ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

PHYSIOLOGICAL INVESTIGATION OF *Rhodobacter sphaeroides*

M.Sc. THESIS by

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Molecular Biology - Genetics & Biotechnology Programme

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Thesis Advisor: Prof. Dr. Zeynep Petek ÇAKAR

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

Rhodobacter sphaeroides'in FİZYOLOJİK İNCELENMESİ

YÜKSEK LİSANS TEZİ

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HAZİRAN 2013

Özge Özmeral, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** 521101124 successfully defended the **thesis** entitled "**Physiological Investigation of *Rhodobacter sphaeroides***", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 03 May 2013
Date of Defense : 10 June 2013

To my family,

FOREWORD

I would like to express my gratitude to my supervisor, Prof. Dr. Zeynep Petek Çakar for giving me a chance to study in this thesis project. Without this oppurtunity, I would never have succeeded at laboratory studies. She became a precious quide giving important advices during my thesis project.

I would like to thank to Ülkü Yılmaz, Ceren Alkım and Burcu Turanlı Yıldız for sharing their knowledge and experience with me.

Also, I would like to thank to current and former members of Yeast Research Group, in particular, Berrak Gülçin Balaban, Arman Akşit, Gökhan Küçükgöze, Hande Tekarslan, Can Holyavkin, Seçil Erbil, Mevlüt Arslan and Elif Engin.

Furthermore, I would like to thank to Murat Kemal Avcı, Büşra Öztürk, Betim Karahoda and Bilal Tetik for their continuous help and friendship.

Finally, I would like to thank to my parents for of all their support during each of my life's endeavor and their understanding and patience. I certainly would not have made it without all of their support.

June 2013

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ABBREVIATIONS

ALA : 5-aminolevulinic acid
AVG : Average
CDW : Cell Dry Weight
CFU : Colony Forming Unit
cyt : Cytochrome
DNA : Deoxyribo Nucleic Acid
DSMZ : Deutsche Sammlung von Mikroorganismen und Zellkulturen
ICM : Intracytoplasmic Membrane
kb : Kilo Base Pairs
LHC : Light Harvesting Complex
ln : Natural Logarithm
μ_{max} : Maximum Sepecific Growth Rate
M27 : Medium 27
Mb : Mega Base Pairs
MPN : Most Probable Number
nm : Nano Meter
OD : Optical Density
PHA : Polyhydroxyalkanoate
PHB : Polyhydroxybutyrate
RC : Reaction Center
SH : Sulfhydryl
w/t : Wild Type

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PHYSIOLOGICAL INVESTIGATION of *Rhodobacter sphaeroides*

SUMMARY

Rhodobacter sphaeroides is an α-3 purple non-sulfur eubacterium with an extensive metabolic organization. Under anaerobic conditions it is able to grow by performing photosynthesis, respiration and fermentation. Photosynthesis occurs only under anaerobic conditions since photosynthetic apparatus of *R. sphaeroides* is harmed in the presence of oxygen. Photosynthesis may be both photoheterotrophic and photoautotrophic. While *R. sphaeroides* performs photoheterotrophic photosynthesis using organic compounds as both a carbon and a reducing source, *R. sphaeroides* performs photoautrophic photosynthesis using carbon dioxide as the sole carbon source and hydrogen as the source of reducing power. In addition, *R. sphaeroides* can grow both chemoheterotrophically and chemoautotrophically.

Regarding biotechnological applications, *R. sphaeroides* is used both in industry and in medicine. Industrial application of *R. sphaeroides* comprises hydrogen gas and polyhydroxybutyrate (PHB) production and bioremediation of heavy metals. *R. sphaeroides* can also be used in the production of vitamin B_{12}, coenzyme Q10, 5-aminolevulinic acid (ALA) and porphyrin.

Like other microorganisms, *R. sphaeroides* has different defence mechanisms to give response against different stress types that occurs frequently in its microenvironment. Defence mechanisms of *R. sphaeroides* vary according to the types of stress. For instance, while under osmotic stress condition, *R. sphaeroides* gives response by increasing cardiopilin levels which is an anionic phospholipid playing an important role in energy conversion in cells of *R. sphaeroides*, as for, under oxidative stress condition it gives response increasing its carotenoid levels. In addition to the protective role of carotenoid for oxidative stress, other components such as superoxide dismutase, catalases and peroxidases play also an important role in eliminating reactive oxygen species from the intracellular environment of *R. sphaeroides*.

R. sphaeroides has improved metal stress resistance strategies to cope with unfavorable effects of heavy metals. These strategies comprise enzymatic transformation, exclusion by permeability barrier, reduction of metals to less toxic forms and efflux of the metal ions from the cell. Another strategy for improving heavy metal stress resistance is to alter the fatty acid composition of their lipid to maintain membrane integrity under heavy metal stress.

Since *R. sphaeroides* is a metabolically highly diverse organism, it can become adapted to a variety of changing conditions. Because of that, it has drawn scientists' attention. *R. sphaeroides* is a promising microorganism for future industrial and medical applications.

In nature, microorganisms, continuously encounter different stress types in their environment. Scientists would like to improve stress resistance of industrially important microorganisms to increase their productivity and yield. A variety of studies on improvement of stress resistance in industrial microorganisms exist in the literature.

In this study, the wild type *R. sphaeroides* R-26 was used as a model organism and its physiological analysis was performed both in the absence and the presence of stress factor. In this thesis study, cobalt tolerance of *R. sphaeroides* has been emphasized particularly, in addition to other heavy metals and other stresses such as NaCl, ethanol, H_3BO_3, H_2O_2 and $CuSO_4$.

Regarding physiological investigation of *R. sphaeroides*, growth curve analysis and cell dry weight determination studies were carried out. The maximum specific growth rate determination was performed using OD_{535} values that were used to obtain the growth curves for both in the absence and in the presence of stress factor. In conclusion, cells of *R. sphaeroides* which were not treated with $CoCl_2$ could grow better than the cells treated with $CoCl_2$. In addition, the results of cell dry weight analysis and determination of maximum specific growth rate confirmed growth rate analysis results, and hence, the inhibitory effect of $CoCl_2$ on growth.

Additionally, phenotypic characterization were performed to observe its resistance and sensitivity against heavy metals. While phenotypic characterization for $CoCl_2$ stress was performed by applying MPN (Most Probable Number) method, spotting assay and monitoring optical density; colony counting or optical density measurement was used for other stresses. Phenotypic characterization results revealed that *R. sphaeroides* showed more tolerance against NaCl, among all other stress factors tested.

This study revealed the general physiological characteristics and stress tolerance levels of *R. sphaeroides* and would be helpful for future studies to improve its stress tolerance.

Rhodobacter sphaeroides' in FİZYOLOJİK İNCELENMESİ

ÖZET

Rhodobacter sphaeroides metabolik olarak geniş bir organizasyona sahip olan α-3 mor non-sülfür bir organizmadır. Oksijensiz ortamda *R. sphaeroides* hücreleri fotosentez, solunum ve fermantasyon yaparak çoğalabilir. *R. sphaeroides* hücrelerinde bulunan fotosentez yapımından sorumlu olan fotosentetik yapıları oksijenin varlığında oksijen tarafından zarara uğratıldığından fotosentez sadece oksijensiz ortamda meydana gelebilir. *R. sphaeroides* hücreleri fotosentezi fotoheterotrofik ve fotoototrofik olarak gerçekleştirebilir. *R. sphaeroides* hücreleri fotoheterotrofik fotosentezi organik bileşikleri hem karbon hem de indirgeyici element olarak kullanarak gerçekleştirirken, fotoototrofik fotosentezi ise karbondioksiti tek karbon kaynağı olarak ve hidrojeni de indirgeyici güç olarak kullanarak gerçekleştirir.

R. sphaeroides'in genomu CI ve CII olmak üzere iki adet kromozomdan ve beş adet endojen plazmitten oluşur. *R. sphaeroides* genomunun ilk fiziksel haritalama neticesinde; CII kromozomunun protein sentezi, amino asit sentezi, yağ asidi metabolizması ve enerji metabolizmasında önemli bir yere sahip olan genleri kodladığı açığa çıkartılmıştır.

R. sphaeroides biyoteknolojide hem endüstriyel, hem de tıbbi alanda kullanımı olan bir organizmadır. *R. sphaeroides*'in endüstriyel uygulamaları hidrojen gazı ve polihidroksibütirat üretimini ve ağır metallerin biyoremediasyonunu kapsar. Ayrıca B_{12} vitamini, coenzim Q10, 5-aminolevulinik asit ve porfirin üretimini de içermektedir.

Diğer organizmalar gibi, *R. sphaeroides* hücreleri de kendi çevrelerinde sürekli olarak karşılaştıkları streslere cevap vermek amacıyla farklı stres çeşitleri için farklı savunma mekanizmalarını geliştirmiştir. *R. sphaeroides*'in kendini korumak için geliştirmiş olduğu savunma mekanizmaları karşılaştığı stres tipine göre değişebilir. Örneğin, hücre içerisinde fazla miktardaki madde yoğunluğu nedeniyle meydana getirilen ozmotik stres koşulu altında, *R. sphaeroides* ozmotik strese karşı bir anyonik fosfolipid olan ve bakteri hücrelerinde enerji dönüşümünde önemli role sahip olan kardiolipin seviyesini artırarak cevap verirken, serbest oksijen tarafından meydana getirilen oksidatif strese karşı ise bakteriye mor rengini veren ve antioksidant olarak da bilinen karotenoid moleküllerinin miktarını artırarak yanıt verir. Oksidatif streste karotenoid moleküllerinin koruyucu fonksiyonunun yanı sıra, süperoksit dismutaz, katalaz ve peroksidaz gibi bileşenler de hücre içindeki reakif oksijen türlerini yok etmede önemli role sahiptirler.

R. sphaeroides ağır metallerin bakteri hücreleri üzerine olan olumsuz etkileriyle başa çıkabilmek için, diğer bazı mikroorganizmalar gibi ağır metal direncini kazanabilmek amaçlı çeşitli stratejiler geliştirmiştir. Bu stratejilerden bazıları; enzimatik transformasyonu, bakterinin geçirgenlik bariyeri ile metallerin hücre dışında bırakılmasını yani bir şekilde bakteri hücreleri tarafından hücre içine

alınmamasını, metallerin daha az toksik olan formlarına indirgenmesini ve metal iyonlarının bazı taşıyıcı proteinler aracılığı ile hücre içinden hücre dışına gönderilmesini kapsar. Bakteri hücreleri tarafından geliştirilen bir diğer strateji ise, ağır metal stresi altında bulunan bakteri hücrelerinin hücre yapısında bulunan lipid molekülündeki yağ asitlerinin bileşimini değiştirmesidir.

R. sphaeroides metabolik olarak oldukça farklı ve neredeyse değişebilen her çevreye uyum gösterebilen bir organizmadır. Bu yüzden *R. sphaeroides* birçok bilim insanının dikkatini çekmiştir ve endüstriyel açıdan da önemli bir mikroorganizmadır.

Ayrıca *R. sphaeroides* ağır metallerin biyoremediasyonu özelliği ile bilinmektedir. *R. sphaeroides*' in bu özelliğinden yola çıkarak, *R. sphaeroides*'in ağır metal direnç mekanizmasını inceleyen çalışmalar literatürde mevcuttur. Ayrıca *R. sphaeroides*'in fotosentez mekanizmasının incelenmesi ve yine *R. sphaeroides*'i kullanarak hidrojen gazı üretimi ve polihidroksibütirat üretimi ile ilgili araştırmalar da söz konusudur.

Doğada mikroorganizmalar, değişen çevre koşullarıyla birlikte sürekli olarak farklı stres çeşitleriyle yüzleşmek zorunda kalırlar. Bu nedenle mikroorganizmaların özellikle de endüstriyel öneme sahip olan mikroorganizmaların, çevrelerinde karşılaştıkları streslerin yüksek düzeylerine karşı dirençli olması istenilen bir özelliktir ve bu özelliği mikroorganizmalara kazandırma yönünde pek çok çalışma gerçekleştirilmektedir.

Bu tez çalışmasında, herhangi bir mutasyon gerçekleştirmeksizin yaban tip *R. sphaeroides* R-26 hücreleri kullanıldı ve fizyolojik analizler, hem stres faktörü varlığı hem de stres faktörü yokluğunda gerçekleştirildi. Bu tez çalışmasında diğer streslerin (NaCl, etanol, H_3BO_3, H_2O_2 and $CuSO_4$ gibi) yanısıra, *R. sphaeroides*'in olası kobalt direnci hakkında bilgi sahibi olabilmek için, özellikle *R.sphaeroides*'in kobalt direncinin belirlenmesi üzerine odaklanıldı.

Fizyolojik incelemelerin parçası olarak mikrobiyal üreme eğrisi analizi ve hücre kuru ağırlığı tespiti, hem stres varlığında hem de stres yokluğunda gerçekleştirilmiştir. Üreme eğrisi grafiğini elde etmek için kullanılan optik yoğunluk değerleri aracılığıyla, maksimum spesifik üreme hızı da yine hem stres varlığı hem de stres yokluğu koşulları için hesaplanmıştır. Elde edilen üreme eğrisi analizi sonucuna göre; *R. sphaeroides* hücreleri herhangi bir stres faktörü içermeyen ortamda, stres faktörünü ($CoCl_2$) içeren ortama göre daha iyi bir üreme göstermiş ve logaritmik evreye daha kısa sürede ulaştığı gözlemlenmiştir. Maksimum spesifik üreme hızı değeri ve hücre kuru ağırlık değeri de beklendiği gibi stres içermeyen ortamda stres içeren ortama göre daha yüksek çıkmıştır. Bu sonuçlar, kobaltın *R. sphaeroides*'in üremesi üzerindeki inhibe edici etkisini göstermektedir.

Ayrıca, *R. sphaeroides*'in direnç mekanizmasını anlamak için ve ağır metallere karşı direnç ve hassasiyetini gözlemlemek için fenotipik karakterizasyon deneyleri gerçekleştirilmiştir. *R. sphaeroides*'in fenotipik karakterizasyonu öncelikli olarak $CoCl_2$ ağır metal stresi için en muhtemel sayı (EMS) yöntemi, damlatma deneyi ve uygun optik dalga boyu değerini kullanarak optik yoğunluğun ölçümü ile gerçekleştirilirken, diğer stresler için ise koloni sayımı veya optik yoğunluk ölçümleriyle gerçekleştirilmiştir. Ağır metallere ve diğer stres faktörlerine karşı fenotipik karakterizasyon çalışmaları neticesinde; $CoCl_2$'nın düşük konsantrasyonlarında *R. sphaeroides* hücrelerinde, yüksek $CoCl_2$'ye kıyasla daha iyi üreme olduğu gözlemlenmiş ve fenotipik karakterizasyonu koloni sayımı yöntemi ile gerçekleştirilen diğer stres faktörlerine kıyasla *R. sphaeroides* hücrelerinin tuz stresine (NaCl) karşı daha dirençli olduğu görülmüştür. Ayrıca *R. sphaeroides*

hücreleri bir ağır metal olan $CoCl_2$ ve diğer stres faktörlerini içermeyen ortamda kendi doğal kırmızı-mor rengini sergilerken, $CoCl_2$'ın yüksek konsantrasyonlarında $CoCl_2$'nın açık pembe rengini almıştır. Bu sonuç, *R. sphaeroides* hücrelerinin $CoCl_2$'yi tuttuğunu düşündürmektedir. Diğer ağır metal stresleri ve ağır metallerin dışındaki streslerin varlığında böyle bir bulguya rastlanmamıştır. Hücre örnekleriyle yapılacak kapsamlı metal tayinleri *R. sphaeroides*'in farklı metallerle olan ilişkisini anlama yönünde önemli bilgiler sağlayabilir.

Bu tez kapsamında yapılan çalışmalar, *R. sphaeroides*'in fizyolojik özelliklerini ve stres direnç düzeylerini açığa çıkarmanın yanı sıra bu mikroorganizmanın stres direncini artırmak yönünde yapılacak olan çalışmalar için de yararlı olabilecektir.

1. INTRODUCTION

1.1 General Information about *Rhodobacter sphaeroides*

R. sphaeroides is a gram negative, purple non-sulfur photosynthetic eubacterium which belongs to the α-3 subgroup of the protebacteria. *R. sphaeroides* is known for its capacity to grow under a wide range of environmental and nutritional conditions (Peuser et al., 2010). *R. sphaeroides* has extensive energy acquiring mechanisms such as aerobic or anaerobic respiration and photosynthesis or chemosynthesis (Kaplan et al., 2007). Because of that, it is a highly diverse organism in terms of its metabolism. The taxonomic classification of *R. sphaeroides* is shown in Table 1.1.

Table 1.1 : Taxonomic classification of *Rhodobacter sphaeroides* (Url-1).

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Alphaproteobacteria
Order	Rhodobacterales
Family	Rhodobacteraceae
Genus	Rhodobacter
Species	*Rhodobacter sphaeroides*

Organic compounds are used as both a source of carbon and reductant for photoheterotrophic and chemoheterotrophic growth. Carbon dioxide is used as the sole carbon source under autotrophic growth conditions. *R. sphaeroides* can also utilize molecular nitrogen as the sole source of organic nitrogen, thus it plays an important role in nitrogen fixation (Mackenzie., 2007). Although the *R. sphaeroides* cell is rod shaped, its shape changes from rod to coccobacillus when it switches from aerobic to

photoheterotrophic growth (Slovak et al., 2005). The electron microscope image of *R. sphaeroides* is shown in Figure 1.1 (Seifert et al., 2010).

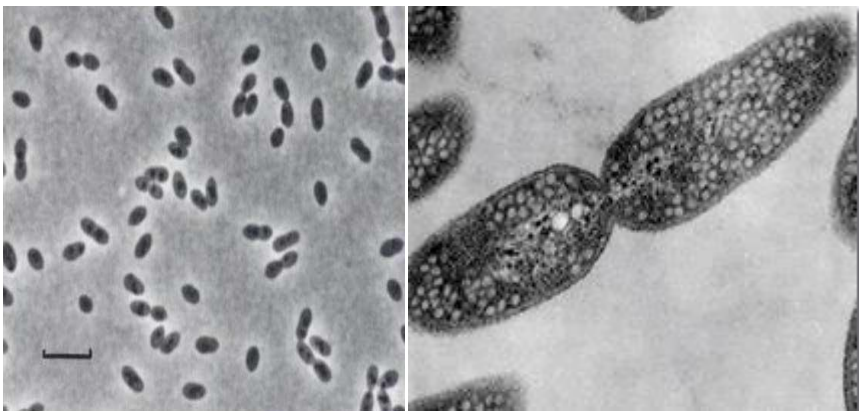

Figure 1.1 : Image of *Rhodobacter sphaeroides*.(Micrographs performed with electron microscope with phase contrast. Tab on the left micrograph equals 5 μm) (Seifert et al., 2010).

The soil, anoxic zones of waters, mud, sludge and organic rich waters comprise the ecological niche of *R. sphaeroides*. Movement of *R. sphaeroides* is achieved via an atypical single subpolar flagellum that provides it rotation in a clockwise direction (Mackenzie et al. 2007).

1.2. Structure of Photosynthetic Apparatus

When oxygen is present in the environment, *R. sphaeroides* performs aerobic respiration for energy generation by means of electron transport chain and ATP machinery components which is present in its cytoplasmic membrane. However, in the absence of oxygen, *R. sphaeroides* develops intracytoplasmic membranes (ICM) which contain photosynthetic apparatus containing the pigment protein complexes and the photosynthetic electron carriers and performs anoxygenic photosynthesis for energy generation (Peuser et al., 2010). The photosynthetic apparatus is composed of three multimeric transmembrane protein complexes: the antenna or light harvesting complexes (LHC), the reaction center (RC) and the cytochrome (cyt) bc_1 complex. Light harvesting complexes are present in two types named as LH1 and LH2 in *R. sphaeroides*, like in most of the purple bacteria. The LH1 forms the RC-LH1 complexes together with RC. The role of the LHCs is to collect incident light binding non–covalently carotenoid and bacteriochlorophyll molecules. After the photon is collected by the LHCs, the excitation reaches the reaction center (RC) and charge

separation occurs here. After a series of electron turnover the reaction is catalyzed by the bc_1 complex (Joliot and Vermeglio, 1999).

1.3. Genome Structure

The genome of *R. sphaeroides* comprises two chromosomes CI and CII (2.9 Mb and 0.9 Mb, respectively) and five endogenous plasmids. Four of these endogenous plasmids range in size from ~114 kb to 100 kb (pRS241a-d). A fifth endogenous plasmid is the smallest with ~42 kb size. Thus, the total genome size of *R. sphaeroides* is ~4.5 Mb. According to the result of first physical mapping of *R. sphaeroides*, it was revealed that CII encodes two ribosomal RNA operons (*rrnB* and *rrnC*) while a third ribosomal RNA operon is encoded by CI. In time, it was found that other highly important genes that encode the important products of metabolism such as protein synthesis, amino acid biosynthesis, fatty acid metabolism, transcriptional regulation and energy metabolism were present on CII. Nevertheless, like *rrn* operons, these genes are in duplicate forms on CI. Some of these duplicate genes include $cbbA_I$ /$cbbA_{II}$ and $cbbP_I$ /$cbbP_{II}$ (enzymes of the reductive Calvin Cycle), *hemA*/*hemT* (5-aminolevulinic acid synthase) and $rpoN_I$ /$rpoN_{II}$ (alternative sigma factors, σ^{54}) (Mackenzie et al., 2001).

1.4. Biotechnological Importance of *Rhodobacter sphaeroides*

R. sphaeroides displays biotechnological importance for several industrial and medical applications, as summarized in sections 1.4.1-1.4.7.

1.4.1. Hydrogen gas (H_2) production by *Rhodobacter sphaeroides*

Hydrogen gas is seen as a future energy carrier since it is renewable, does not increase greenhouse effect and is easily converted to electricity by fuel cells (Seifert et al., 2010). It is known that hydrogen can be produced from a variety of feed stocks including fossil resources such as natural gas and coal as well as renewable resources. However, since fossil resources do not provide infinite energy resource for generating hydrogen gas for energy industry, there is a strict need for development of renewable and clean energy resources. A variety of process technologies can be used including biological, photo electrochemical and thermochemical processes in hydrogen production. Biological hydrogen production by photosynthetic microorganisms that used in hydrogen production has more several advantages among other processes. For example, biological hydrogen production by photosynthetic microorganisms requires the use of simple reactor with low energy

requirements and the cost can be reduced by using of cheaper substrates for microorganism such as waste water originating from different industries (Seifert et al., 2010).

R. sphaeroides, which is a photosynthetic and metabolically highly diverse organism that can be cultured both in the presence and absence of light, has an ability to decompose vast variety of organic substances of waste industry originating from different industries such as food, dairy, sugar or alcohol–distilling. In consequence of this metabolic process it produces hydrogen and CO_2. *R. sphaeroides* is one of the most active hydrogen producers among the photosynthetic organisms known as a hydrogen producer (Seifert et al., 2010).

1.4.2. Polyhydroxybutyrate (PHB) production by *Rhodobacter sphaeroides*

Polyhydroxybutyrate (PHB) is the most common polyhdroxyalkanoate (PHA) and various bacterial strains among archaebacteria, Gram positive and Gram negative bacteria and photosynthetic bacteria including *R. sphaeroides,* synthesize and deposit it as an cytoplasmic inclusion under unbalanced growth conditions both aerobically and anaerobically. When the bacteria come across nutrient–depleted environment, PHBs are degraded by bacteria in order to get energy to maintain its life. PHB is biodegradable, biocompatible and not cytotoxic to cells thus, it has great potential in several fields such as pharmacology, transplantology and tissue engineering etc. (Yang et al., 2005). Polyhydroxybutyrate (PHB) granules formed by *R. sphaeroides* are shown in Figure 1.2 **(**Bebien, M. et al., 2001).

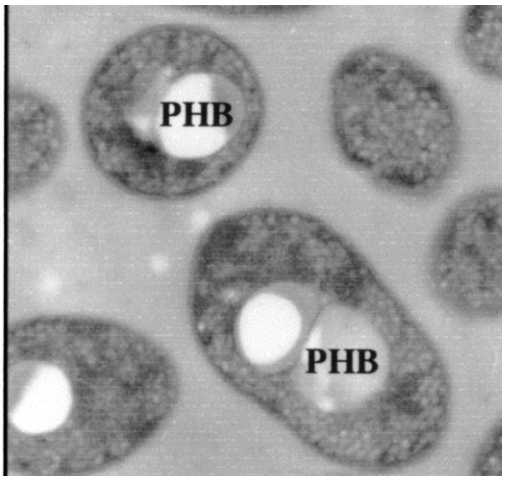

Figure 1.2 : Thin–section micrograph of polyhydroxybutyrate (PHB) granules inside *R.sphaeroides* cells (Bebien, M. et al., 2001).

1.4.3 Bioremediation of heavy metals by *Rhodobacter sphaeroides*

Bioremediation which detoxify and degrade environmental contaminants, requires the use of microbes to solve environmental problems such as contamination of soil or groundwater. Photosynthetic organisms are particularly recommended to be employed in bioremediation process for degradation or recovering of pollutants from contaminated environments (Giotta et al., 2005). Like most microorganisms, *R. sphaeroides* has an ability to reduce oxyanions and sequester metals (Pisani et al., 2008). *R. sphaeroides* known for its ability to tolerate high concentrations of heavy metals, to bioaccumulate cobalt and nickel and to reduce oxyanions as tellurite, selenite and chromate which are more toxic, even at low concentration for other microorganisms (Italiano et al., 2012). Giotta *et al.* have reported that *R. sphaeroides* showed high level of tolerance against some heavy metals such as Hg^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, MoO_4^{2-} by applying increasing mM concentrations (Giotta et al., 2005). With respect to their results, *R. sphaeroides* R-26 showed more tolerance to molybdate among other metal factors applied. Even, there was no substantial decrease of cellular mass at its high concentrations (60 mM). Moreover, Giotta *et al.* have also remarked that *R. sphaeroides* R 26 was more tolerant to heavy metals than *E. coli*. Tolerance towards heavy metals allows to propose the employment of *R. sphaeroides* in metal contaminated environments (Giotta et al., 2005).

1.4.4. Production of vitamin B_{12} by *Rhodobacter sphaeroides*

Application of vitamin B_{12} comprises its use for treating anemia and neuritis, as health food supplement and as a growth-promoting factor in feedstock for animals. Vitamin B_{12} is unique among the other vitamins since it is the only one produced by prokaryotes (Kang et al., 2012). While total chemical synthesis of vitamin B_{12} requires more than 60 steps, only some members of archaea and eubacteria have the ability to produce vitamin B_{12} through two routes, containing approximately 30 enzyme-mediated steps. It means that in nature, synthesis of vitamin B_{12} involves less complex steps than chemical synthesis of vitamin B_{12}. Synthesis of vitamin $B_{12,}$ which shares a common pathway with porphyrin, chlorophyll and heme via 5-aminolevulinic acid (ALA) can be performed both aerobically and anaerobically by *R. sphaeroides. R. sphaeroides* is one of the most important vitamin B_{12} producer microorganism. It was shown that under aerobic dark culture conditions *R. capsulatus* and *R. gelanitosa* produced 21- 33 μg/g dry weight of vitamin B_{12}, respectively. On the contrary, it was reported that under aerobic dark culture conditions *R. sphaeroides* P47 could produce 75 μg/g dry weight of vitamin B_{12} while under anaerobic light culture conditions *R.*

sphaeroides P47 produced 87 µg/g dry cells of vitamin B_{12}. Production of vitamin B_{12} by *R. sphaeroides* was almost the twice when compared with that of produced by *R. capsulatus* and *R. gelanitosa* under aerobic dark culture conditions (Sasaki et al., 2005). However, the regulation mechanisms of both aerobic and anaerobic synthesis pathways of vitamin B_{12} are still unclear (Kang et al., 2012).

1.4.5. Production of coenzyme Q10 by *Rhodobacter sphaeroides*

Coenzyme Q10, found in the membranes of many organelles such as mitochondrion, endoplasmic reticulum, peroxisomes etc., is a powerful antioxidant that neutralizes the free radicals. Coenzyme Q10 has been used for treating heart diseases such as heart failure for more than 30 years. Coenzyme Q10 is an important physiological regulator since it regulates important physiological events such as high blood pressure. Also, recently coenzyme Q10 has been used as a food supplement. The commercial production of coenzyme Q10 started about 25 years ago by using photosynthetic bacteria. It was reported that *R. sphaeroides* produced coenzyme Q10 in the anaerobic light conditions as two fold as in the aerobic dark conditions (Sasaki et al., 2005).

1.4.6 Production of 5-aminolevulinic acid (ALA) by *Rhodobacter sphaeroides*

5-aminolevulic acid (ALA), which is a non-protein amino acid, is found in bacteria, fungi, animals and plants. Since ALA is a well-known intermediate of tetrapyrrole biosynthesis such as porphyrin, heme and vitamin B_{12}, it has drawn great attention. ALA was first used in the field of agriculture as a selective biodegradable herbicide or insecticide because of its nontoxicity to crops, animals and humans. Moreover, ALA was shown to increase cold temperature tolerance and salt tolerance in plants (Kang et al., 2012). In medical field, ALA was used for the diagnosis of heavy-metal poisoning and cancer treatment by laser irradiation technology. So far, chemical synthesis of ALA has been performed with low yield and high price. Restrictive events for chemical synthesis of ALA such as environmental pollution, global warming etc. led to the need for renewable resources such as microbes to produce high value industrial chemicals. When it was compared with the chemical methods, microbial production of ALA has many advantages over chemical production due to being a green, sustainable, renewable and inexpensive method. Biological production of ALA was previously reported in some reports in 1980s. Also, large amounts production of ALA by the photosynthetic bacterium *R. sphaeroides* with the intermittent addition of levulinic acid was reported before (Sasaki et al., 2005).

1.4.7. Production of porphyrin by *Rhodobacter sphaeroides*

Porphyrin is a commercial medicine for liver diseases, cancer diagnosis and cancer treatments. Before microbial production of porphyrin has become popular, porphyrin production was carried out from animal blood for medical applications. As for the microbial production of porphyrin, by using photosynthetic bacteium *R. sphaeroides* which is a most potent microorganism in which porphyrin production was achieved with the amount of 10-100 *mg*/*l* of porphyrin extracellularly (Sasaki et al., 2005).

1.5. Stress Response of *Rhodobacter sphaeroides*

Microorganisms have different defense mechanisms according to each stress type that they encounter in their surroundings. Miscellaneous bacterial mechanisms recognise different environmental changes and reply them with an appropriate response. When *R. sphaeroides* reacts against stresses, the cells may change the level of synthesis of certain cell components, like other microorganisms (Tsuzuki et al., 2011). Proteins whose expression is changed due to different environmental stress factors are called stress proteins. Microorganisms have two types of stress proteins, general stress proteins and specific stress proteins. General stress proteins are described as those proteins the concentrations of which are altered by two or more stress factors (Nepple and Bachofen, 1997). GroEL which belongs to the family of heat shock proteins can be given as an example for general stress proteins. When microorganisms are exposed to different physical and chemical stress types, stress proteins including GroEL are rapidly synthesized (Duncan et al., 1999; Tsuzuki et al., 2011).

Both well-known and the most encountered stress types of *R. sphaeroides* include osmotic stress, oxidative stress and heavy metal stress. For example, regarding osmotic stress which is generated by high amount of NaCl or other substrates inside the cells, microorganisms have evolved two mechanisms against this stress. One of these mechanisms involves the synthesis of intracellular osmoprotectants known as compatible solutes such as the amino acids glutamate, proline, and the amino acid derivative glycine-betaine and the sugar trehalose. The second common mechanism involves altering membrane composition including both changes in fatty acid saturation and phospholipid composition to overcome osmotic stress. *R. sphaeroides* 2.4.1 can adapt to concentrations of NaCl up to about 0.4 M, but higher concentrations of NaCl prevent cell growth. Another strain closely related to strain 2.4.1, *R. sphaeroides* f. sp. *denitrificans*, synthesizes trehalose as an osmoprotectant in

response to salt stress. *R. sphaeroides* f. sp. *denitrificans* also accumulates potassium cations to stabilize the sodium levels inside the cells (Tsuzuki et al., 2011). It was also reported that *R. sphaeroides* R-26 increased the cardiolipin levels under osmotic stress which is an anionic phopholipid playing an important role in energy conversion in *R. sphaeroides* R-26 cells (Catucci et al., 2004).

The presence of different stress factors such as heavy metal stress can cause the oxidative stress inside the cells of an microorganism. *R. sphaeroides* can grow both under aerobic and anaerobic conditions. Under aerobic conditions photosynthesis genes of *R. sphaeroides* are repressed by the PpsR protein in *R. sphaeroides*. However, several studies showed that O_2 is generated in the reaction center (RC) of the *R. sphaeroides* photosynthetic apparatus when light and oxygen are simultaneously present. Therefore, the generated stress by the presence of these two factors is termed photooxidative stress. *R. sphaeroides* exhibits a specific photooxidative stress response, which is initiated by the release of sigma factor RpoE. As well as RpoE, RpoHII that conroled by RpoE is also activate the defense mechanism in the presence of oxidative stress (Berghoff et al., 2011).

Additionally, carotenoids of *R. sphaeroides* play an important role in response to oxidative stress which is generated by singlet oxygen (O_2) (Nuss et al., 2008). It was reported that while *R. sphaeroides* that has carotenoids could survive, carotenoid-deficient mutant was rapidly killed when oxygen is present since oxygen has unfavorable impacts on different cell components including DNA, proteins and lipids (Zeller et al., 2005). Except this defense system against oxidative stress, *R. sphaeroides* has some components to protect the cells to remain under oxidative stress including superoxide dismutase, catalases and peroxidases which are responsible for significantly dispelling oxidative stress (Markowicz et al., 2010).

The presence of heavy metals in surrounding environment of microorganisms is another common stress factor. Microorganisms have evolved metal stress resistance strategies such as exclusion by permeability barrier, enzymatic transformation, reduction of metal to less toxic forms and efflux of the metal ions from the cell. Another one of evolving strategies, in the result of metal exposure of the cells, microorganisms can alter the fatty acid composition of their lipids to maintain membrane integrity (Markowicz et al., 2010).

1.6. General Effects of Metal Stress on Microorganisms

The interaction between metal ions and microorganisms was widely investigated in the last decades (Giotta et al., 2005) Metal contaminants are commonly found in soil, sediments and water. Environmental metal pollution can occur via industrial processes such as mining, refining etc. Metals are non-biodegradable, but can be transformed by a different way such as methylation. Some metals, such as Ca, Co, Ni and Zn, are essential and serve both as micronutrients for microorganisms and as important functions in enzyme productivity as cofactor. Among them, Co^{2+} was found to have an interesting effect on *R. sphaeroides* at increasing ion concentrations by reducing the cellular content of bacteriochlorophyll (Italiano et al., 2011).

On the other hand, many other metals, such as Hg, Pb, Cd and Au have no biological functions on microorganisms and they are toxic to microorganisms even if they are exposed at their minimal concentration. Metals which are both nonessential and essential at higher concentrations have an unfavorable impact on microorganisms by blocking the functional groups of important molecules and transport channels for required nutrient ions, damaging cell membranes and DNA structure and altering enzyme specificity. For instance; mercury, cadmium and silver bind to SH groups and this ends up with an inhibition of the sensitive enzymes (Anyanvu et al., 2011). Additionally, morphological changes of cells can be generated by the presence of some heavy metals. It was shown by Italiano et al. that *R. sphaeroides* R26 cells that were exposed to chromate (CrO_4) were enlarged and elongated by the pressure of chromate (Italiano et al., 2012).

1.7 The Aim of This Study

Microorganisms encounter different stress types including nutrient starvation, temperature and pH changes, heavy metal stress, oxidative stress, osmotic stress etc. in their surroundings and have to cope with these stresses in nature. For that reason, bacteria need adaptational strategies to survive to grow in adverse conditions. Since *R. sphaeroides* is an industrially important microorgnism, it is important to investigatate *R. sphaeroides* in terms of resistance to different stress types that are encountered mostly.Thus, the aim of this study was to physiologically investigate wild type *R. sphaeroides* R-26 for resistance to heavy metal stress and other stress factors such as NaCl, H_2O_2, H_3BO_3, $CuSO_4$ and ethanol.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Strain name

Wild type *R. sphaeroides* strain R-26, which was kindly provided by Dr. Massimo Trotta from University of Bari, Italy, was used in this study.

2.1.2 Growth media

Liquid and solid M27 were prepared and used throughout the study.

Chemical composition of Medium 27 of the DSMZ is shown in Table 2.1

Table 2.1 : Chemical composition of Medium 27 of the DSMZ (Italiano et al., 2009).

Chemical	**Concentration**
Macronutrients	**(mg l^{-1})**
KH_2PO_4	500
$MgSO_4.7H_2O$	800
NaCl	400
NH_4Cl	400
$CaCl_2.2H_2O$	50
D,L malic acid	1500
Yeast extract	2000
p-Amino benzoic acid	1
Iron(II)citrate	18

Table 2.1 (continued) : Chemical composition of Medium 27 of the DSMZ.

Micronutrients	**(mg l^{-1})**
$ZnSO_4.7H_2O$	100
$MnCl_2.4H_2O$	30
H_3BO_3	300
$CoCl_2.6H_2O$	200
$CuCl_2.2H_2O$	10
$NiCl_2.6H_2O$	20
$Na_2MoO_4.2H_2O$	30
ddH_2O was added up to final volume 1000 mL	
pH was adjusted to pH 6.8 using NaOH solution	

To prepare the solid media, 20g agar was added into 1000 mL M27 throughout the study.

2.1.3 Laboratory equipment

The list of laboratory equipments used in this study is given in Table 2.2.

Table 2.2 : Laboratory equipments used in this study.

Equipment	**Supplier**	**Country**
Autoclave	Tommy SX700E	China
Benchhop Centrifuge	Beckman Coulter Allegra 25R	USA
Deep Freezer	-80 ^oC Sanyo Ultra Low MDT-U40865	Japan
Desiccator	Finemech Bola-Star Vitrium Desiccator	USA
Refrigerator	-20 ^oC Arçelik 3011 NY	Turkey
Incubator	Nüve EN400-EN500	Turkey
Laminar Flow Hood	Biolab Faster BH-EU 2003	Italy
Light Microscope	Olympus CH30	USA
Magnetic Stirrer	Labworld	Germany
Microbalance	Precisa 620C SCS	Switzerland
Microfuge	Eppendorf Microcentrifuge-5424	Germany

Table 2.2 (continued) : Laboratory equipments used in this study.

Micropipettes	Eppendorf	Germany
pH Meter	Mettler Toledo MP220	Switzerland
Transilluminator	Vilber Lourmat	
UV-Visible Spectrophotometer	Shimadzu UV-1700	Japan
Vortex Mixer	Heidolph	Germany

2.1.4. Chemicals, buffers-solutions and software –websites

The chemicals, buffers-solutions and software-websites are given in Table 2.3, 2.4 and 2.5, respectively.

Table 2.3 : List of chemicals used in this study.

Chemicals	**Supplier**	**Country**
Agar	BD Difco™	USA
p-Amino benzoic acid	Fluka	USA
Ammonium chloride, NH_4Cl	Sigma ALDRICH	USA
Boric acid, H_3BO_3		
Calcium chloride dihydrate, $CaCl_2.2H_2O$	Carlo Erba	Italy
Cobalt chloride hexahydrate, $CoCl_2.6H_2O$	Fluka	USA
Copper chloride dihydrate, $CuCl_2.2H_2O$	Sigma ALDRICH	USA
Ethanol, C_2H_6O	J.T. Baker	The Netherlands
Glycerol, $C_3H_8O_3$	Duchefa Bochemie	The Netherlands
Iron(II)citrate		
Magnesium sulfite heptahydrate, $MgSO_4.7H_2O$	MERCK	Germany
D,L malic acid	MERCK	Germany
Manganese chloride tetrahydrate, $MnCl_2.4H_2O$	MERCK	Germany

Table 2.3 (continued) : List of chemicals used in this study.

Nickel chloride hexahydrate, $NiCl_2.6H_2O$	MERCK	Germany
Sodium chloride, NaCl	MERCK	Germany
Sodium hydroxide, NaOH	MERCK	Germany
Sodium molybdate, $Na_2Mo_4.2H_2O$	MERCK	Germany
Yeast Extract	MERCK	Germany
Zinc sulfate heptahydrate, $ZnSO_4.7H_2O$	MERCK	Germany

Table 2.4 : List of solutions and buffers used in this study.

Component	**Amount**
$CoCl_2.6H_2O$ Stock Solution	500 mM Stock Solution
NiCl2.6H2O Stock Solution	100 mM Stock Solution
ZnCl2.6H2O Stock Solution	100 mM Stock Solution
CuSO4.5H2O Stock Solution	100 mM Stock Solution
NaCl Stock Solution	4 M Stock Solution
H_3BO_3 Stock Solution	2 M Stock Solution
Glycerol	30% v/v
Ethanol	70% v/v
NaOH Stock Solution	10 M Stock Solution

Table 2.5 : Software and websites used in this study.

Microsoft Office Program 2010	
A five – tube MPN table	http://www.jlindquist.net/generalmicro/102dil3a.html

2.2 Methods

2.2.1 Preparetion of growth media

To prepare the liquid media, all chemical constituents, shown in the section 2.1.2 were mixed with distilled water in given amounts to 1000 mL. To prepare the solid media, 2% (w/v) agar was added into the liquid medium in the flask and shaken to disperse all contents in both liquid and solid media. Before autoclaving, the pH of media was adjusted to pH 6.8 using NaOH solution. Autoclave was performed at 121 $^{\circ}$C and 1 atmosphere for 15 minutes.

2.2.2 Cultivation and storage conditions

Culture was cultivated in 50 mL test tubes containing 17 mL Medium 27 (M27) of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) at pH 6.8 providing semiaerobic condition at 37 $^{\circ}$C, 170 rpm throughout this study, unless otherwise indicated. One mL of cells of *R. sphaeroides* strain R-26 at exponential growth phase were harvested in 1.5 mL microfuge tube by centrifuging at 13000 *g* for 4 min. The supernatant was thrown away and the pellet was resuspended in 30 % (v/v) glycerol. The stock solution of the culture was conserved at -80 $^{\circ}$C.

2.2.3 Determination of resistance to $CoCl_2$

Stress resistance of bacterial cells were determined by most probable number (MPN) methodology, spotting assay and measuring optical density at 535 nm.

2.2.3.1 Determination of cell survival by means of the most probable number (MPN) method

Most probable number method is a useful statistical method in estimating microbial population concentrations in a liquid medium with 95% confidence. For this reason, resistance to $CoCl_2$ was estimated by most probable number method. Firstly, *R. sphaeroides* strain R-26 was incubated in 50 mL test tube containing 17 mL M27 at 37 $^{\circ}$C, 170 rpm overnight as preculture. After bacterial cells reached their logarithmic phase of growth, 20 µl bacterial cells were inoculated into 96 well plates containing 180 µl M27 treated with 5 mM and 8 mM $CoCl_2$ as well as non-stress (control) condition with five repetition by preparing their serial dilutons from 10^1 fold to 10^8 fold (Appendix A.1.). These 96-well plates were incubated at 37 $^{\circ}$C, 170 rpm for 72 hours. The viable cell numbers were calculated using the MPN table at the end of each incubation day for both $CoCl_2$-treated and non-treated cultures. The survival ratio was calculated by dividing the number of cells treated with $CoCl_2$ to that of non- treated with $CoCl_2$ stress.

2.2.3.2 Spotting assay

To do spotting assay, *R. sphaeroides* R-26 cells were inoculated into 50 mL test tube containing 17 mL M27 and incubated overnight as preculture. Precultures were inoculated into new 50 mL test tube containing M27 with an initial OD_{535} of 0.1. The cultures were grown until their logarithmic phase of growth. Cells were concentrated to 4 OD_{535} units of cells and harvested in 1.5 mL microfuge tube. Samples were centifuged at 13000 *g* for 4 minutes. Supernatant was thrown away and 50 µl M27 was added to the pellet for resuspension. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of bacterial cells were prepared and 3µl of each dilution was spotted onto M27 plates that containing different concentrations of $CoCl_2$ (0 mM, 2.5 mM, 5 mM, 10 mM, 20 mM, 25 mM $CoCl_2$). Plates were incubated at 37 $^{\circ}$C for 72 hours.

2.2.3.3. Monitoring OD_{535} for determining the $CoCl_2$ resistance

After obtaining precultures of *R. sphaeroides* strain R-26, bacterial cells from preculture were inoculated into 50 mL test tube containing M27 with 0 mM, 8 mM and 24 mM $CoCl_2$ concentrations in a volume of 17 mL. These test tubes containing M27 and different concentrations of $CoCl_2$ were incubated at 37 $^{\circ}$C, 170 rpm for 72 hours. At the end of each day, optical density measument at 535 nm were performed for each test tube and survival ratio of cultures for each concentration of $CoCl_2$ was determined by dividing the optical density measurement results of samples treated with $CoCl_2$ to that of the control group (M27 with 0 mM $CoCl_2$) for each day.

2.2.4 Physiological analysis of wild type

2.2.4.1 Growth curve analysis of wild type in the presence and absence of $CoCl_2$

Firstly, wild type bacterial cells were inoculated into 50 mL test tube in which 17 mL M27 was present and incubated at 37 $^{\circ}$C, 170 rpm overnight as preculture. Preculture was used to inoculate into 250 mL flask both containing 100 mL M27 and containing 100 mL M27 with appropriate concentration of $CoCl_2$ (8 mM $CoCl_2$) with an initial OD_{535} of 0.05. The cultures were incubated at 37 $^{\circ}$C, 170 rpm for 32 hours. During incubation period, samples were taken from the cultures at different time intervals and OD_{535} values of samples were monitored. With respect to optical density results, growth curves of wild type in the presence and absence of $CoCl_2$ were obtained.

2.2.4.2 Maximum specific growth rate determination

Maximum specific growh rates (μ_{max}) of wild type in the absence and presence of $CoCl_2$ were calculated using the natural logarithm of the OD_{535} values which corresponds to the maximum slope from this logarithmic representation.

2.2.4.3. Cell dry weight analysis

1.5 mL microfuge tubes were dried in 80 $^{\circ}$C oven for 48 hours and weighed by using microbalance (Precisa 620C SCS, Switzerland). They were placed in a desiccator (Finemech Bola-Star Vitrium, USA) for 15 minutes before determination of their weights. 1 mL culture was added to preweighted microfuge tubes and centrifuged at 13000 *g* for 4 minutes. Pellets were obtained by throwing away the supernatant and the tubes with the pellets were dried in 80 $^{\circ}$C oven for 48 hours. At the end of 48 hours each tube was placed to desiccator again for 15 minutes to remove moisture of tubes and then weighted. Real values of dried cells were obtained by subtracting the second weight from the first weight of each tube.

2.2.5 Analysis of cross resistance to other stress types

To investigate cross resistance of *R. sphaeroides* for different stress types, the precultures of bacterial cells were inoculated into M27 non-stress (control) condition and M27 containing 1 M NaCl, 5 % (v/v) of ethanol, 1 mM $CuSO_4$, 4 mM H_2O_2 and 100 mM H_3BO_3 stress factors. After incubation the test tubes containing the stresses as well as the control group were incubated at 37 $^{\circ}$C, 170 rpm for 48 h. The preculture of *R. sphaeroides* was spread onto plates containing M27 preparing their serial dilutions according to their OD_{535} results for 24 h and 48 h. After 3 days of incubation on plates, colonies were counted directly.

Cross resistance of wild type *R. sphaeroides* was investigated for 1 mM $NiCl_2$, 10 mM $ZnCl_2$ and 24 mM $CoCl_2$ stress factors performing the assays in triplicates by measuring OD_{535} for 24, 48 and 72 h by batch culturing. To perform the assay bacterial preculture was inoculated into 50 mL test tubes containing 1 mM $NiCl_2$, 10 mM $ZnCl_2$ and 24 mM $CoCl_2$ stress factors together with a non-stress (control) group and incubated at 37 $^{\circ}$C, 170 rpm during 72 h. At the end of each incubation day, growing of bacterial cells was checked by measuring optical density values at 535 nm and their survial ratios were calculated by dividing optical density result of samples treated with different stress factors to that of non-stress condition which serves as a control group and their survival values were given as a percent value.

3. RESULTS and DISCUSSION

3.1 Determination of $CoCl_2$ Resistance of *Rhodobacter sphaeroides* by MPN Method

$CoCl_2$ stress tolerance of *R. sphaeroides* was tested with 5-tube MPN methodology. MPN method was performed applying 5 mM and 8 mM $CoCl_2$ stress concentrations. Both cultures with and without stress application were incubated at 37 $^{\circ}$C, 170 rpm. At the end of the incubation time, survival ratio was determined for both 5 mM and 8 mM $CoCl_2$ stress concentrations by dividing number of "cells/mL" of the stress applied samples to number of "cells/mL" of the control group. The results of percent survival ratio of wild type for MPN assay are given in Table 3.1 and Figure 3.1, respectively.

Table 3.1 : Percent survival ratio of wild type after 72nd hour incubation.

Stress Concentrations	Number of cells/mL	Percent Survival
0 mM	91000000	---
5 mM	170	0.0002
8 mM	94	0.0001

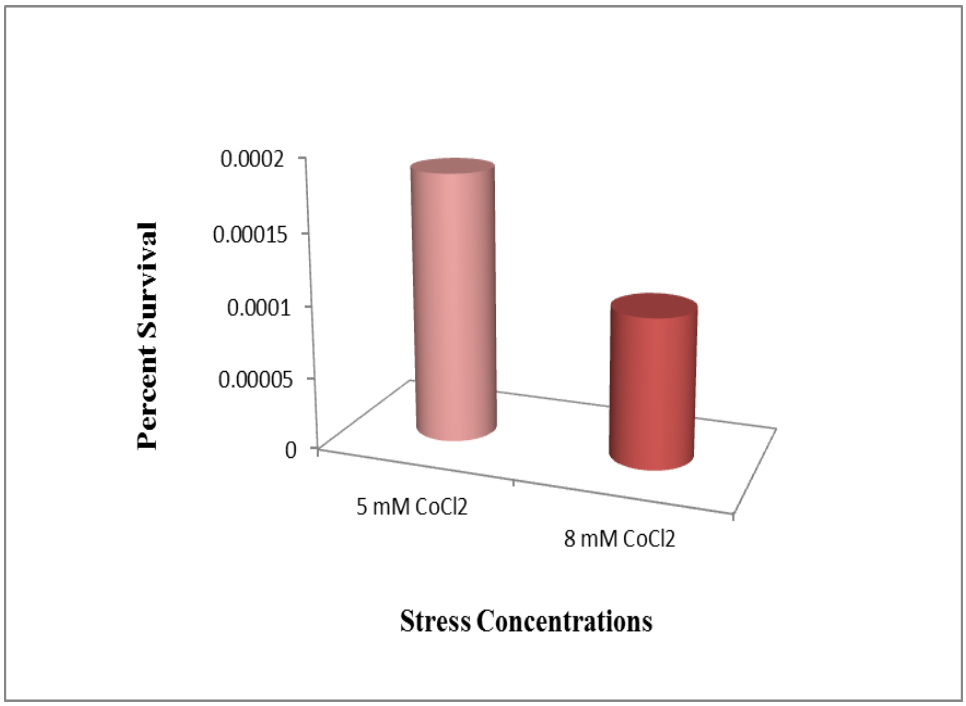

Figure 3.1 : The graphic of percent survival ratio result of wild type at 72nd hour.

The percent survival ratio of wild type at 5 mM $CoCl_2$ was twice that of at 8 mM $CoCl_2$ for its 72nd hour result. Moreover, it indicates that survival values were so low and observed to be insignificant when bacterial cells when exposed to 5 mM and 8 mM $CoCl_2$ concentrations. However, more significant result would be obtained at lower concentrations of $CoCl_2$. Stress concentrations were too high to be applied by MPN method for *R. sphaeroides*.

3.2. Determination of Stress Resistance of *Rhodobacter sphaeroides* to $CoCl_2$ by Spotting Assay

Spotting assay was carried out in the presence and absence of $CoCl_2$ in order to observe stress tolerance of wild type *R. sphaeroides* to $CoCl_2$. The images belonging to the spotting assay are shown in Figure 3.2.

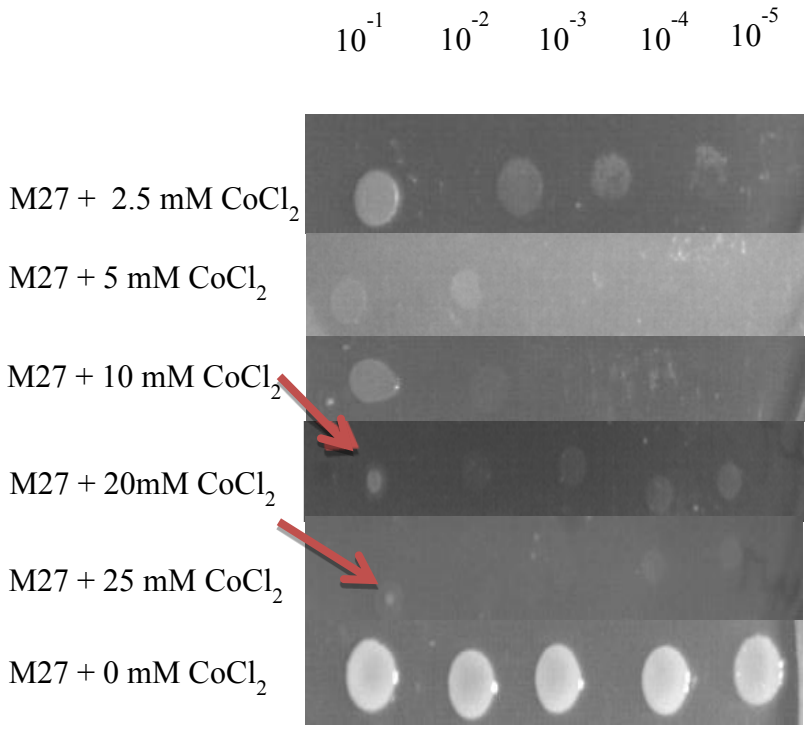

Figure 3.2 : Spot assay results of wild type on M27 plate and M27 containing $CoCl_2$ (72 h results).

Wild type *R. sphaeroides* could grow well in the absence of cobalt, however, at increasing concentrations of $CoCl_2$, growth of wild type *R. sphaeroides* was inhibited. The best growth was observed on the plate containing 2.5 mM $CoCl_2$ but toward 10^{-5} dilution, especially at 10^{-5} dilution, growth of wild type *R. sphaeroides* was not observed. The growth on the plate containing 5 mM $CoCl_2$ was observed barely up to 10^{-2} dilution. Growth of wild type *R. sphaeroides* on plates containing 10 mM, 20 mM and 25 mM $CoCl_2$ were observed only at 10^{-1} dilution but at increasing concentrations of $CoCl_2$ the diameter of the spot showing the growth area was reduced. The most important observation concerning spotting assay of wild type *R. sphaeroides* was the occurrence of colour change of bacterial cells. Bacterial cells on the plate containing 0 mM $CoCl_2$ were in red colour like their natural color, at low concentrations of $CoCl_2$ (2.5 mM an 5 mM $CoCl_2$) the colour of bacterial cells were white. This may indicate that the presence of stress factor may have inhibited pigment production of *R. sphaeroides*. However, at increasing $CoCl_2$ concentrations (10 mM, 20 mM and 25 mM $CoCl_2$) the bacterial cells were in pink colour, like the colour of $CoCl_2$ solution. It can be suggested that *Rhodobacter sphaeroides* R-26 would be keeping $CoCl_2$ inside the cell at 10 mM, 20 mM and 25 mM $CoCl_2$ concentrations.

3.3 Determination of Stress Resistance of *Rhodobacter sphaeroides* to $CoCl_2$ by measuring OD_{535}

Alternatively, $CoCl_2$ stress tolerance of *R. sphaeroides* was investigated by applying 8 mM and 24 mM $CoCl_2$ stress through batch culturing. Optical density measurements of two independent repeats for each culture at 535 nm were performed. Survival ratio of each stress concentration was determined by dividing the result of optical density measurement at 535 nm of treated with $CoCl_2$ to that of non-treated with $CoCl_2$ for each day. The OD_{535} results and percent survival ratio of wild type for 0 mM, 8 mM, 24 mM $CoCl_2$ are given in Table 3.2 and Figure 3.3, respectively.

Table 3.2 : Optical density results of wild type for 0 mM, 8 mM, 24 mM $CoCl_2$.

Time	AVG of 0 mM $CoCl_2$ OD_{535}	AVG of 8 mM $CoCl_2$ OD_{535}	AVG of 24 mM $CoCl_2$ OD_{535}	Percent Survival at 8 mM $CoCl_2$	Std Dev	Percent Survival at 24 mM $CoCl_2$	Std Dev
24 hour	2.76	0.13	0.05	4.7	0.0046	2.0	0.0023
48 hour	4.60	0.15	0.14	3.4	0.0014	3.0	0.0091
72 hour	5.65	0.16	0.20	2.9	0.0037	3.6	0.0008

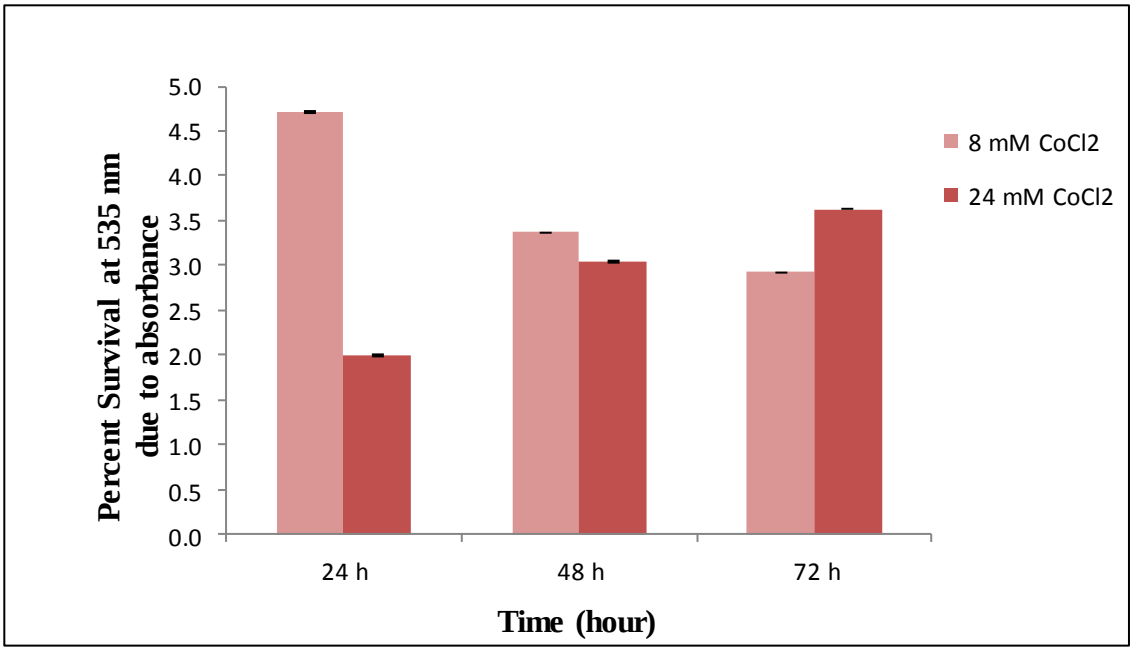

Figure 3.3 : The percent survival values of wild type for 8 mM, 24 mM $CoCl_2$ after 24h, 48h and 72h of incubation.

Bacterial cells could grow better at 8 mM $CoCl_2$ than at 24 mM $CoCl_2$ for 24 and 48 hours. At their 72nd hour incubation the survival ratio of cells treated-24 mM $CoCl_2$ increased significantly. The results of percent survival values of bacterial cells obtained by MPN method and spectrophotometric method were different from each other. The percent survival values obtained by MPN method were so low compared to that obtained spectrophotometrically. The reason might be related with assay conditions. To perform MPN methodology bacterial cells were incubated in 96 well plates; however, in order to perform spectrophotometric measurements of bacterial culture, the culture was incubated in 50 mL culture tubes as described in Section 2.2.2. Oxygen amount might affect the growth of bacterial cells, since oxygen amount was different for each methodology. Additionally, MPN method and monitoring optical densities by spectrophotometrically can not distinguish alive and dead cells. With the difference of these two methods, spotting assay gives visual results and shows alive cells visually, since on plates only alive cells can be observed.

3.4 Physiological Analysis of Wild Type

In order to observe the physiological differences between the conditions in the absence and presence of $CoCl_2$ for wild type bacterium, physiological characterization was performed. As physiological experiments; growth curve analysis, maximum specific growth rate determination and cell dry weight analysis were carried out.

3.4.1 Growth curve analysis of wild type in the presence and absence of cobalt

By using the optical density measurement results, growth curves of wild type in the presence and absence of 8 mM $CoCl_2$ were drawn and growth rate differences between them were observed for wild type bacterial cells. The OD_{535} results in the absence and presence of 8 mM $CoCl_2$ are shown in Table 3.3 and Figure 3.4, respectively.

Table 3.3 : OD_{535} results of growth curve analysis for wild type in the presence and absence of 8 mM $CoCl_2$.

Time (hour)	Wt Non-Stress				Wt Stress			
	OD_{535} 1	OD_{535} 2	AVG	Std Dev	OD_{535} 1	OD_{535} 2	AVG	Std Dev
0	0.07	0.07	0.07	0.001	0.08	0.08	0.08	0.0007
45'	0.18	0.16	0.17	0.014	0.09	0.09	0.09	0.0000
1 h 15'	0.32	0.32	0.32	0.000	0.10	0.10	0.10	0.0000
1 h 45'	0.89	0.85	0.87	0.030	0.10	0.09	0.10	0.0100
2 h 35'	1.90	1.82	1.86	0.060	0.11	0.11	0.11	0.0000
4 h 20'	2.88	2.77	2.83	0.080	0.12	0.12	0.12	0.0000
5 h 50'	3.48	3.39	3.44	0.063	0.19	0.18	0.19	0.0100
8 h	3.38	3.27	3.33	0.080	0.20	0.19	0.20	0.0100
9 h 30'	4.01	3.91	3.96	0.070	0.26	0.24	0.25	0.0141
24 h	3.96	3.80	3.88	0.113	0.35	0.30	0.33	0.0400
32 h	3.27	3.26	3.27	0.010	0.65	0.57	0.61	0.0600

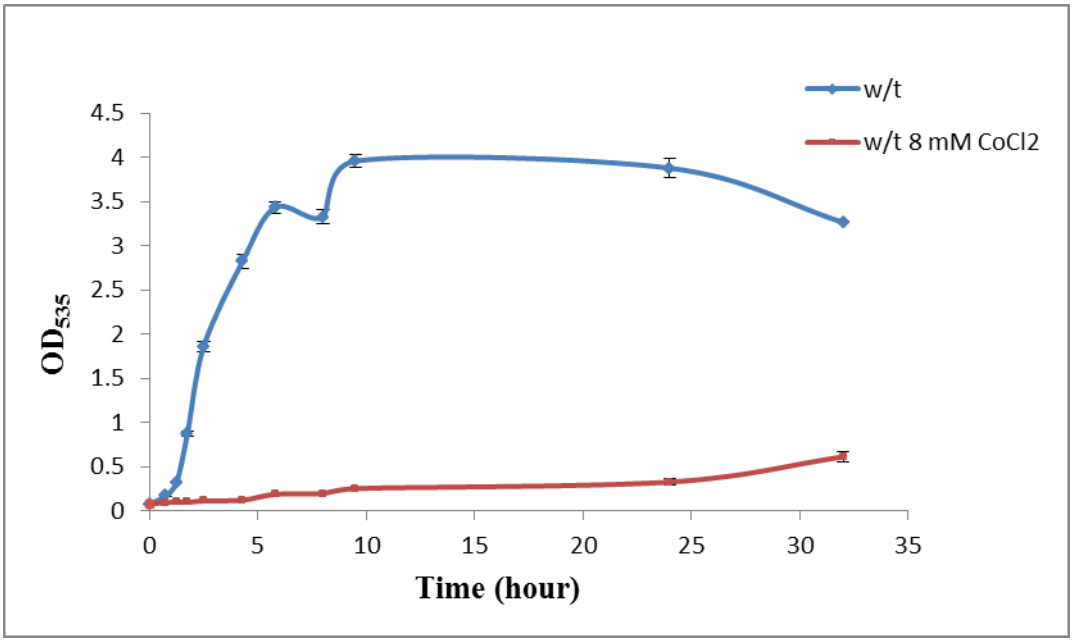

Figure 3.4 : Growth curve with respect to OD_{535} measurements of wild type in the absence and presence of 8 mM $CoCl_2$.

As expected, cobalt affected the growth negatively when compared to control condition. With respect to growth curve graphic, wild type *R. sphaeroides* reached its logarithmic growth phase earlier at non-stress condition. Thus, presence of cobalt retarded the growth of *R. sphaeroides* and the duration to logarithmic growth phase.

In order to calculate the maximum specific growth rates for conditions in the absence and presence of $CoCl_2$ for wild type bacterium, natural logarithm (ln) of OD_{535} values were

calculated. Maximum specific growth rates of wild type in the absence and presence of 8 mM $CoCl_2$ are given in Figure 3.5.

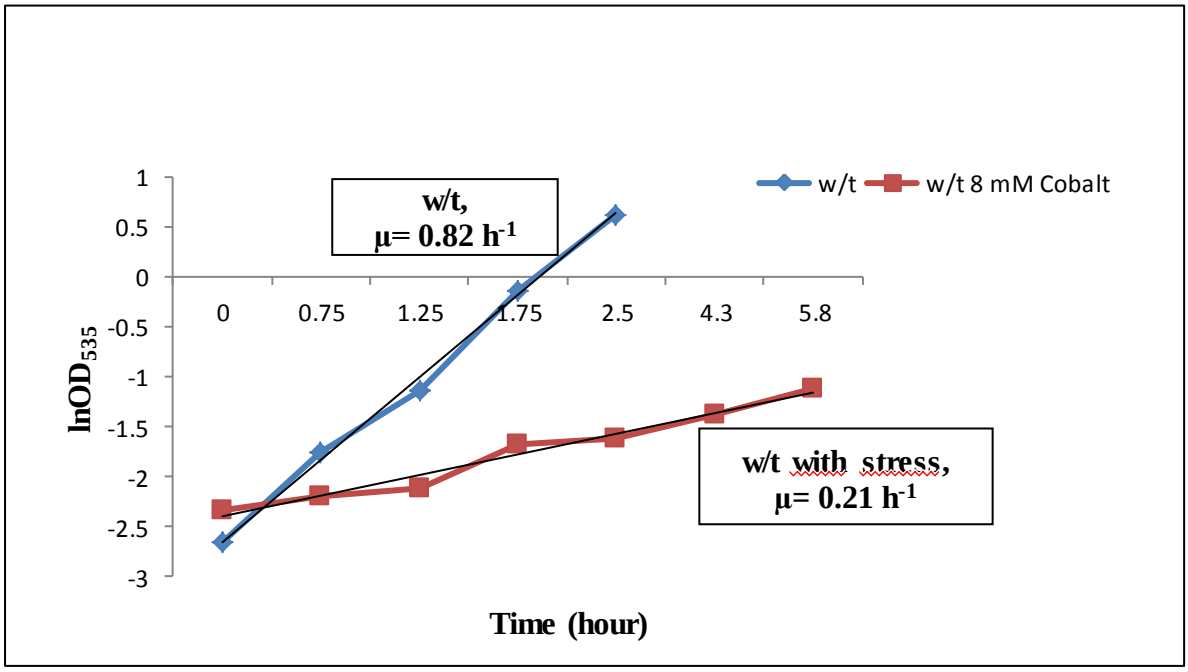

Figure 3.5 : Maximum specific growth rate graphic of wild type cells grown in the absence and presence of $CoCl_2$.

As concluded from the graphic, maximum specific growth rate of wild type *R. sphaeroides* was lower in the presence of stress than in the absence. This result was an expected result since cobalt adversely affected the growth of bacterial cells, as shown in Figures 3.1, 3.2, 3.3 and 3.4, respectively.

3.4.2 Cell dry weight analysis

Cell dry weight values of the samples of wild type cells were determined in the absence and presence of $CoCl_2$. Cell dry weight determination was performed in triplicates. Additionally, cell dry weight samples with high deviation were omitted. The cell dry weight results are given in Table 3.4 and Figure 3.6, respectively.

Table 3.4 : Cell dry weight results of wild type cells grown in the absence and presence of 8 mM $CoCl_2$.

Time (hour)	AVG of WT Non-Stress as mg/mL	Std. Dev.	AVG of WT Stress as mg/mL	Std. Dev.
2.5	0.80	0.17	----	----
4.3	1.30	0.28	0.30	0.14
5.8	1.23	0.11	0.67	0.11
8	1.25	0.07	0.45	0.07
24	1.13	0.00	0.50	0.00
27	1.25	0.07	0.65	0.07
32	1.43	0.15	0.83	0.05

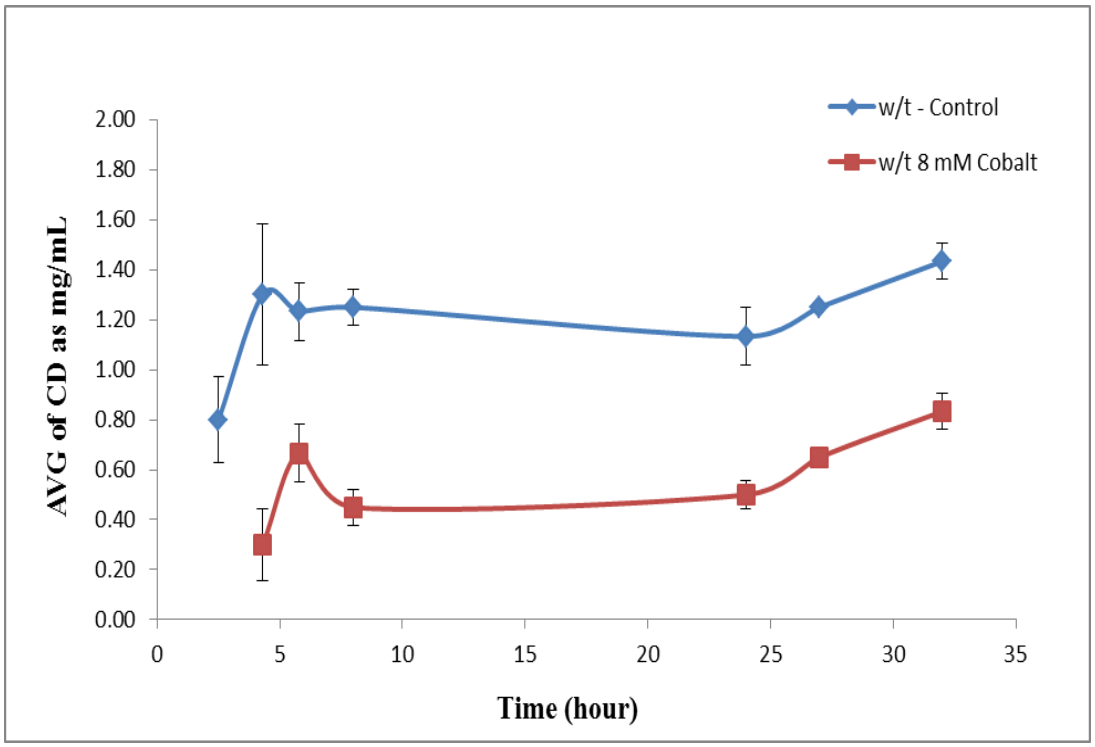

Figure 3.6 : Average cell dry weight as mg/mL graph of wild type cells in the absence and presence of 8 mM $CoCl_2$.

It was observed that wild type in non-stress conditions had more biomass than in stress conditions. The result of cell dry weight analysis showed that the results of growth curve analysis was parallel with the result of cell dry weight analysis.

3.5 Determination of Cross Resistance to Other Stress Types

Cross resistance of wild type *R. sphaeroides* to other stress types was investigated for 1 M NaCl, 5 % (v/v) of ethanol, 1 mM $CuSO_4$, 4 mM H_2O_2 and 100 mM H_3BO_3 stress factors by colony counting methodology. The figures showing the CFU values of wild type 24 h and 48 h results for different stress types are given in Figure 3.7, 3.8, 3.9, 3.10 and 3.11, respectively.

Table 3.5 : CFU results of stress-treated and non-treated wild type for 24 hours ($CFU_{1,2}$ imply to the two independent measurements of CFU; AVG refers to the arithmetic mean value of two independend CFU measurements. Std. Dev. indicates standard deviation of two measurements).

Stress Types	**AVG of Stress Samples as CFU/mL**	**AVG of Control Samples as CFU/mL**	**Percent Survival as CFU/mL**	**Std. Dev.**
Ethanol-24 hour	$25x10^5$	$42x10^6$	5.95	0.0040
NaCl-24 hour	$184x10^5$	$68.5x10^6$	26.86	0.0137
$CuSO_4$-24 hour	No Growing	$42x10^6$	0	----
H_2O_2-24 hour	$32.5x10^6$	$42x10^6$	77.38	0.0354
H_3BO_3-24 hour	No Growing	$63x10^6$	0	----

CFU values of wild type after 24 hours incubation for ethanol, NaCl, $CuSO_4$, H_2O_2 and H_3BO_3 stress factors, respectively are given in Table 3.5.

Table 3.6 : CFU results of stress-treated and non-treated wild type for 48 hours ($CFU_{1,2}$ imply to the two independent measurements of CFU; AVG refers to the arithmetic mean value of two independent CFU measurements. Std. Dev. indicates standard deviation of two measurements).

Stress Types	AVG of Stress Samples as CFU/mL	AVG of Control Samples as CFU/mL	Percent Survival as CFU/mL	Std. Dev.
Ethanol-48 hour	$25x10^5$	$63x10^6$	5.95	0.0001
NaCl-48 hour	$212.5x10^6$	$147x10^6$	144.55	0.0529
$CuSO_4$-48 hour	$35.5x10^5$	$63x10^6$	5.634920635	0.0014
H_2O_2-48 hour	$32x10^6$	$63x10^6$	50.79	0.0228
H_3BO_3-48 hour	$38.5x10^6$	$63x10^6$	61.11	0.0162

CFU values of wild type after 48 hours incubation for ethanol, NaCl, $CuSO_4$, H_2O_2 and H_3BO_3 stress factors, respectively are given in Table 3.6.

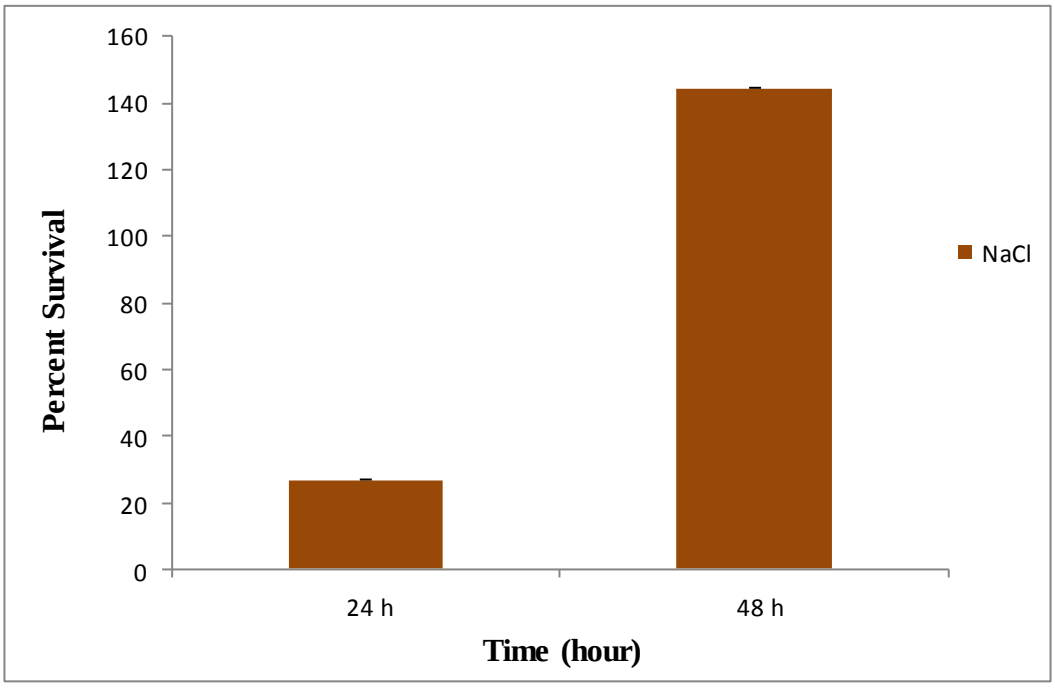

Figure 3.7 : Percent survival of wild type as CFU/mL for NaCl. Survival ratio for each stress factor was performed taking account of CFU/mL values.

According to 48 hour results of colony count methodology, *R. sphaeroides* R-26 showed high tolerance to 1 M NaCl. It was reported that *R. sphaeroides* 2.4.1 could survive about 0.4 M NaCl, but higher concentrations of NaCl prevent cell growth (Tsuzuki et al., 2011). These results pointed out that *R. sphaeroides* strain R-26 showed higher tolerance, when compared to other *R. sphaeroides* strains under 1M NaCl stress.

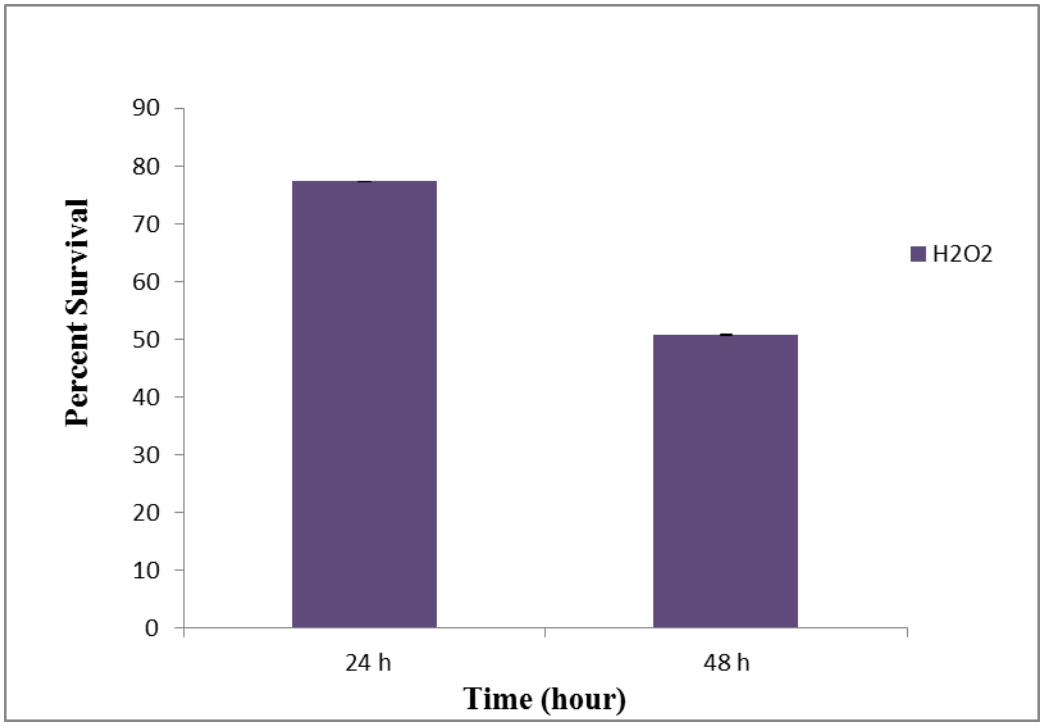

Figure 3.8 : Percent survival of wild type as CFU/mL for H_2O_2. Survival ratio for each stress factor was performed taking account of CFU/mL values.

To generate oxidative stress, 4 mM H_2O_2 was applied. With respect to the Figure 3.8 showing colony counting results for H_2O_2 stress factor, percent survival value decreased at 48th hour of incubation. Zellerand and Klug. explained that based on the results of zone inhibition test for *R. sphaeroides* 2.4.1 and *R. capsulatus* SB1003 that *R. sphaeroides* 2.4.1 did not show any inhibition zone at 12.5 mM of H_2O_2 concentration. Additionally, they have added to their explanation concerning H_2O_2 resistance that *R. sphaeroides* showed more tolerance than *R. capsulatus* which is related with *R. sphaeroides* (Zeller and Klug, 2004). Since the percent value after 48 hours of incubation is around 50 %, this shows that *R. sphaeroides* R-26 could survive higher concentrations than 4 mM H_2O_2.

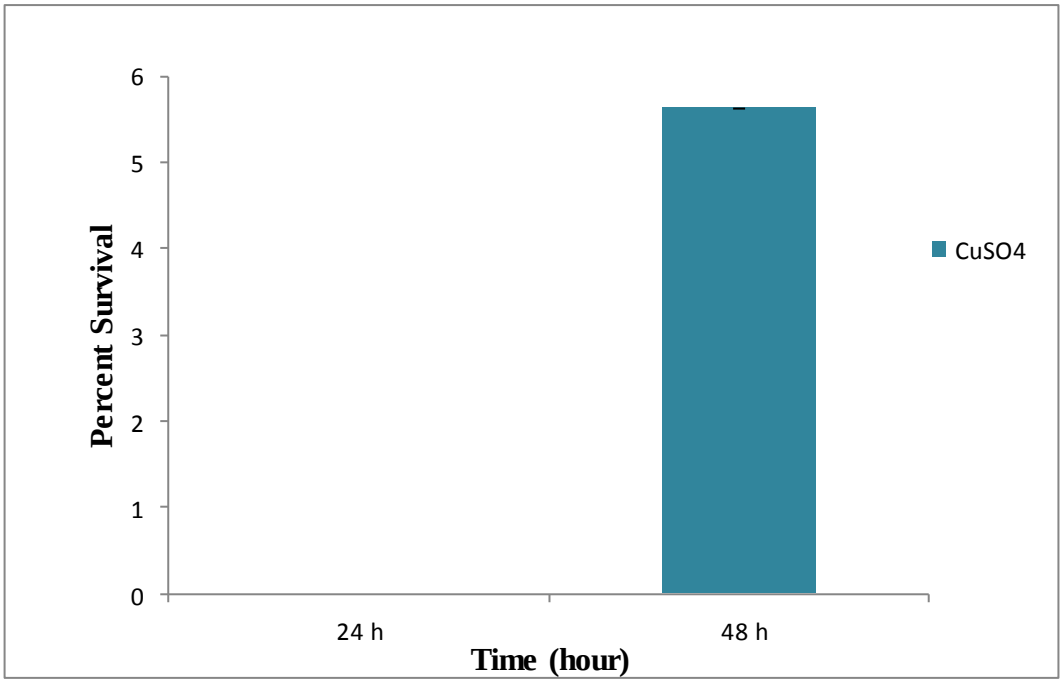

Figure 3.9 : Percent survival of wild type as CFU/mL for $CuSO_4$. Survival ratio for each stress factor was performed taking account of CFU/mL values.

Copper, which is an essential element for biological activities of living organisms, was applied at 1 mM $CuSO_4$ as a stress factor. Figure 3.9 shows the result of this assay for *R. sphaeroides.* It is known that *Saccharomyces cerevisiae* is relatively resistant to copper and able to grow up to 2 mM $CuSO_4$. However, *Candida albicans* is a more copper resistant organism and it grows up to 20 mM $CuSO_4$. Even, in rich medium *C. albicans* showed tolerance up to 50 mM $CuSO_4$ (Weissman et al., 2000).

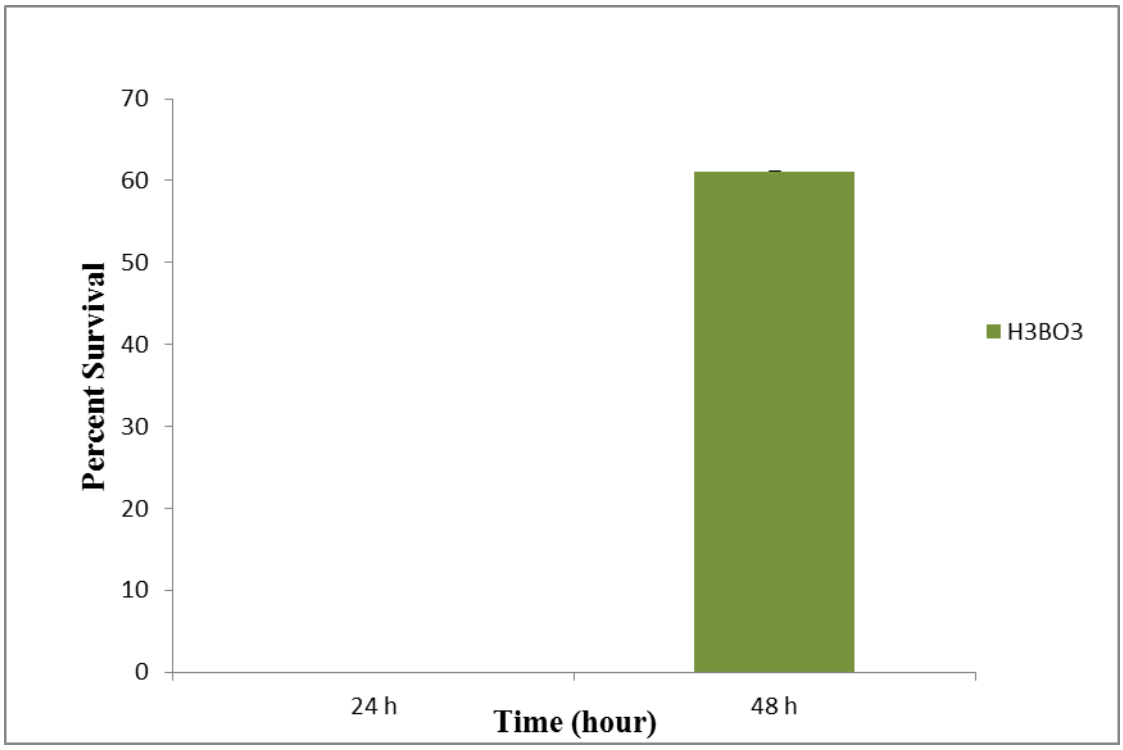

Figure 3.10 : Percent survival of wild type as CFU/mL for H_3BO_3. Survival ratio for each stress factor was performed taking account of CFU/mL values.

As for H_3BO_3, 100 mM concentration of H_3BO_3 was applied and the result is shown in the Figure 3.10. Hereof, Sen *et al.* impled that *Bacillus boronophilus* could survive up to about 180 mmol l^{-1} H_3BO_3. However, higher concentrations of H_3BO_3 prevented cell growth (Sen et al., 2011).

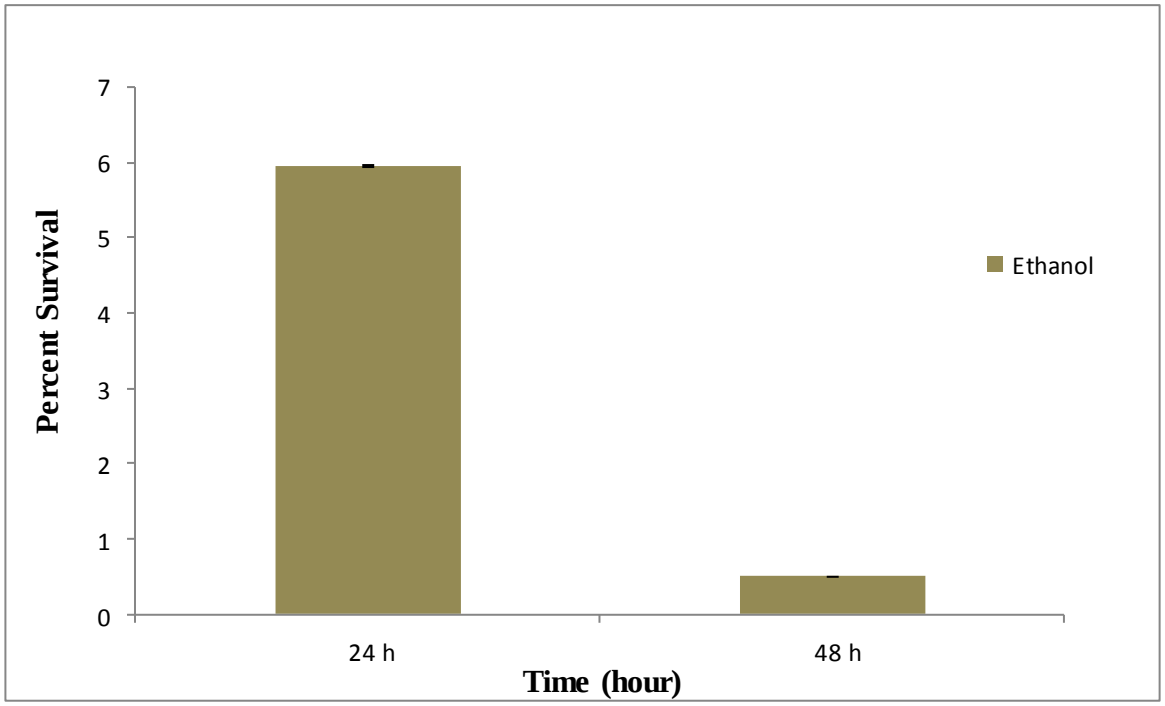

Figure 3.11 : Percent survival of wild type as CFU/mL for ethanol. Survival ratio for each stress factor was performed taking account of CFU/mL values.

Five % (v/v) of ethanol was applied as a stress factor and significantly low percent values were obtained. Kumar *et al.* showed in the result of alcohol tolerance test that *S. cerevisiae* could tolerate up to 15 % (v/v) of ethanol (Kumar et al., 2011).

The graphics showing the colony count results implies that growth of bacterial cells were observed under ethanol, NaCl, H_2O_2 stress factors after 24 hours of incubation. On the contrary, under $CuSO_4$ and H_3BO_3 stress conditions no growth was observed after 24 hours of incubation. In contrast to 24 hour results of $CuSO_4$ and H_3BO_3 stress factors,within 48 hours growth of *R. sphaeroides* occurred under these stress conditions.

Cross resistance of wild type *R. sphaeroides* was investigated for 1 mM $NiCl_2$, 10 mM $ZnCl_2$, 24 mM $CoCl_2$ stress factors performing the assays in triplicates by measuring OD_{535} as batch culturing.

The results of wild type OD_{535} for three experiment for 24 mM $CoCl_2$ after 24 h, 48 h and 72 h incubation are given in Table 3.7.

Table 3.7 : OD_{535} results of wild type for 24 mM $CoCl_2$ and its corresponding control condition for 24 h, 48 h and 72 h, respectively.

24 h	**AVG of Control OD_{535}**	**AVG of 24 mM $CoCl_2$ OD_{535}**	**Percent Survival**	**Std. Dev.**
First Experiment	3.47	0.12	4.64	0.015
Second Experiment	3.57	0.22		
Third Experiment	3.40	0.14		
48 h	**AVG of Control OD_{535}**	**AVG of 24 mM $CoCl_2$ OD_{535}**	**Percent Survival**	**Std. Dev.**
First Experiment	3.67	0.56	15.08	0.003
Second Experiment	3.32	0.5		
Third Experiment	3.44	0.51		
72 h	**AVG of Control OD_{535}**	**AVG of 24 mM $CoCl_2$ OD_{535}**	**Percent Survival**	**Std. Dev.**
First Experiment	3.15	0.8	19.89	0.199
Second Experiment	3.21	0.36		
Third Experiment	2.96	0.68		

The results of wild type OD_{535} for three experiment for 1 mM $NiCl_2$ after 24 h, 48 h and 72 h incubation are given in Table 3.8.

Table 3.8 : OD_{535} results of wild type for 1 mM $NiCl_2$ and its corresponding control condition for 24 h, 48 h and 72 h, respectively.

24 h	**AVG of Control OD_{535}**	**AVG of 1 mM $NiCl_2$ OD_{535}**	**Percent Survival**	**Std. Dev.**
First Experiment	3.47	0.09	2.69	0.001
Second Experiment	3.57	0.07		
Third Experiment	3.40	0.11		
48 h	**AVG of Control OD_{535}**	**AVG of 1 mM $NiCl_2$ OD_{535}**	**Percent Survival**	**Std. Dev.**
First Experiment	3.67	0.1	3.40	0.003
Second Experiment	3.32	0.13		
Third Experiment	3.44	0.11		
72 h	**AVG of Control OD_{535}**	**AVG of 1 mM $NiCl_2$ OD_{535}**	**Percent Survival**	**Std. Dev.**
First Experiment	3.15	0.13	4.39	0.001
Second Experiment	3.22	0.13		
Third Experiment	2.96	0.14		

The results of wild type OD_{535} for three experiment for 10 mM $ZnCl_2$ after 24 h, 48 h and 72 h incubation are given in Table 3.9.

Table 3.9 : OD_{535} results of wild type for 10 mM $ZnCl_2$ and its corresponding control condition for 24 h, 48 h and 72 h, respectively.

24 h	**AVG of Control OD_{535}**	**AVG of 10 mM $ZnCl_2$ OD_{535}**	**Percent Survival**	**Std. Dev.**
First Experiment	3.47	0.83	22.55	0.035
Second Experiment	3.57	0.66		
Third Experiment	3.40	0.92		
48 h	**AVG of Control OD_{535}**	**AVG of 10 mM $ZnCl_2$ OD_{535}**	**Percent Survival**	**Std. Dev**
First Experiment	3.67	0.75	17.81	0.582
Second Experiment	3.32	0.59		
Third Experiment	3.44	0.55		
72 h	**AVG of Control OD_{535}**	**AVG of 10 mM $ZnCl_2$ OD_{535}**	**Percent Survival**	**Std. Dev.**
First Experiment	3.15	0.8	21.75	0.22
Second Experiment	3.22	0.63		
Third Experiment	2.96	0.65		

The Figures 3.12, 3.13 and 3.14 were obtained by using the values given on the tables in Table 3.7, 3.8, 3.9, respectively.

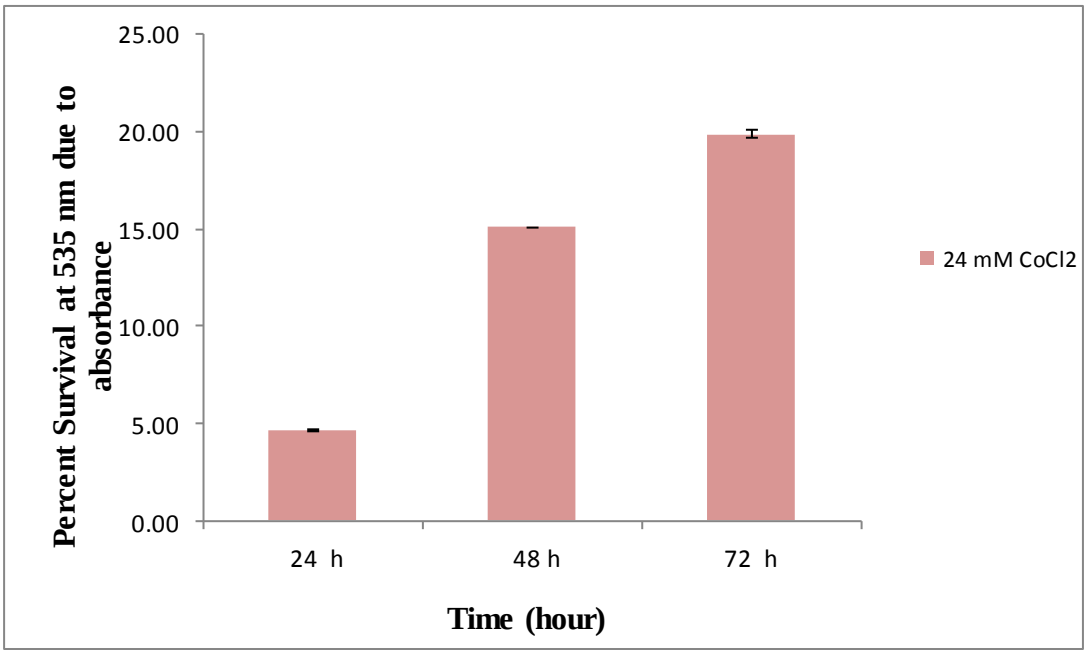

Figure 3.12 : Percent survival values of wild type for 24 mM $CoCl_2$ after 24 h, 48 h and 72 h of incubation.

R. sphaeroides was shown to have 20 % survival for 24 mM $CoCl_2$ after 72 hours of incubation. Rubikas *et al*. showed that minimal inhibitory concentration of $CoCl_2$ for *Escherichia coli* V38 was 0.5 mM. (Rubikas et al., 1997). However, at high concentrations of $CoCl_2$ (up to 24 mM $CoCl_2$) growth of *R. sphaeroides* cells were observed. Based on the results shown in Figure 3.12 and previous studies on other microorganisms, *R. sphaeroides* was shown to be more resistant to 24 mM $CoCl_2$ than most microorganisms.

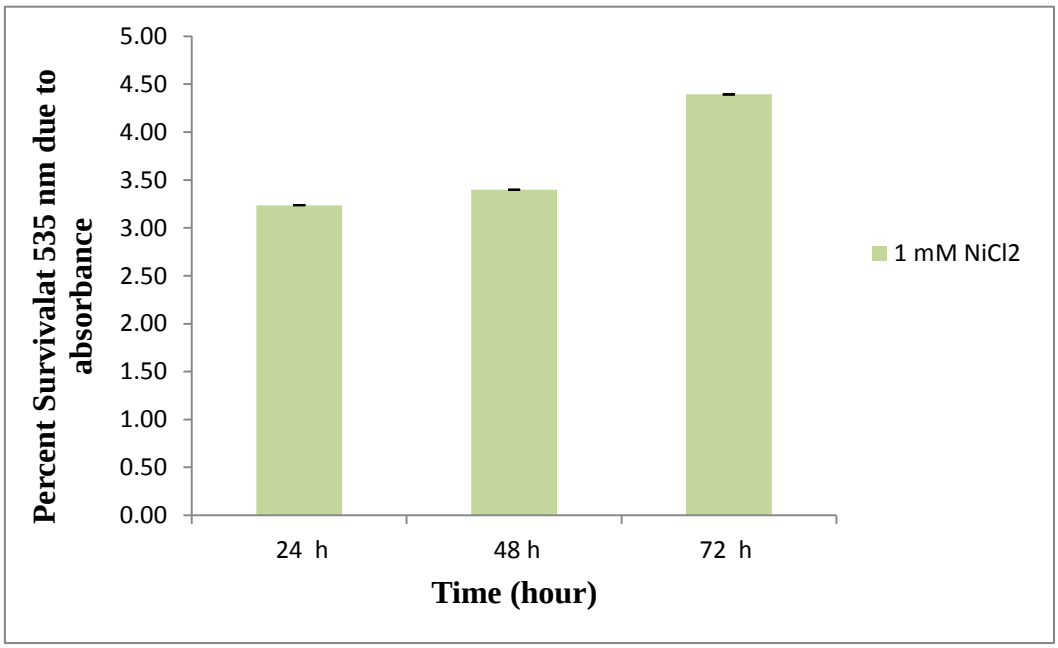

Figure 3.13 : Percent survival values of wild type for 1 mM $NiCl_2$ after 24 h, 48 h and 72 h of incubation.

R. sphaeroides had 4.5 % survival for 1 mM $NiCl_2$ after 72 hours incubation. Rubikas *et al*. showed that *Escherichia coli* V38 is resistant to 4 mM $NiCl_2$. Moreover, cell growth at higher concentrations of $NiCl_2$ (up to 6 mM $NiCl_2$) was determined by Rubikas *et al*. They also determined that 6.5 mM $NiCl_2$ prevent the growth of *E. coli* V38 cells (Rubikas et al,). Herein 1 mM $NiCl_2$ was applied. Higher concentrations of $NiCl_2$ should be applied to determine whether *R. sphaeroides* R 26 will be able to survive or not for future studies.

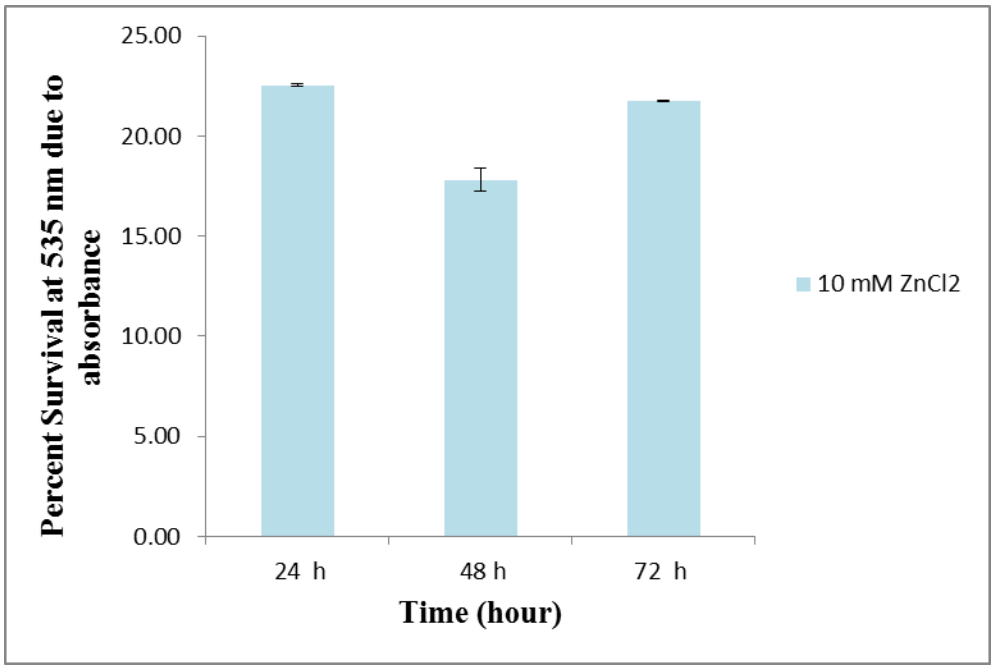

Figure 3.14 : Percent survival values of wild type for 10 mM $ZnCl_2$ after 24 h, 48 h and 72 h of incubation.

As shown in the Figure 3.14 *R. sphaeroides* had approximately 22 % survival after 72 hours of incubation at 10 mM $ZnCl_2$ stresss conditions. Panwichian *et al* explained that the minimal inhibition concentration of $ZnCl_2$ for *R. sphaeroides* KMS24 was 1.5 mM (Panwichian et al, 2011). Additionally, experiments of Majzlik *et al.* showed that minimal inhibition concentration of $ZnCl_2$ for *Streptomyces flavovirens* ON3 was 1.8 mM and 0.6 mM for solid and liquid conditions, respectively (Majzlik et al., 2011). The result presented in this study showed that the wild type *R. sphaeroides* R-26 was relatively more resistant to $ZnCl_2$ than the other microorganisms mentioned above.

4. CONCLUSIONS

In this study wild type *R. sphaeroides* strain R-26 was used for physiological analysis. In this context, growth curve analysis both in the absence and presence of stress and stress tolerance tests to different heavy metals and other stress factors were performed. Stress resistance determination against $CoCl_2$ was performed first by MPN method, spotting assay and monitoring OD_{535} values. Analysis of 1 M NaCl, 5 % (v/v) of ethanol, 1 mM $CuSO_4$, 4 mM H_2O_2 and 100 mM H_3BO_3 stress factors were performed by colony counting method. However, stress analysis of 1 mM $NiCl_2$, 10 mM $ZnCl_2$, 24 mM $CoCl_2$ stress types was performed monitoring OD_{535} values.

According to the MPN analysis of wild type, wild type could grow better at 5 mM $CoCl_2$ containing medium compared to that 8 mM $CoCl_2$ for 72^{nd} hour incubation. However, it was observed that percent survival values of bacterial cells for each concentration of $CoCl_2$ was so low. It indicates that the stress level applied was too high to determine $CoCl_2$ effect on the growth of *R. sphaeroides* cells.

In spotting assay, the presence of cobalt affected the growth of bacterial cells negatively. The increasing $CoCl_2$ amounts inhibited the bacterial growth. Moreover, at increasing concentrations of $CoCl_2$ colour change of bacterial cells was observed on plates. According to the observations in the result of colour change, the colour of bacterial cells on plates that containing only M27 were red-purple just as its natural colour. At low concentrations of $CoCl_2$ the colour of bacterial cells were white. It may show that the presence of stress factor inhibited the production of pigment molecules. However, on the plate that contained high concentrations of $CoCl_2$ such as 10, 20 and 25 mM $CoCl_2$ concentrations, the colour of bacterial cells were pink, the colour that observed was like the colour of $CoCl_2$. It could be suggested that *Rhodobacter sphaeroides* strain R-26 would keep $CoCl_2$ inside the cells. Atomic absorption spectrophotometry can be used in order to demonstrate whether *R. sphaeroides* could keep $CoCl_2$ inside the cells.

Monitoring optical densities of bacterial cells at proper OD value is an alternative method to determine the viability of cells under stress conditions. 1mM $NiCl_2$, 10 mM $ZnCl_2$, 8 mM $CoCl_2$ and 24 mM $CoCl_2$ stress types were analyzed measuring optical densities at 535 nm of bacterial culture. Among those heavy metals, bacterial cells showed more tolerance to 10 mM $ZnCl_2$ and 8-24 mM $CoCl_2$ than most microorganisms based on literature data. Wild type bacterial culture showed low tolerance to 1 mM $NiCl_2$ than other microorganisms.

Another strategy to determine cross-resistance levels of wild type was colony counting method. With this method, 1 M NaCl, 5 % (v/v) of ethanol, 1 mM $CuSO_4$, 4 mM H_2O_2 and 100 mM H_3BO_3 stress factors were analyzed. With respect to results after 24 hours incubation, bacterial cells could only grow on the plates that contained H_2O_2, NaCl and ethanol. However, after 48 hours incubation, bacterial cells could grow on the plates containing all types of stress factors. *R. sphaeroides* R-26 was shown to be resistant to 1 M NaCl. Because, it was determined that *R. sphaeroides* 2.4.1 which is an another strain of *R. sphaeroides*, could survive 0.4 M NaCl. Additionaly, it was reported that no growth of bacterial cells occurred above this concentration.

Growth curve analysis was performed in the absence and presence of 8 mM $CoCl_2$. The result of growth curve analysis together with cell dry weight analysis and maximum specific growth rate measurement showed that the presence of stress retarded the *R. sphaeroides* to reach its logarithmic phase of growth.

As part of this thesis study, general physiological investigation of wild type *R. sphaeroides* was performed. It provided us a foreknowledge concerning its general stress tolerance. With this information, firstly, atomic absorption spectrophotometry studies should be applied to determine resistance type of *R. sphaeroides* to $CoCl_2$ and to other heavy metals. Then, molecular and proteomic analysis should be applied for both control and stress conditions and difference between these two conditons should be evaluated. After that, to obtain resistant microorganism to stress factor of interest instead of using classical recombinant DNA technology methods, evolutionary engineering strategy should be applied since evolutionary engineering methodology is advantageous for improving the stress tolerance of microorganisms. Finally, to obtain further information, molecular analyses of *R. sphaeroides* mutant(s) obtained by evolutionary engineering should be performed for further studies.

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APPENDICES

APPENDIX A : MPN Score Table for Five Tubes

APPENDIX A

Table A.1 : MPN Score Table for Five Tubes (Url-2).

Number of Positive Tubes in			MPN in the inoculum of the middle set of tubes
First set	**Middle set**	**Last set**	
0	0	0	‹0.01
0	0	1	0.02
0	1	0	0.02
0	2	0	0.04
1	0	0	0.02
1	0	1	0.04
1	1	0	0.04
1	1	1	0.06
1	2	0	0.06
2	0	0	0.05
2	0	1	0.07
2	1	0	0.07
2	1	1	0.09
2	2	0	0.09
2	3	0	0.12
3	0	0	0.08
3	0	1	0.11
3	1	0	0.11
3	1	1	0.14
3	2	0	0.14
3	2	1	0.17
4	0	0	0.13
4	0	1	0.17
4	1	0	0.17
4	1	1	0.21
4	1	2	0.26
4	2	0	0.22
4	2	1	0.26
4	3	0	0.27
4	3	1	0.33
4	4	0	0.34
5	0	0	0.23
5	0	1	0.31
5	0	2	0.43
5	1	0	0.33
5	1	1	0.46
5	1	2	0.63
5	2	0	0.49
5	2	1	0.7
5	2	2	0.94
5	3	0	0.79
5	3	1	1.1
5	3	2	1.4
5	3	3	1.8
5	4	0	1.3
5	4	1	1.7
5	4	2	2.2
5	4	3	2.8
5	4	4	3.5
5	5	0	2.4
5	5	1	3.5
5	5	2	5.4
5	5	3	9.2
5	5	4	16
5	5	5	›24

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