on the membrane potential in Elodea densa and Helianthus annuus root and H+ extrusion of suspension-cultured Daucus carota cells", *Plant Physiol.*, vol. 90, pp. 280–284, 1989.

[35] T. Yamauchi, T. Hara, and Y. Sonoda, "Effects of boron deficiency and calcium supply on calcium metabolism in tomato plants", *Plant soils*, vol. 93, pp. 223–230, 1986.

[36] R. N. Sah and P. H. Brown, "Boron determination- a review of analytical methods", *Microchem. J. 56*, , pp. 285–304, 1997.

[37] Y. S. Bai and C. D. Hunt, "Dietary boron enhances efficacy of cholecalciferol in broiler chicks", *J. Trace Elem. Exp. Med.*, vol. 9, pp. 117–132, 1996.

[38] M. Hegsted, M. J. Keenan, F. Siver, and P. Wozniak, "Effect of boron on vitamin D deficient rats", *Biol. Trace Elem. Res.*, vol. 28, pp. 243–255, 1991.

[39] F. H. Nielsen, C. D. Hunt, L. M. Mullen, and J. . R. Hunt, "Effect of dietary boron on mineral, estrogen and testosterone metabolism in post-menopausal women", *FASEB J.*, vol. 1, pp. 394–397, 1987.

[40] M. Park, Q. Li, N. Shcheynikov, W. Z. Zeng, and S. Muallem, "NaBC1 is an ubiquitous electrogenic Na+-coupled borate transporter essential for cellular boron homeostasis and cell growth and proliferation", *Mol. Cell*, vol. 16, pp. 331–341, 2004.

[41] M. Park, Q. Li, N. Shcheynikov, S. Muallem, and W. Z. Zeng, "Borate transport and cell growth and proliferation: not only in plants", *Cell Cycle*, vol. 4, pp. 24–26, 2005.

[42] W. B. Clarke, C. E. Weber, M. Koekebakker, and R. D. Barr, "Lithium and boron in human blood", *J. Lab. Clin. Med.*, vol. 109, pp. 155–158, 1987.

[43] F. H. Nielsen, "Boron- an overlooked element of potential nutritional importance", *Nutr. Today*, vol. January/Fe, pp. 4–7, 1988.

[44] M. R. Naghii and S. Samman, "The effect of boron supplementation on the distribution of boron in selected tissues and on testosterone synthesis in rats", *J. Nutr. Biochem.*, vol. 7, pp. 507–512, 1996.

[45] J. G. Penland, “Dietary boron brain function, and cognitive performance”, *Environ. Heal. Perspect*, vol. 102, pp. 65–72, 1994.

[46] A. Bennett, R. I. Rowe, N. Soch, and C. D. Eckhert, “Boron stimulates yeast (*Saccharomyces cerevisiae*) growth”, *J. Nutr.*, vol. 129, pp. 2236–2238, 1999.

[47] I. Bonilla, M. Garcia-Gonzalez, and P. Mateo, “Boron requirement in Cyanobacteria. Its possible role in the early evolution of photosyntetic organisms”, *Plant Physiol.*, vol. 94, pp. 1554–1560, 1990.

[48] X. Hou and B. T. Jones, “Inductively Coupled Plasma/Optical Emission Spectrometry” in *Encyclopedia of Analytical Chemistry*, John Wiley., R. A. Meyers, Ed. 2000, pp. 9468–9485.

[49] D. Skoog, F. J. Holler, and S. R. Crouch, “Principles of Instrumental Analysis”, 6th Edition, Thomson Brooks/Cole, 2007, pp. 254–256.

[50] J. Nolte, *ICP Emission Spectrometry, A Practical Guide*. Weinheim, Germany: Wiley-VCH, 2003.

[51] R. Thomas, “Practical Guide to ICP-MS”, Geithersburg, Maryland, USA: Marcel Dekker Inc., 2004, pp. 1–7.

[52] K. L. Linge and K. E. Jarvis, “Quadrupole ICP-MS: introduction to instrumentation, measurement techniques and analytical capabilities”, *Geostand. Geoanalytical Res.*, vol. 33, pp. 445–467, 2009.

[53] B. R. LaFreniere, R. S. Houk, and V. A. Fassel, “Direct detection of vacuum ultraviolet radiation through an optical sampling orifice: analytical figures of merit for the nonmetals, metalloids, and selected metals by inductively coupled plasma-atomic emission-spectrometry”, *Anal. Chem.*, vol. 59, pp. 2276–2282, 1987.

[54] P. Y. Lee, J. Costumbrado, C. Y. Hsu, and Y. H. Kim, “Agarose gel electrophoresis for the separation of DNA fragments”, *J .Vis. Exp.*, vol. 62, 2012.

[55] D. Voytas, “Resolution and recovery of large DNA fragments” in *Current Protocols in Molecular Biology*, vol. 51, John Wiley and Sons Inc., 2000.

[56] R. B. Helling, H. M. Goodman, and H. W. Boyer, "Analysis of R. EcoRI fragments of DNA from lambdoid bacteriophages and other viruses by agarose gel electrophoresis", *J. Virol.*, vol. 14, pp. 1235–1244, 1974.

[57] M. B. Başkan and A. Pala, "İçme sularında arsenik kirliliği: ülkemiz açısından bir değerlendirme", *J. Eng. Sci.*, vol. 15, pp. 69–79, 2009.

[58] H. S. J. Chou and C. T. D. Rosa, "Case studies-arsenic", *J. Hyg. Env. Heal.*, vol. 206, pp. 381–386, 2003.

[59] U.S. EPA, "Workshop on managing arsenic risks to the environment: characterization of waste, chemistry, and treatment and disposal", EPA/625/R-03/010, 2003.

[60] J. Matschullat, "Arsenic in the geosphere-a review", *Sci. Total Env.*, vol. 249, pp. 297–312, 2000.

[61] M. Bissen and F. H. Frimmel, "Arsenic–a review part I: occurrence, toxicity, speciation, mobility", *Acta Hydrochim. Hydrobiol.*, vol. 1, pp. 9–18, 2003.

[62] U.S. EPA, "Arsenic treatment technologies for soil, waste, and water", EPA-542-R-02-004, Cincinnati, 2002.

[63] F. Habashi, "Principles of extractive metallurgy", London: CRC Press, 1969, p. 325.

[64] WHO, "Arsenic and arsenic compunds", *Environ. Heal. Criteria 224*, 2001.

[65] S. Wang and C. Mulligan, "Occurrence of arsenic contamination in Canada: sources, behavior and distribution", *Sci .Total Environ.*, vol. 366, pp. 701–721, 2006.

[66] U.S. Environmental Protection Agency, "Technical fact sheet: final rule for arsenic in drinking water", 2001.

[67] M. Kumaresan and P. Riyazuddin, "Overview of speciation chemistry of arsenic", *Curr. Sci.*, vol. 80, pp. 837–846, 2001.

[68] Federal Register, “National primary drinking water regulations; arsenic and clarifications to compliance and new source contaminants monitoring”, 2001. [Online]. Available: https://www.federalregister.gov/articles/2001/01/22/01-1668/national-primary-drinking-water-regulations-arsenic-and-clarifications-to-compliance-and-new-source. [Accessed: 22-Jul-2016].

[69] G. P. Bienert, M. D. Schüssler, and T. P. Jahn, “Metalloids: essential, beneficial or toxic? Major intrinsic proteins sort it out”, *Trends Biochem. Sci.*, vol. 33, pp. 20–26, 2007.

[70] D. Chakraborti, S. Mukherjee, S. Pati, M. Sengupta, M. Rahman, U. Chowdhury, D. Lodh, C. Chanda, A. Chakraborti, and G. Basu, “Arsenic groundwater contamination in middle Ganga plain, Bihar, India: a future danger”, *Env. Heal. Perspect*, vol. 111, pp. 1194–1201, 2003.

[71] R. Zangi and M. Filella, “Transport routes of metalloids into and out of the cell: A review of the current knowledge”, *Chem. Biol. Interact.*, vol. 197, pp. 47–57, 2012.

[72] G. P. Bienert, M. D. Schüssler, T. P. Jahn, M. D. Schussler, and T. P. Jahn, “Metalloids: essential, beneficial or toxic? Major intrinsic proteins sort it out”, *Trends Biochem. Sci.*, vol. 33, no. 1, pp. 20–26, 2008.

[73] T. Kaize, S. Watanebe, and K. Itoh, “The acute toxicity of arsenobetaine”, *Chemosphere*, vol. 14, pp. 1327–1332, 1985.

[74] J. W. Harrison, E. W. Packman, and D. D. Abbott, “Acute oral toxicity and chemical and physical properties of arsenic trioxides”, *AMA Arch. Ind .Health.*, vol. 17, pp. 118–123, 1958.

[75] E. Yıldırım, “Multielement speciation using HPLC-ICPMS”, PhD Thesis, 2015.

[76] D. N. Mazumder, J. Das Gupta, A. K. Chakraborty, A. Chatterjee, D. Das, and D. Chakraborti, “Environmental pollution and chronic arsenicosis in South Calcutta”, *Bull World Heal. Organ.*, vol. 70, pp. 481–485, 1992.

[77] J. Chung, S. Yu, and Y. Hong, “Environmental source of arsenic exposure”, *J. Prev. Med. Public Heal.*, vol. 47, pp. 253–257, 2014.

[78] ATSDR, “Arsenic toxicity”, 2011. [Online]. Available: http://www.atsdr.cdc.gov/csem/csem.asp?csem=1&po=8. [Accessed: 17-Jul-2016].

[79] WHO, “Exposure to arsenic: a major public health concern”, 2010. [Online]. Available: http://www.who.int/ipcs/features/arsenic.pdf. [Accessed: 21-Jul-2016].

[80] International Agency for Research on Cancer, “Arsenic, metals, fibres, and dusts”, *IARC Monogr. Eval. Carcinog. Risks Hum.100C,* pp. 1–526, 2012.

[81] E. Bustaffa, A. Stoccoro, F. Bianchi, and L. Migliore, “Genotoxic and epigenetic mechanisms in arsenic carcinogenicity”, *Arch. Toxicol.,* vol. 88, pp. 1043–1067, 2014.

[82] I. Litwin, T. Bocer, D. Dziadkowiec, and R. Wysocki, “Oxidative stress and replication-independent DNA breakage induced by arsenic in *Saccharomyces cerevisiae*”, *Plos Genet.,* vol. 9, no. 7, pp. 1–14, 2013.

[83] W. J. Jo, A. Loguinov, H. Wintz, M. Chang, and A. H. Smith, “Comperative functional genomic analysis identifies distinct and overlapping sets of genes required for resistance to monomethylarsonous acid (MMA(III)) and arsenite (As(III)) in yeast”, *Toxicol .Sci .,* vol. 111, pp. 424–436, 2009.

[84] L. Du, Y. Yong, J. Chen, Y. Liu, Y. Xia, Q. Chen, and X. Liu, “Arsenic induces caspase- and mitochondria-mediated apoptosis in *Saccharomyces cerevisiae*”, *FEMS Yeast Res.,* vol. 7, pp. 860–865, 2007.

[85] D. J. Betteridge, “What is oxidative stress?”, *Metabolism,* vol. 49, pp. 3–8, 2000.

[86] R. Wysocki and M. J. Tamás, “How *Saccharomyces cerevisiae* copes with toxic metals and metalloids.”, *FEMS Microbiol. Rev.,* vol. 34, no. 6, pp. 925–951, 2010.

[87] H. V. Aphoshian and M. M. Aposhian, “Arsenic toxicology: five questions”, *Chem. Res. Toxicol.,* vol. 9, pp. 1–15, 2006.

[88] C. Huang, Q. Ke, M. Costa, and X. Shi, “Molecular mechanisms of arsenic carcinogenesis”, *Mol .Cell Biochem.,* vol. 255, pp. 57–66, 2004.

[89] J. Qin, Y. Nannan, Y. Linfen, L. Dayu, F. Yinsing, W. Wei, M. Xiaojun, and L. Bingcheng, "Simultaneous and ultrarapid determination of reactive oxygen species and reduced glutathione in apoptotic leukemia cells by microchip electrophoresis", *Electrophoresis*, vol. 26, pp. 1155–1162, 2005.

[90] L. Zhuheng, L. Zhen, W. Yunbiao, W. Chengke, X. Jingbo, and W. Zhenxin, "Studying cytotoxicity of low concentration arsenic on PC 12 cell line", *Anal. Methods*, vol. 6, no. 6, pp. 1709–1713, 2014.

[91] M. F. Hughes, M. D. Beck, Y. Chen, A. S. Lewis, and D. J. Thomas, "Arsenic exposure and toxicology: a historical perspective", *Toxicol .Sci .*, vol. 123, pp. 305–332, 2011.

[92] K. F. Akter, G. Owens, D. E. Davey, and R. Naidu, "Arsenic speciation and toxicity in biological systems", *Rev. Environ. Contam. Toxicol.*, vol. 184, pp. 97–149, 2005.

[93] D. Sinha and M. Roy, "Antagonistic role of tea against sodium arsenite-induced oxidative DNA damage and inhibition of DNA repair in Swiss albino mice", *J. Environ. Pathol. Toxicol. Oncol.*, vol. 30, pp. 311–322, 2011.

[94] Z. Uçkun, A. Üstündağ, M. Korkmaz, and Y. Duydu, "Micronucleus levels and sister chromatide exchange frequencies in boron exposed and control individuals", *J.Fac.Pharm.*, vol. 38, no. 2, pp. 79–88, 2009.

[95] H. Turkez, F. Geyikoglu, A. Tatar, M. S. Keles, and I. Kaplan, "The effects of some boron compounds against heavy metal toxicity in human blood", *Exp. Toxicol. Pathol.*, vol. 64, pp. 93–101, 2012.

[96] I. Kucukkurt, S. Ince, H. H. Demirel, R. Turkmen, E. Akbel, and Y. Celik, "The effects of boron on arsenic-induced lipid peroxidation and antioxidant status in male and female rat", *J. Biochem. Mol. Toxicol.*, vol. 0, pp. 1–8, 2015.

[97] P. Sharma, A. B. Jha, R. S. Dubey, and M. Pessarakli, "Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions", *J. Bot.*, vol. 2012, pp. 1–26, 2012.

[98] L. W. Bergman, "Growth and maintenance of yeast", *Methods Mol. Biol.*, vol. 177, pp. 9–14, 2001.

[99] "Bacterial Growth Curve," *The Pennsylvania State University*. [Online]. Available: https://online.science.psu.edu/micrb106_wd/node/6122. [Accessed: 19-Jul-2016].

[100] "Plant/Fungi DNA isolation kit product insert," *Norgen Biotek Corp.* [Online]. Available: https://norgenbiotek.com/sites/default/files/resources/Plant-Fungi-DNA-Kit-Insert-PI26200-14-M14.pdf. [Accessed: 20-Jul-2016].

[101] M. Saghbini, D. Hoekstra, and J. Gautsch, "Media formulations for various two-hybrid systems", *Methods Mol. Biol.*, vol. 177, pp. 15–39, 2001.

[102] A. Üstündağ, C. Behm, M. Föllmann, Y. Duydu, and G. H. Degen, "Protective effect of boric acid on lead and cadmium induced genotoxicity in V79 cells", *Arch. Toxicol.*, vol. 88, no. 6, pp. 1281–1289, 2014.

[103] Y. Matsumoto, R. Fishel, and R. B. Wickner, "Circular single-stranded RNA replicon in *Saccharomyces cerevisiae*", *Proc. Natl. Acad. Sci. USA*, vol. 87, pp. 7628-7632, 1990.