FACTORS AFFECTING COILING AND UNCOILING OF *Spirulina platensis* C1

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Abstract

Spirulina platensis C1 is a cyanobacterium that has helical trichome. The morphological transformation to straight trichome of this strain has been observed. To determine the environmental factors which enhance this transformation, effect of nutrient concentration, high temperature and high light intensity were studied. The results showed that these factors cannot enhance the morphological transformation to straight trichome under the study conditions. However, it was found that the factors mentioned have an effect on the helicity of cell. In the study of UV irradiation, the condition cannot induce the mutation thus the straight form was not found. Furthermore, the straight forms were found when the cultures were grown under prolonged static condition at room temperature. It could be proposed that the morphological change from helical to straight trichome is spontaneous mutation.

No difference in growth rate, biochemical compositions and photosynthetic characteristic between the helix and the straight trichomes was found. The total

protein extracted from both forms was separated by Two-dimensional gel electrophoresis (2D-electrophoresis). The protein profiles of both forms were found to be similar. One hundred forty eight and one hundred fifty protein spots were detected in helix and straight form, respectively. Sixty seven protein spots have different spot density and 2 unique protein spots were detected only in the straight form. When the spot density of the 67 proteins were compared, it was found that 15 spots and 52 spots of the 67 spots in the helical form have higher and lower spot density, respectively, than that of the straight form. Therefore, the 69 protein spots probably related to the morphological transformation of trichome.

Keywords : *Spirulina platensis* / Two dimensional gel electrophoresis

หัวข้อวิทยานิพนธ์	การศึกษาปัจจัยที่มีผลต่อกระบวนการ coiling และ uncoiling ของ *Spirulina platensis* C1
หน่วยกิตของวิทยานิพนธ์	12 หน่วย
โดย	นางสาว ศรีธนา ธรรมาธร
อาจารย์ที่ปรึกษา	รศ. บุษยา บุนนาค
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สายวิชา	เทคโนโลยีชีวภาพ
ปีการศึกษา	2544

บทคัดย่อ

Spirulina platensis C1 เป็นไซยาโนแบคทีเรียสายพันธุ์ที่มีรูปร่างเกลียวและพบว่ามีการเปลี่ยนรูปร่างเป็นเส้นตรง ซึ่งเป็นการเปลี่ยนแปลงแบบถาวร (irreversible) งานวิจัยนี้ได้ทำการศึกษาผลของความเข้มข้นสารอาหาร อุณหภูมิสูง และความเข้มแสงสูงที่มีต่อรูปร่างเกลียว จากการศึกษาไม่พบว่าปัจจัยดังกล่าวมีส่วนช่วยให้เกิดการเปลี่ยนแปลงรูปร่างเป็นเส้นตรง แต่พบว่ามีผลต่อลักษณะเกลียวของเซลล์ สำหรับผลของ UV ซึ่งไม่พบเซลล์รูปร่างตรง เนื่องจากภายใต้สภาวะที่ทำการศึกษาไม่ใช่สภาวะที่จะกระตุ้นให้เกิดการกลายพันธุ์ได้ อย่างไรก็ตามเมื่อตั้งเซลล์ไว้เป็นเวลานานที่อุณหภูมิห้อง พบเซลล์รูปร่างตรงเกิดขึ้น จึงคาดว่าการเปลี่ยนแปลงของเซลล์จากรูปร่างเกลียวเป็นรูปร่างตรงนั้น เป็นการกลายพันธุ์ที่เกิดขึ้นเองในธรรมชาติ (Spontaneous mutation)

จากการเปรียบเทียบอัตราการเจริญ องค์ประกอบทางชีวเคมี รวมถึงคุณลักษณะการสังเคราะห์แสงของเซลล์รูปร่างเกลียวและรูปร่างตรง ไม่พบว่ามีความแตกต่างกัน แต่เมื่อทำการเปรียบเทียบโปรตีนที่สกัดได้จากเซลล์ทั้งสองรูปร่างโดยวิธี Two dimensional electrophoresis พบว่าโปรตีนในเซลล์ของทั้ง 2 รูปร่างมีลักษณะคล้ายกันโดยพบโปรตีนจำนวน 148 จุดและ150 จุด ในเซลล์รูปร่างเกลียวและรูปร่างตรง ตามลำดับ อย่างไรก็ตามพบว่าโปรตีนที่แตกต่างกันมี 69 จุดโดยที่โปรตีน 67 จุดเป็นโปรตีนที่มีความเข้มของจุดแตกต่างกันระหว่าง 2 รูปร่างและโปรตีนจำเพาะ 2 จุด ซึ่งพบได้เฉพาะในเซลล์รูปร่างตรงเท่านั้น จากการเปรียบเทียบความเข้มจุดของโปรตีน พบว่าโปรตีน 15 จุดในเซลล์รูปร่างเกลียวมีความเข้มของจุดสูงกว่า และ โปรตีน 52 จุดมีความเข้มของจุดต่ำกว่าโปรตีนเดียวกันซึ่งพบได้ในเซลล์รูปร่างตรง ดังนั้นจึงคาดว่าโปรตีนทั้ง 69 จุดนี้อาจมีส่วนเกี่ยวข้องกับการเปลี่ยนแปลงรูปร่างของเซลล์

คำสำคัญ (Keywords) : *Spirulina platensis* / Two dimensional electrophoresis

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Page

List of Symbols

β	beta
γ	gamma
ω	omega
g	gram
mg	milligram
µg	microgram
m	meter
cm	centimeter
mm	millimeter
µm	micrometer
nm	nanameter
l	litre
ml	millilitre
µl	microlitre
µmol	micromole
µE	microeinstein
^{o}C	degree centigrade
m	specific growth rate
h	hour
min	minute
s	second
w/v	weight per volume

w/w	weight per weight
kDa	kilodalton
Chl	chlorophyll
GLA	gamma linolenic acid
rpm	round per minute
ppm	part per million
O.D.	optical density
Ala	alanine
Glu	glutamic acid
Orn	ornithine
Gly	glycine
DAP	diaminopimelic acid
Mw	molecular weight
IEF	isoelectric focusing
IPG	immobiline pH gradient
pI	isoelectric point
DTT	dithiothreitol
SDS	sodium dodecyl sulfate
PMSF	phenyl methyl sulphonyl fluoride
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethananesulfonic acid
GlcNAc	N-acetyl glucosamine
MuNAc	N-acetyl muramic acid
PVDF	Polyvinylidene difluoride

TEMED	N,N,N’,N’-tetramethylethylene diamine
Tris	Tris(hydroxymethyl)-aminoethane

Chapter 1

Introduction

1.1 Background

Spirulina (*Arthrospira*) is a filamentous cyanobacterium which consisted of cylindrical cells arranged in an unbranched trichome. The trichome forms an open left – handed helix along the entire length and motiles by gliding movement [59]. *Spirulina* filament has 6 to 16 μm wide, the helix pitch between 12 to 72 μm long, and the helix diameter range from 30 to 70 μm. *Spirulina* has been isolated from a wide range of aquatic environments. It is a dominant species in alkaline, saline water with pH up to 11.

Spirulina has been commercially cultivated for production of food supplement in human diets and animal feed for fish and shrimp. *Spirulina* is a rich source of protein (50 – 70% of dry weight). It has potential as a source of valuable chemical compounds such as β-carotene and phycocyanin which are used as colorant in food and cosmetic, essential fatty acids, γ-linolenic acid (GLA, 18:3 ω6) that has cholesterol lowering ability. Due to the absence of cellulose cell wall, no chemical or physical processing steps is necessary to break *Spirulina* cells. Thus, the steps of processing the harvested cells can be decreased comparing to other green algae. Consequently, *Spirulina* is cultivated in several countries as shown in Table 1.1.

Table 1.1 Commercial production of *Spirulina* in 1995 [65].

Company	Country	Total pond area (m^2)	Productivity (tonnes per year)
Ballarpur Industries Ltd.	India	54,000	25
Cyanotech Corporation	USA	100,000	250
Earthrise Farms	USA	150,000	360
Myanma Microalga Biotechnology	Myanmar	130,000	32
Siam Algae Co., Ltd.	Thailand	44,000	125
Wuhan Microalgal Biotechnology Company	China	20.000	25
Nan Pao Resin Chemical Co.,Ltd.	Taiwan	50,000	70

Spirulina (*Arthrospira*) are recognizable by the main morphological feature of the genus: the arrangement of the multicellular cylindrical trichomes in an open-hand helix along the entire length. However, there are many reports of the morphological change in *Spirulina*. Cells in liquid medium have helix shape and change to spiral shape when transferred to solid medium. The reversible transition from helix to spiral shape was more rapid than transition from spiral to helix shape[63]. Furthermore, it was found that environmental factors affected helicity of trichome. These environmental factors are light intensity, nutrient concentration, growth temperature and salinity of medium. It was reported that light intensity and nutrient concentration initiated three morphological variants of *S. fusiformis*; trichomes forming loose helices, trichomes having tight coiled and trichome

increased coiling to very tight variant. These forms can reverse to another form under study conditions; high light intensity and high nutrient concentration which caused the transformation from loose helices to the tighter coil. While the high light intensity and low nutrient concentration led to forming of very tight trichome [24]. Another factor is growth temperature, the increasing of temperature gave rise to more tightly coiled trichome of *Spirulina* [62]. In addition, cellular morphology as also found to be affected by salinity of medium and component anions. *Spirulina* cells have loosely coiled when grown in high salinity. At the same salinity level, cultures grown in sulfate rich medium have more loose helices than cells grown in bicarbonate and chloride rich medium [26]. It is more likely that the degree of coiling (helicity) is the reversible transformation.

Moreover, the straight trichomes were frequently found in laboratory and outdoor cultivation. This form does not revert back to the helical form (irreversible transformation). Lewin [31] found that the uncoiled variant arose in *S. platensis* culture which grown under constant light (200 lux) and agitation on a shaker at 32 ^{o}C. Straight trichomes have been also reported in culture of *S. fusiformis*. Although, a small number of this straight trichomes was found in laboratory condition, over 50% was found after transfer to open-air condition [23]. Besides, the straight trichomes were appeared in the commercial production of *Spirulina* especially appeared in non-aerated or less aerated pond (personal communication).

The helical shape of trichome is a unique characteristic of the genus *Spirulina*. Morphological features such as cell size and shape have generally used to distinguish *Spirulina* from another genus in the Oscillatoriaceae. Thus the occurrence of straight trichome in *Spirulina* culture may cause a misunderstanding of this

species with the species of *Oscillatoria*. In addition, the present of straight variants in the culture might raise a question in purity of the product.

S. platensis strain C1, a laboratory strain which has helical shape. This strain was found to have cellular morphology reverted from helical to straight form as it was found in the commercial production strain. Therefore, it was used as model to examine the response to various environmental conditions that may enhance or lead to the formation of straight trichome. These informations may be used for preventation of morphological change of *Spirulina*. Comparison of the two morphones in growth, pigment contents, biochemical compounds, photosynthetic characteristic, protein profiles of the two morphological forms may help us identify the gene involved in the morphological change and to give initial information to better understand the process in the transformation of the morphology.

1.2 Objectives

The objectives are to study the effect of environmental factors on cellular morphology of *S. platensis* C1, and comparison in growth, biochemical compounds, photosynthetic characteristic and protein profiles of two morphological forms.

1.3 Scopes

The pure helical form of *S. platensis* C1 was used for studying the effect of nutrient concentrations (Zarrouk's medium 1x, 2x, 0.5x), high temperature (40 ^{o}C), high light intensity (300 $\mu E \bullet m^{-2} \bullet s^{-1}$), UV radiation and combined factors on cellular morphology.

Characterization of helical and straight forms, both forms were analyzed for growth, biochemical compositions, photosynthetic characteristics and protein profiles from 2D-electrophoresis.

1.4 Expected result

The results obtained may be used as a guideline for prevention of morphological change of *Spirulina*. Knowledge of protein profiles may help us to study the gene(s) involved in the morphological change.

Chapter 2

Literature review

2.1 *Spirulina*

Spirulina is a cyanophyta that isolated from a wide range of environments such as soil, seawater, marshes, brackish water and fresh water. It can be found dominating in the saline water with pH up to 11, an unfavorable environment for most algal species. *Spirulina* is one of the most commonly found in many alkaline lakes in Africa and America [31].

2.1.1 Morphology

Spirulina (*Arthrospira)* is a multicellular filamentous cyanobacterium. It was composed of cylindrical cells arranged in unbranced filament, called trichome. A trichome forms an opened left-hand helix along the entire length and motiles by gliding movement. Under the light microscopy, it appears as a blue-green non-heterocyst filament. Some species have gas vacuole in their cells. The photosynthetic pigments; chlorophyll a, phycocyanin, allophycocyanin and carotenoid are located in thylakoid membrane.

The helicity of trichomes are varied with the species (Fig. 2.1). The helix pitch varies from 12 to 72 μm, the helix diameter between 30 to 70 μm and width of the trichome range from 6 to 16 μm [59]. Desikachary and Jeeji Bai [10] divided twenty four cultivated strains and natural samples of *Arthrospira* into four groups. The criteria for separation of species are; (1) helicity of trichomes and

pattern of trichomes coiling (2) attenuation of trichome ends and (3) end cell morphology (Fig. 2.2).

However, the straight trichome of *Spirulina* were often found in laboratory and outdoor cultivation [31, 23]. Most member of Oscillatoriaceae have unbranched, cylindrical filaments. Thus, helicity of trichome may not be used as a taxonomic criterion for separation of *Spirulina* from another genus in the Oscillatoriaceae.

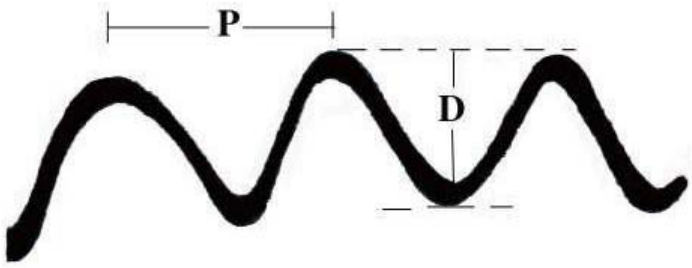


Fig. 2.1 The helical trichome of *Spirulina platensis*; where p represents the pitch and d represents the outer diameter of the helix (adapted from Van Eykelenburg, 1979 [62]).

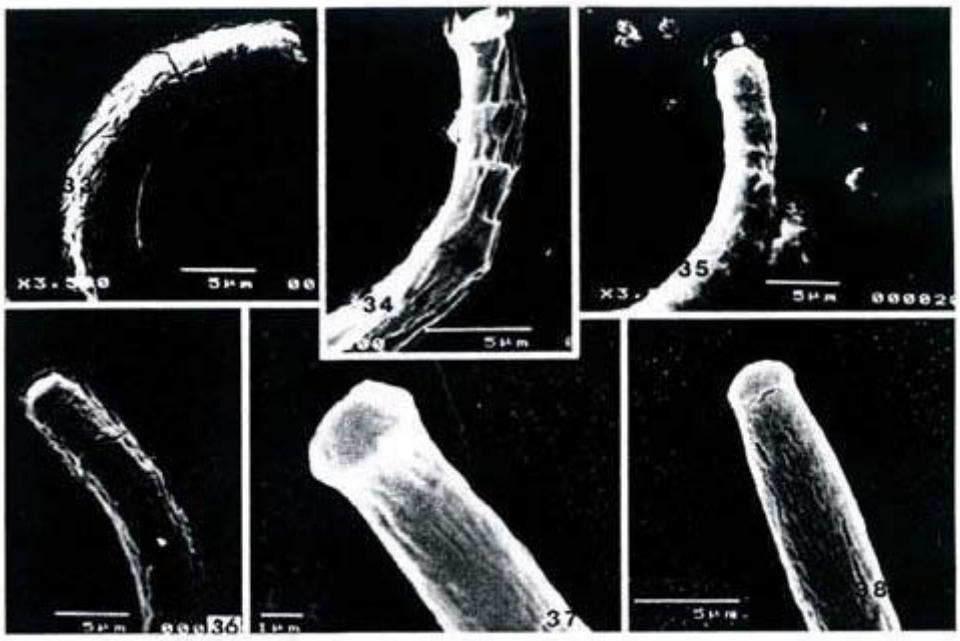


Fig. 2.2 Scanning electron micrograph of end cell. Top row; (left) rounded end cell, (middle) rounded end cell, (right) discoid calyptr on rounded end cell. Lower row; (left) rounded end cell, (middle) capitate end cell, (right) capitate end cell [10].

2.1.2 Life cycle

Spirulina are reproduced by fragmentation. A new filament is initiated when a part of a filament breaks off. The mature trichome is broken to form a specialized cell, necridia (Fig. 2.3). Then the necridia undergoes detached with formation of short and motile filaments called hormogonia. These hormogonia break off from the parent filament at the transverse rings of pore through the peptidoglycan layer of cell wall [18]. The hormogonia cells undergo enlargement and maturation.

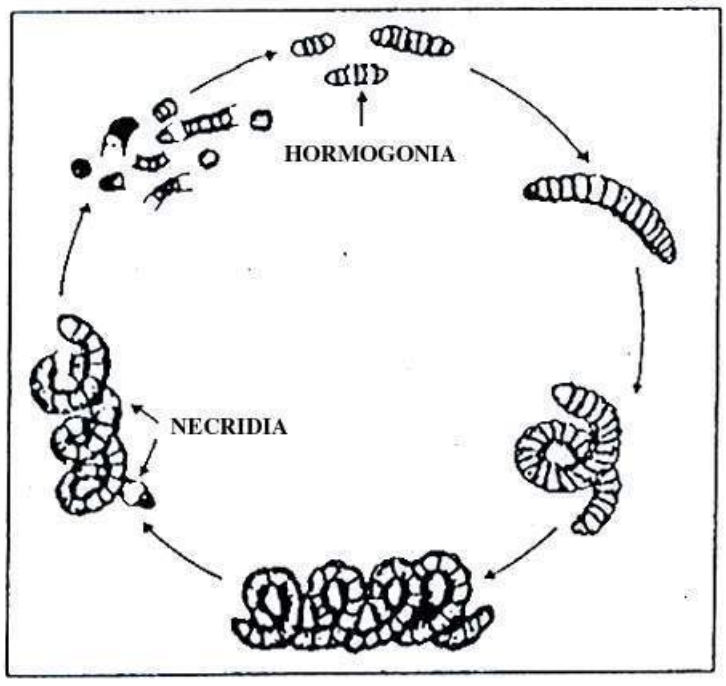


Fig.2.3 Life cycle of *Spirulina* [48].

2.1.3 Cell wall

The wall of Cyanophyta processes many similarities with gram-negative bacteria [57]. It is located between the cytoplasmic membrane and the outer membrane. The cell wall of *Spirulina* consists of four layers; L-I to L-IV (Fig. 2.4). Van Eykenlenburg [61] shows that the L-I and L-III layers are electron transparent and contained fibrilar material whereas the L-II and L-IV layers are electron-dense. All layers are 10 to 15 nm thick and therefore the whole wall ranges in thickness up to about 60 nm. The septum wall consists of three layers; the L-II layer sandwiched between two of the L-I layer. In the L-II layer, it contains peptidoglycan layer which maintains the structure and shape of the cell.

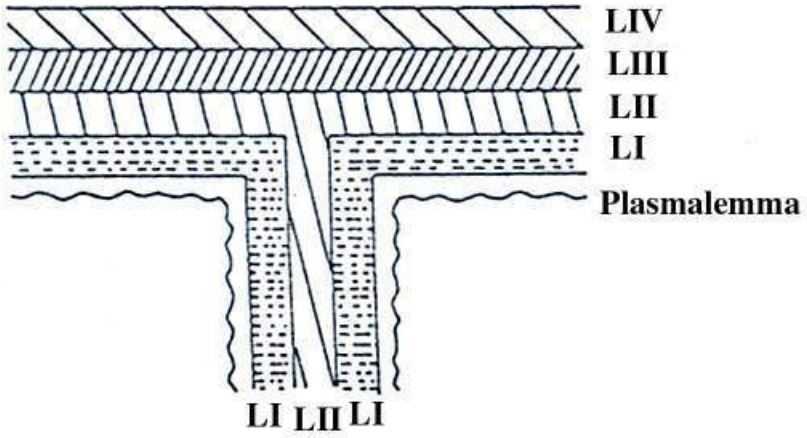


Fig. 2.4 Schematic diagram of the cell wall and septum of *S. platensis*. The cell wall is divided into four layers and the septum is divided into three layers [61].

2.1.3.1 Chemical compositions and structure of peptidoglycan

Peptidoglycan (murein) is a complex polymeric substance that composed of amino sugars and amino acids. The glycan strand is made of the amino sugars N-acetyl glucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) which are linked together by β-1,4 glycosidic bond. The pentapeptide chain is connected to the carboxyl group of MurNAc (Fig.2.5). The chemistry of the glycan strands show only few variations among different bacteria, such as O or N acetylation, whereas that of the peptides vary a lot [19]. The cross-linking peptide bond is formed between the amino group of meso-diaminopimelic acid in one chain and the terminal alanine of another chain (Fig. 2.6).

Investigation of the peptidoglycan chemical composition has been made possible by two analysis methods. First, murein was completely degraded with specific enzymes, such as muramidase that degraded the glycan strands or amidase that cleaved off the peptides from the glycans, yielding the murein degradation product called muropeptides. The structures of all identified monomeric muropeptides were shown in Fig. 2.7A. The 1,6-anhydromuramic acid is a natural tag at one end of the glycans. It was used to calculated the average length of all glycan strands in the murein by determining the ratio of all muropeptides to all 1,6-anhydromuropeptides [19]. The murein was cross-linked not only by dimeric peptide bridges connecting two glycan strands but also by trimeric peptide bridges connecting three glycan strands (Fig. 2.7B). Then these muropeptides were analyzed by high performance liquid chromatography (HPLC) or mass spectrometry (MS) techniques.

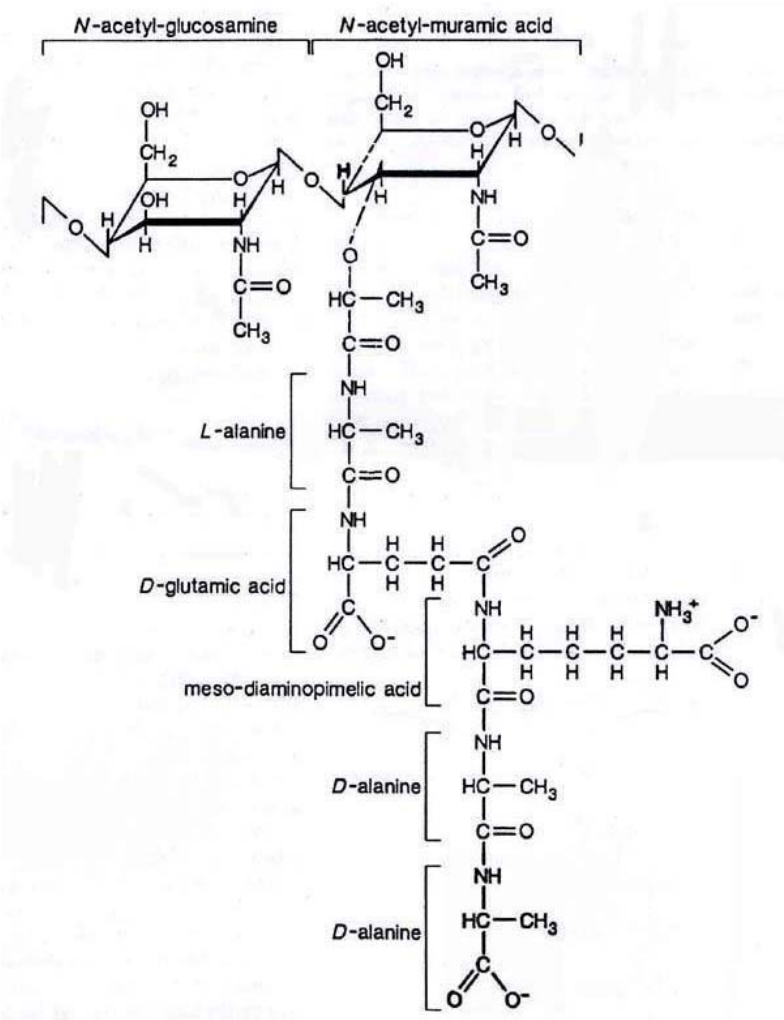


Fig. 2.5 The basic unit of peptidoglycan in the bacterial cell wall [27].

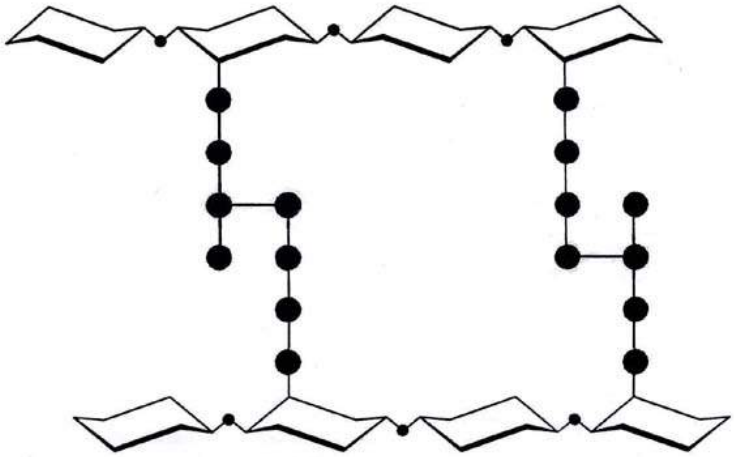


Fig. 2.6 Cross-links between peptidoglycan units. The sugar units are joined end-to-end (β-1,4 glycosidic bond) to form long, straight glycan chains. The peptide bond creates cross-links when the amino group of a meso-diaminopimilic acid in one chain replaces the terminal alanine of another chain [27].

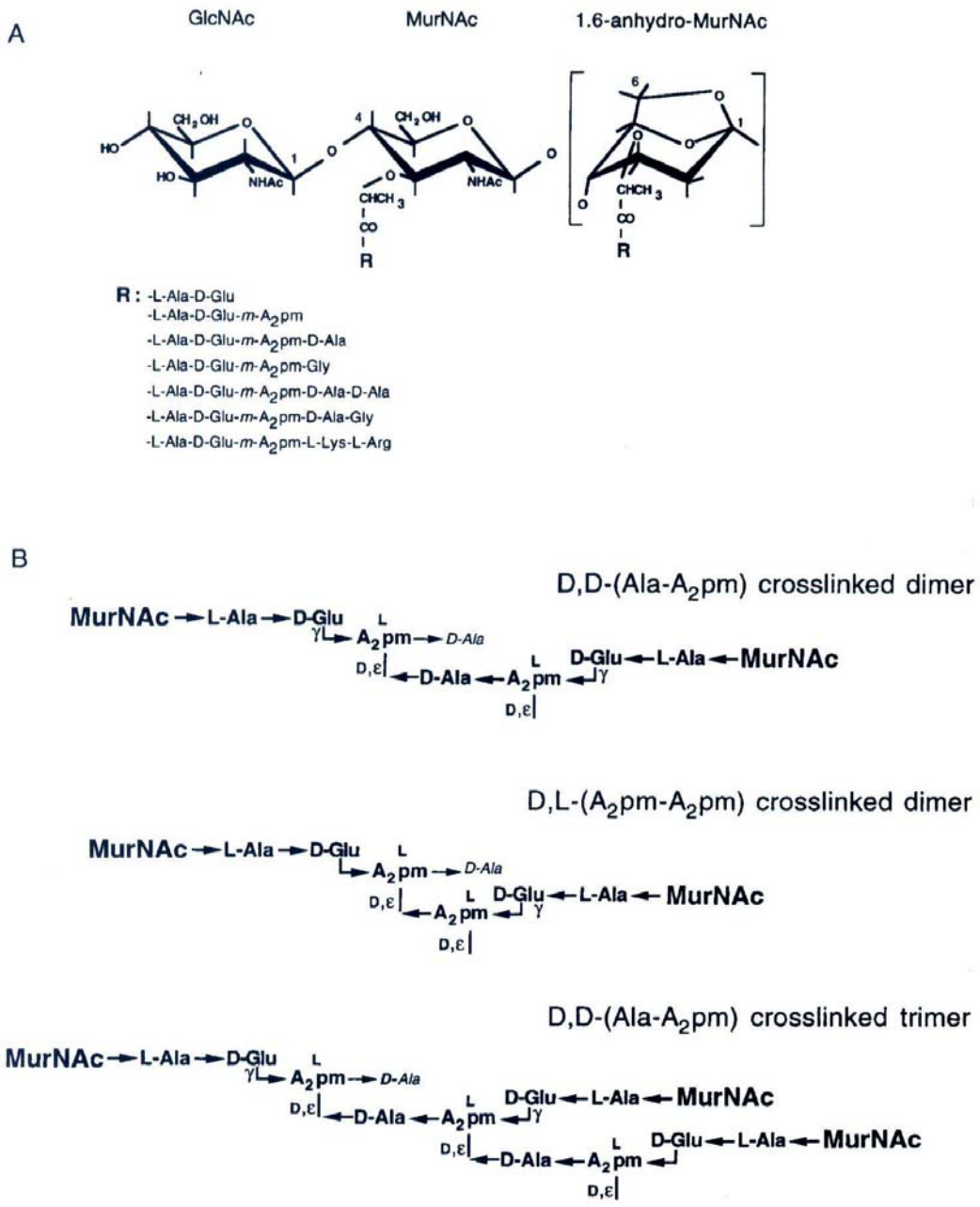


Fig. 2.7 Muropeptide structures. (A) Monomeric muropeptide structure. The different peptide moieties (R) that can substitute the lactyl group of MurNAc are listed. (B) Chemistry of the major cross-linked dimer and trimers, A_2pm, diaminopimelic acid [19].

Quintela *et al*. [44] demonstrated the muropeptide compositions of murein from ten species of Gram-negative bacteria; *Aeromonas sp*., *Acinetobacter acetoaceticus*, *Agrobacterium tumefaciens*, *Enterobacter cloacae*, *Proteus morganii*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Vibrio parahaemolyticus*, *Yersinia enterocolitica* and *Escherichia coli*. The muropeptides were determined by HPLC technique. Most species have mureins which build up from N-acetylglucosamine-(β-1,4)-N-acetyl-muramyl-L-Ala-D-Glu-mA2pm-D-Ala-D-Ala monomeric subunits. The contribution of LD-A2pm-A2pm cross-linked muropeptides was extremely variables from 1 to 45% of cross-linked muropeptides. The results indicated that murein of Gram-negative bacteria was homogeneous in composition down to the muropeptide level, however, structurally heterogeneous.

Quintela *et al*. [45] studied the murein properties of the extreme thermophilic eubacterium *Thermus thermophilus*. It has a flexible rod shape that is negative for the Gram's reaction. Murein properties of this thermophile is the first described. Compositions of murein component were compared with the murein of *E. coli*. It was found that the murein from *T. thermophilus* was more complex than that from *E. coli*. The basic monomeric subunit consisted of N-acetylglucosamine-N-acetylmuramic acid-L-Ala-D-Glu-L-Orn-D-Ala-D-Ala. Diaminopimelic acid which is typical in gram-negative cell wall was not found. The di-Gly peptide bridges mediated the cross-linking. The results indicated that the murein of *T. thermophilus* presented an intermediate complexity between gram-positive and gram-negative organisms. The murein composition and peptide cross-bridges were typical for a gram-positive bacterium. However, the degree of cross-linkage and glycan length in murein were closed to gram-negative bacterium.

Many bacteria can converse their morphology when exposed to sub-optimal conditions. They enter into a state of nonculturability. Bacteria lose culturability on growth media, however, remain viable and demonstrate metabolic activity. This state is known as viable but nonculturable state (VBNC). Signoretto *et al*. [54] studied the biochemical composition of the cell wall of *Enterococcus faecalis* in VBNC state in comparison of growing cells. The VBNC state of enterococcal cells appeared as slightly elongated cell. The results have shown the differences in the peptidoglycan chemical composition of two forms. The VBNC cell was shown an increase in total cross-linking which rose from 39% in growing cells to 48% in VBNC cells. The oligomers of muropeptide have a higher order of the total cross-linking than that of the dimers. They suggested that all change in the cell wall of VBNC enterococci are specific to particular physiological state (Signoretto *et al*.) [54].

Costa *et al*. [7] have shown the peptidoglycan composition of spiral and coccoid form of *Helicobacter pylori*. This organism is a gram-negative bacterium that has spiral shape. *H. pylori* was transformed to coccoid form when cultured under adverse conditions. The results indicated that *H. pylori* mureins were made up of amino acids; meso-diaminopimelic acid, Glu and Ala which were the same as found in *E. coli*. No qualitative differences in peptidoglycan chemical compositions of spiral and coccoid cells were detected, however, quantitative alterations were found. Muropeptides with disaccharide-dipeptides and (1,6) anhydro muramic acid were increased in coccal cell of *H. pylori*.

These observations suggest that peptidoglycan is the main supporting structure of the cell. Mureins from different bacterial species vary in chemical structure. As mentioned above, variation of cross-linking peptide bond led to difference in bacterial morphology. Thus, chemical structure of peptidoglycan plays a major role in morphological alteration.

2.1.3.2 Biosynthesis of peptidoglycan

The process of biosynthesis can divided into three stages; (i) synthesis of precursors (subunits) in the cytoplasm, (ii) translocation of the subunits as lipid-linked compounds across the cell membrane and (iii) polymerization of subunits in the periplasm [34].

In the first stage, the condensation of N-acetylglucosamine α-1-phosphate with UTP catalyzed by a uridyltransferase (or pyrophosphorylase) leads to the formation of UDP-GlcNAc (Fig. 2.8, reaction 1). Then the reduction of the pyruvyl group by an NADPH-linked reductase leads to the formation of N-acetylmuramic acid (Fig. 2.8, reaction 2). The murein precursor UDP-MuNAc-pentapeptide is synthesized by the sequential addition of the amino acids (Fig. 2.8, reaction 3 to 8); alanine, glutamic acid and meso-diaminopimelic acid. Biosynthesis pathway of these amino acids are shown in Fig. 2.9 to Fig. 2.11. These murein subunits are transferred to the membrane in the second stage. Both MurNAc-pentapeptide and GlcNAc are attached to the cell membrane carrier lipid called bactoprenol or undecaprenol phosphate. This carrier lipid is a long chain hydrocarbon that can enter cytoplasmic membrane and carries hydrophobic molecules into or through the lipid bilayer [43]. The last stage, murein subunits are

transferred to the periplasmic space and incorporated in the newly synthesized peptidoglycan.

2.1.3.3 Elasticity of peptidoglycan

Murein sacculus is a bag-shaped macromolecule that surrounds the cell and maintains the morphology of the bacterium (Fig. 2.12). The entire wall is held together by covalent chemical bonds. It is composed of two kinds of covalent bonds; one is the β-1,4-glycosidic bond that connects the sugar units end-to-end into straight strands of stiff glycan, the other is the cross-linked peptide bond that formed between the glycan strands. The peptides are highly flexible [8]. The murein sacculus can expand or contract in response to change in environmental ionic strength or pH. In the absence of salt, extreme acid or alkaline pH, peptidoglycan complexes expand because of electrostatic repulsion [9].

Koch and Woeste [28] demonstrated that the murein sacculus of *E. coli* can expand at extreme pH or in the chemically modified sacculi (Fig. 2.13). Two peptides were not linked but they pointed above or below the plane of the figure. Thus, sacculus seem to be expanded. Furthermore, the environmental stress may affect the peptide chains. The unfolding peptide chains led to the expansion of wall fabric as shown in Fig. 2.14. The glycan chains are less elastic than the peptides but the sugar units can be pulled out of their straight-line arrangement [27]. Thus, breakage or reformation of chemical bonds can alter the shape of cell wall [69].

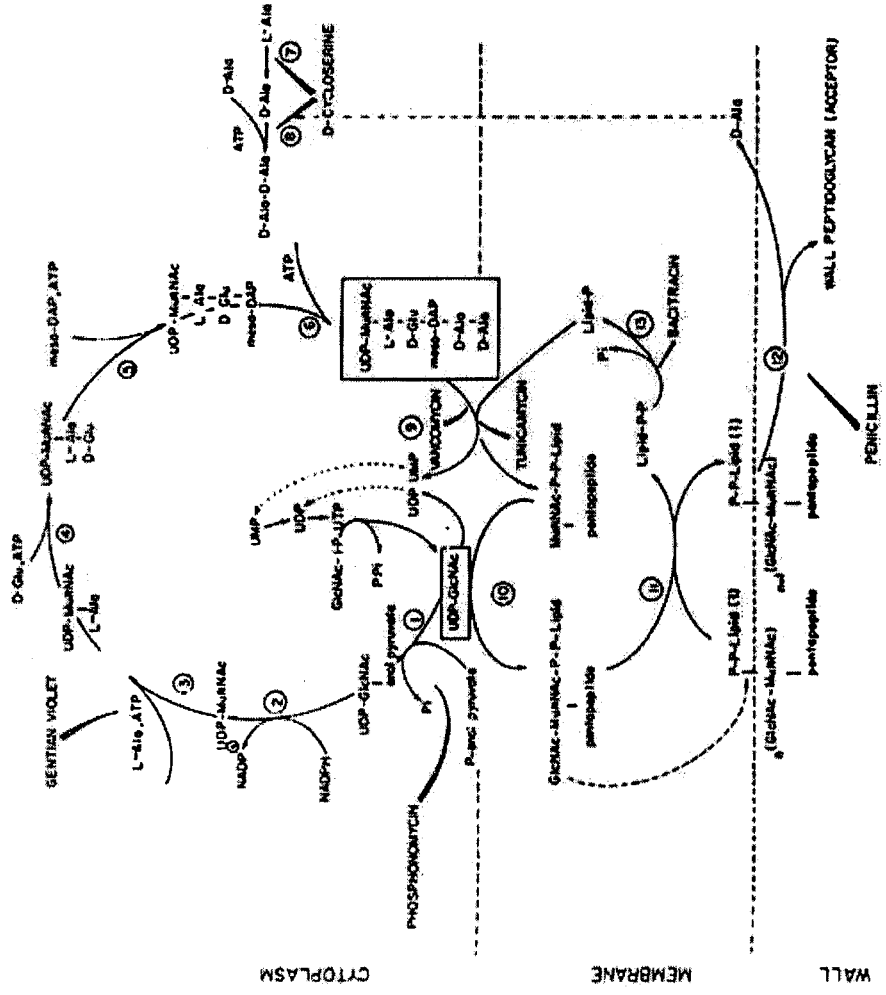


Fig. 2.8 Biosynthesis of *E. coli* peptidoglycan. The stages of synthesis that occur in cytoplasm, in the membrane and outside the membrane are separated by dashed lines [34].

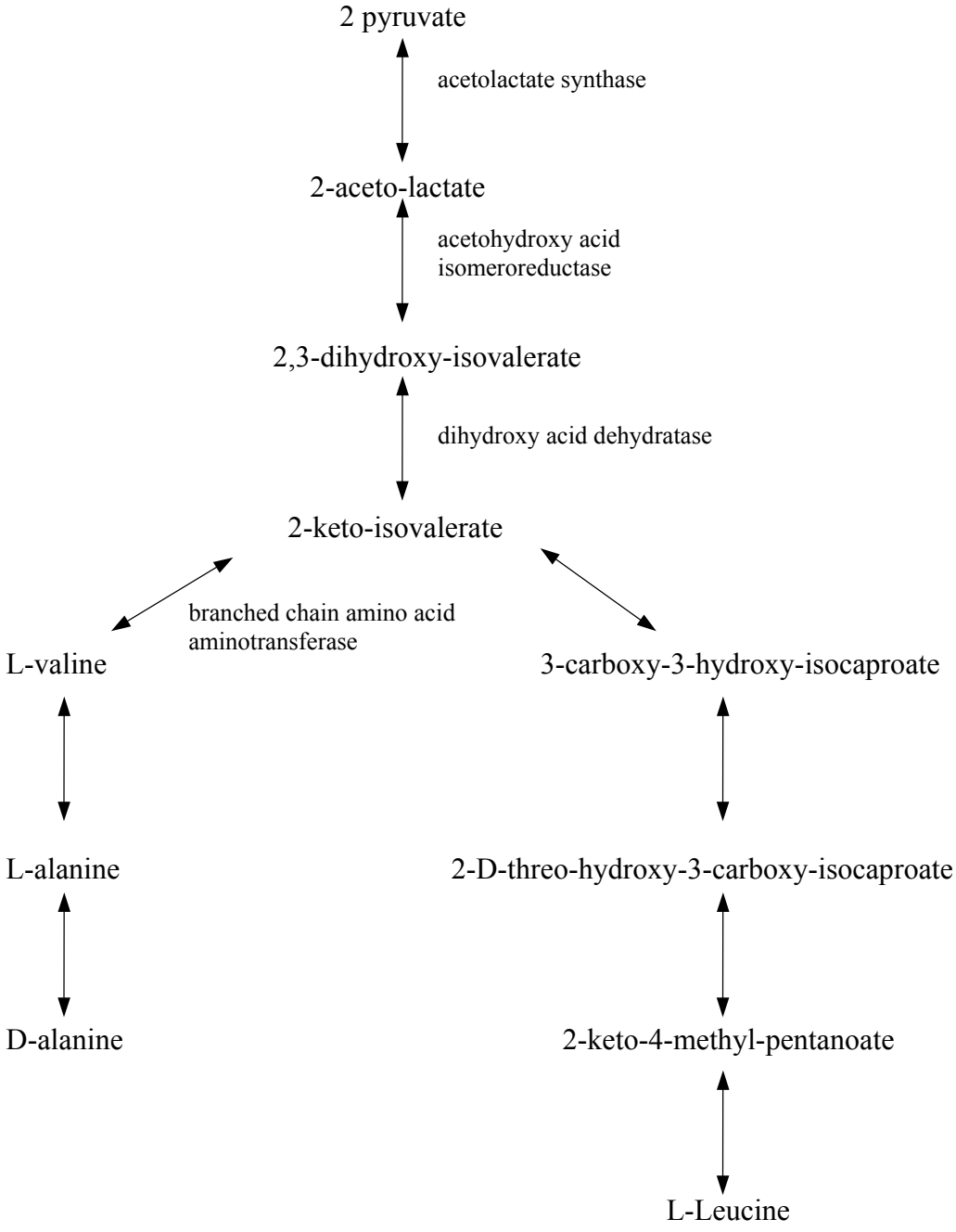


Fig. 2.9 Biosynthesis of valine, alanine and leucine [20].

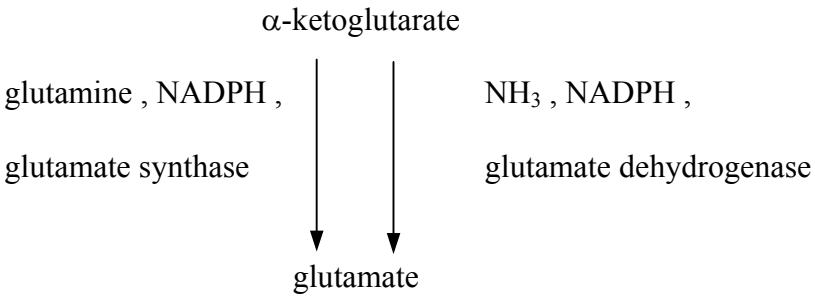


Fig. 2.10 Biosynthesis of glutamic acid (glutamate) [33].

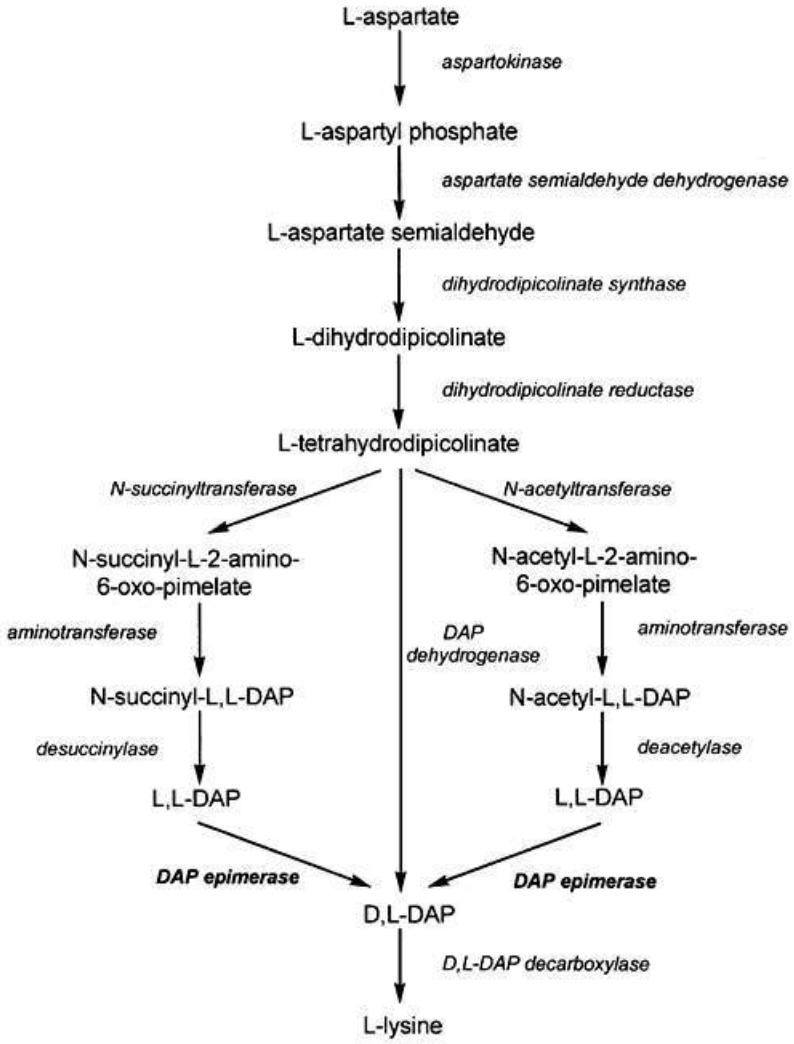


Fig. 2.11 Biosynthesis of Diaminopimelic acid [70].

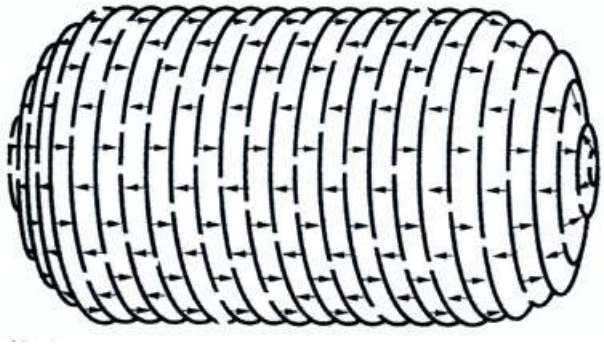


Fig. 2.12 Schematic drawing of the architecture of the murein sacculus. The parallel lines represent a few of the vast number of glycan strands and the arrow indicate the peptide bridges [19].

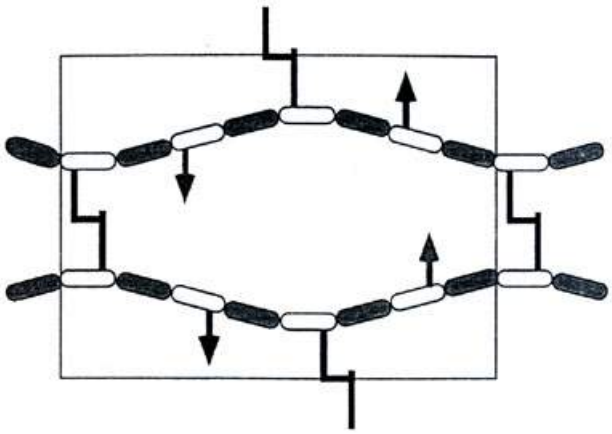


Fig. 2.13 Structure of a stressed unit of the wall fabric. The structure as depicted is for the conformation of ring units of isolated sacculi at extreame pH or in the chemically modified sacculi [28].

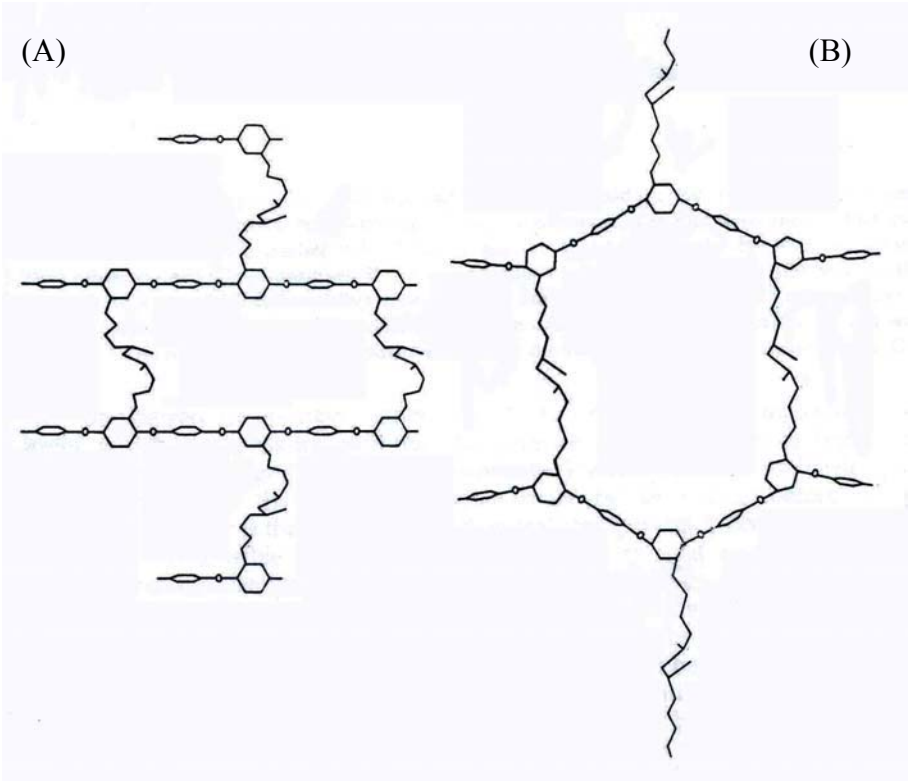


Fig. 2.14 Peptidoglycan polymer of the bacterial sacculus. In the relaxed configuration (A), the sugar units are thought to form straight and parallel chains. Under stress (B), the peptide chains unfold allowing the fabric to expand to cover a much larger area [27].

2.1.3.4 Bacterial cell wall recycling

During bacterial growth, cell wall peptides are released from the murein and reutilized for the synthesis of new cell wall material [14]. *Escherichia coli* and most other gram-negative bacteria release the turnover material at the rate of 40 to 50% of its murein sacculus per generation. The turnover products are occurred by the breakdown of murein in the periplasm with lytic transglycosylases and endopeptidases (Fig. 2.15). The two major products are murein tripeptide, L-Ala-D-Glu-DAP and the large muropeptide, 1,6-anhydro-MurNAc-β-1,4-GlcNAc-tetrapeptide. They are accumulated in the periplasm then transported into the cytoplasm by transporter proteins (the periplasmic permeases). The uptake of cell wall peptides is mediated by two ways. In the first case, the large muropeptide is transported into the cell by the AmpG transporter. Then it is degraded by an amidase (AmpD) and then tripeptide, which is then reused for the synthesis of murein precursor molecules, is released. In the other case, tripeptide in the periplasm is specifically taken up by the cell through the oligopeptide permease transport system (Opp) or murein peptide permease (Mpp).

Biosynthesis of peptidoglycan may be divided into two major pathways; *de novo* pathway and salvage pathway. The *de novo* pathway is the pathway of synthesis of amino sugars and amino acids. Then they are assembled to form the murein layer. Whereas the salvage pathway is a recycle pathway, which some units obtained from peptidoglycan degradation are utilized as precursors for the synthesis of new cell wall. The recycling pathway relates to a group of transporter proteins that play a role in transportation of turnover products into the cell.

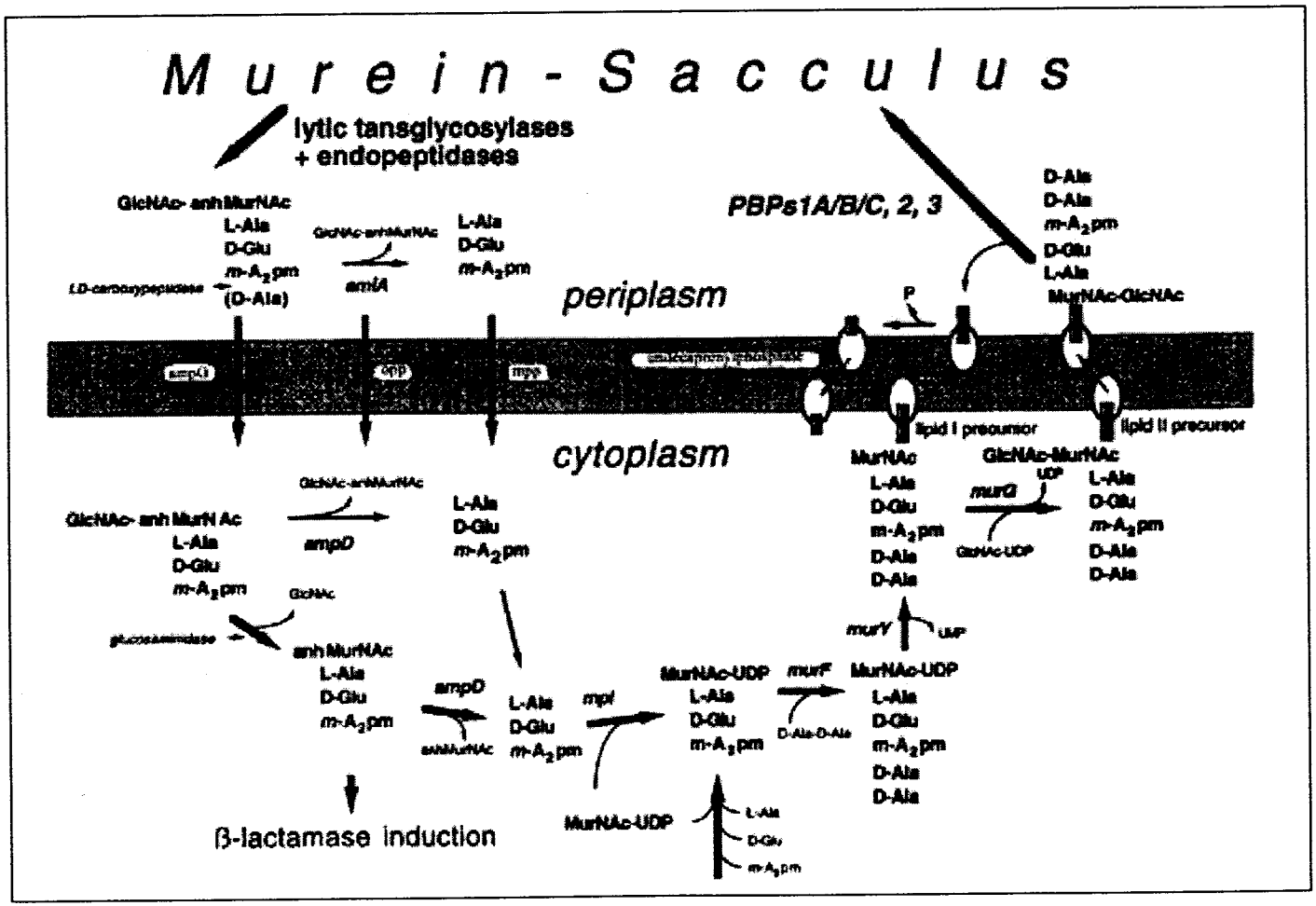


Fig. 2.15 Recycling pathway of murein turnover products in *E.coli* [19].

The ATP binding cassette (ABC) superfamily is a large group of proteins that translocates a wide range of molecules across the cytoplasmic membrane. Substrates assigned to member of this large family of transporters include compounds as varied as peptides, sugar, lipids, heavy metal chelates, polysaccharides, alkaloids, steroids, glutatione conjugates and inorganic acids [58]. The range of substrates transported by an individual member of a family can be narrow or broad, depend on the transporter. Members of ABC transporter not only uptake nutrients but also involve in a large variety of processes such as signal transduction, protein secretion, antigen presentation, bacterial pathogenesis, sporulation, drug and antibiotic resistance.

ABC transporter is composed of two parts; two membrane-integral domains and two ATP-hydrolyzing domains (referred to as ABC subunits/domains) (Fig. 2.16). The membrane-integral domains are highly hydrophobic and each domain is normally consisted of six membrane spanning segments. The ATP-binding proteins are located at the cytoplasmic membrane and utilize ATP as a source of energy. The substrate binding proteins or receptors of ABC transporters generally determine the substrate specificity of the system. In Gram-negative bacteria, the binding proteins were located in the periplasm while in Gram-positive bacteria they were associated to the cell membrane (Fig. 2.17).

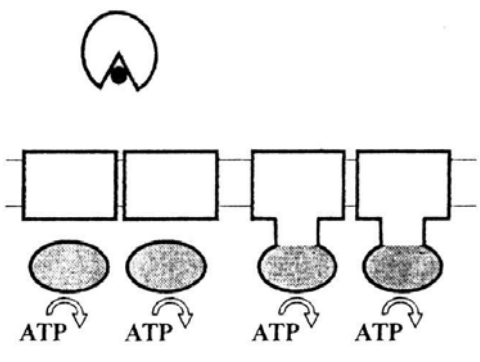


Fig. 2.16 Schematic diagram of the four structural domains of ABC transporters. A is bacterial import system and B is bacterial export system. (Adapted from Schneider, E. and Hunke, S., 1998 [53]).

The ABC transporters are classified into three major groups; (1) bacterial importers (the periplasmic permeases), (2) bacterial exporters and (3) eukaryotic transporters [12]. Several bacterial ABC transporters carries out a wide range of transport functions (Table 2.1). Many of them transport a large variety of molecules such as sugars, amino acids, peptides, inorganic ions and vitamins into the cell in association with the binding proteins (BP). Some of exporters involve in protein secretion which requires two accessory envelope proteins. One is an inner membrane protein that classified into the membrane fusion protein family (MFP). The second accessory protein is an outer membrane protein (OM). In addition, some of exporters efflux drug molecules or polysaccharides across the cytoplasmic membrane. The unique feature of the bacterial importers is that they have the specific binding protein that bind to its substrate. Then the substrates are imported into the cytoplasmic membrane.

Some of the bacterial importers are involved in translocation of muropeptide of the bacterial cell wall. The function of these transporters is uptaking of breakdown products in the cell wall recycling pathway. After cell wall degradation the tripeptides for synthesis of new murein are reutilized. The recycling pathway presumably supplies a constant level of tripeptide in the cytoplasm [42]. Thus, ABC transporter is related to the synthesis of peptidoglycan.

The peptidoglycan synthesis in bacteria that are altered from their normal shapes to the other shapes have been studied. *E. coli*, which were grown in mecillinam treatment, its typical rod shape was conversed to spherical shape. In the other organism, *K. pneumoniae*, which is a pH dependent bacterium that has rounded cell at pH 7 and rod shape at pH 5.8. It was found that the rate of incorporation of nascent peptidoglycan into the wall of spherical cell was lower than the rod shape cell [4, 51]. The results of these studies suggest that the bacterial morphology is affected by the rate of peptidoglycan synthesis.

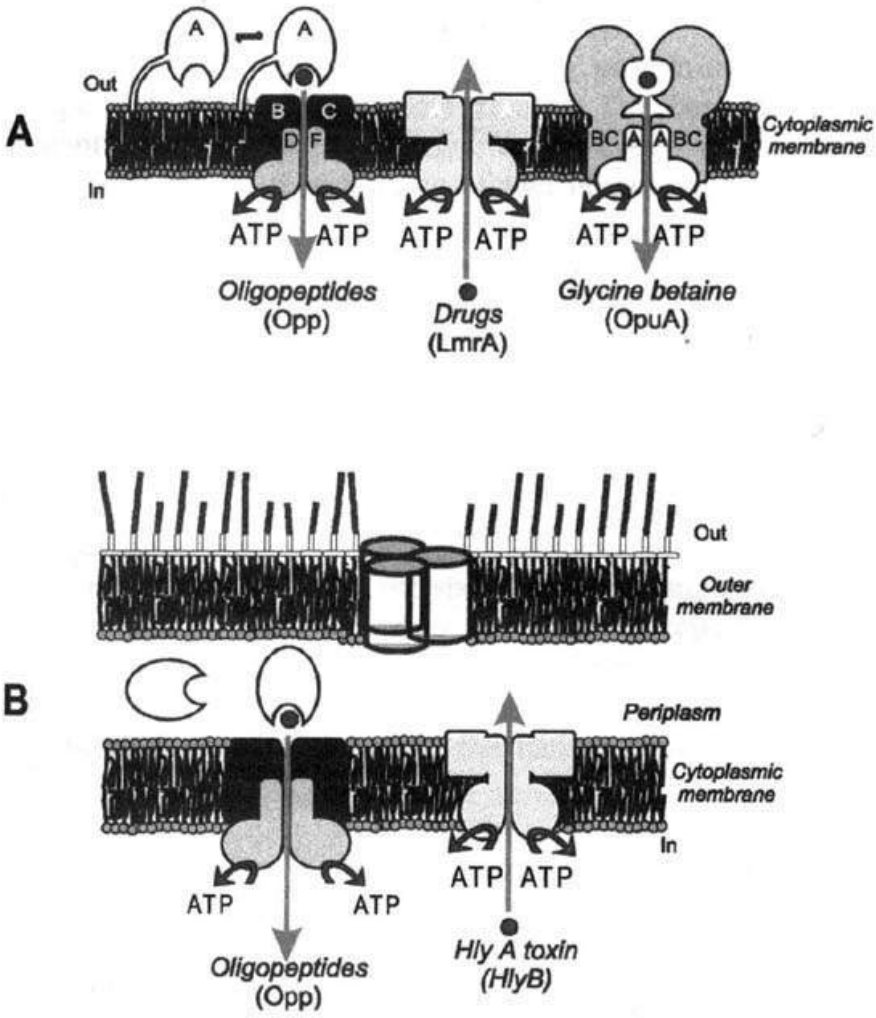


Fig. 2.17 Domain organization of ABC transporters in Gram-positive (A) and Gram-negative (B) bacteria (adapted from Frank, J.M. *et. al.* [22]).

Table 2.1 Examples of bacterial ABC transporters [40].

Substrate	Bacteria	ATPase	Channel	BP	MFP	OM channel
ABC Transporters for import						
Maltose	*Escherichia coli*	MalK	MalF, G	MalE		
Histidine	*Salmonella typimurium*	HisP	HisM, Q	HisJ		
Oligipeptides	*S. typhimurium*	OppD, F	OppB, C	OppA		
Galactose	*E. coli*	MglA	MglC	MrlB		
Ribose	*E. coli*	RbsA	RbsC	RbsB		
Arabinose	*E. coli*	AraG	AraH	AraF		
Phosphate	*E. coli*	PstB	PstA, C	PstS		
Ribose	*E. coli*	RbsA	RbsC	RbsC		
Leucine/ isoleucine/ valine	*E. coli*	LivF, G	LivH, M	LivJ		
Leucine/ isoleucine/ Valine-I	*Pseudomonas aeruginosa*	BraF, G	BraD, E	BraC		
Vitamin B_{12}	*E. coli*	BtuD	BtuC	BtuE		
Fe-enterobactin	*E. coli*	FecC	FepD, G	FepB		
Fe-hydroxamate	*E. coli*	FhuC	FhuB	FhuD		
Fe-dicitrate	*E. coli*	FecE	FecC, D	FecB		
Glycerol 3-phosphate	*E. coli*	Ugpc	UgpA, E	UgpE		
ABC transporters for protein export						
Hemolysin	*E. coli*	HlyB (C)	HlyB (N)	-	HlyD	Tolc
Cyclolysin	*Bordetella pertussi*	CyaB (C)	CyaB (N)	-	CyaD	CyaE
Proteases A, B, C	*Erwinia chrysanthemi*	Prt (C)	PrtD (N)	-	PrtE	PrtF
Colicin V	*E. coli*	CvaB (C)	CvaB (N)	-	CvaA	TolC
ABC Transporter for export						
Daunomycin	*Streptomyces peucetius*	DrrA	DrrB	-		
Capsular polysaccharide	*E. coli*	KpsT	KpsM	-		
Capsular polysaccharide	*Haemophilus influenzae*	BexA	BexB	-		
Capsular polysaccharide	*Neisseria meningitidis*	CtrD	CtrC	-		
Lipooligosaccharide	*Rhizobium meliloti*	NodI	NodJ	-		

2.3 Morphological alteration of bacteria

Many bacteria have been reported in the occurrence of difference in morphological forms. They have their shapes altered in response to several sub-optimal conditions. Some of the changes are reversible while another are irreversible transformation. The transformation from one form to the other form occurs under variation of various environmental factors such as temperature, nutrient and pH. Some of the studies on the transformation of morphological forms are summarized as follows:

2.3.1 Reversible transformation

Satta *et al*. [51] have shown the effect of pH medium on the morphology of *Klebsiella pneumoniae*. This bacterium has pH-dependent property. Cultures have rod shape at pH 5.8 and coccoid shaped when expose to pH 7 culture medium. Moreover, the coccoid cell can be reversed to the rod shape when the pH is downshifted from 7 to 5.8.

The rod shape is the typical morphology of *Vibrio vulnificus*. Nilsson *et al*. [39] found that the cells were easily changed to coccoid shape when the culture was incubated at low temperature (5 ^{o}C). Furthermore, it would be converted to the original cell shape when it was exposed to an upshift in temperature (23 ^{o}C). The authors suggested that upshift temperature might be the determining factor in the resuscitation of this bacterium.

Vibrio parahaemolyticus is reported for morphological transformation. Jiang and Chai [25] shown that morphological change of this organism occurred when subjected to starvation at low temperature. This bacterium has rod shape at 35 ^{o}C. The result indicated that *V. paramaemolyticus* converted to

spherical cell in 50 to 80 days during starvation at 3.5 ^{o}C. After temperature upshift, the cultures in spherical shape were represented the regrowth and the rod shaped cells were found. They suggested that the cultures in spherical form during exposed to low temperature would grow and multiply when the temperature was upshifted. Furthermore, *V. cholerae* was shown to have morphological conversion under low temperature. This bacterium has a reversible morphological change which coccoid form develops into rod shape in response to the temperature upshift condition [47].

Spirulina is also shown morphological changes under various growth conditions. Van Eykelenburg and Fuchs [63] had been observed the rapid reversible morphological change in *S. platensis*. There were two difference processes that involve in morphological conversion. The first is the change from helix to spiral shape. The other is the morphological change from spiral to helix which occur very fast. When the spiral cell is rewetted by slowly dropping water on the surface of the cell, it converts to the helical shape. They proposed that the change in relative humidity led to the change in cellular morphology.

Kebede [26] studied the growth of *S. platensis* which isolated from Lake Chitu, Ethiopia in response to variation of salinity and ionic component. Salinity of media was tested in the range of 13 to 88 g•l^{-1} by addition of $NaHCO_3$, NaCl and Na_2SO_4. It was found that the increasing in salinity of culture medium led to reduction in growth. Moreover, the cells in high salinity medium shown the difference in the length of trichomes and the degree of coiling (helicity). The cells with long trichomes, formed aggregate in the medium with the lowest salinity (13 g•l^{-1}), while the cells have loosely coil in the high saline medium. At the same salinity level in the present of sulphate, the cells changed in the degree of

coiling compared to bicarbonate and chloride rich medium. The results showed that salinity may not be the only factor that affects cellular morphology. The morphology also depends on the anion components.

Light intensity and nutrient concentration have shown the effect on the morphology of *Spirulina fusiformis*. Jeeji Bai and Seshadri [24] demonstrated that the culture, which was grown in nutrient deficiency condition, increased in degree of coiling causing tight trichome. While the cells in high nutrient have loose trichome. Under high light intensity and high nutrient concentration condition the morphological conversion from loose helices to tight coil is induced whereas the high light intensity and low nutrient concentration lead to the formation of very tight trichome.

Van Eykelenburg [62] also showed the morphological change of *S. platensis* which isolated from Lake Nakuru, Africa in relation to temperature. When growth temperature increased from 13.5 to 40 ^{o}C, the increase in sheath material formation and the decrease in cyanophycin and polyglucan granule concentration were observed. Increasing of temperature affected cellular morphology. It caused the cells conversion to tight and short trichome. However, there was no significant change in thickness of the peptidoglycan layer in the culture grown at temperature ranged from 13.5 to 38.5 ^{o}C. The results suggested that the morphological change may be resulted from the arrangement of peptidoglycan layer of the cell wall (Van Eykelenburg) [62].

2.3.2 Irreversible transformation

Helicobacter pylori is a gram-negative bacterium that causes the pathogenesis of gastric epithelium. The morphological conversion from the spiral, which is the typical shape, to the coccoid form has been studied. Either physical or chemical stress led to transformation into the coccoid form. Sorbery *et al*. [56] found that the coccoid form has a 1000-fold lower ATP level per cell than that of the spiral form. The result indicated that the coccoid form decreased metabolic activity in their cells. Moreover, the conversion from coccoid to spiral form during prolonged incubation for four weeks was not found.

Tominaga *et al*. [60] studied the effect of various growth conditions; oxygen concentration, pH and temperature on morphology of *H. pylori*. Under the standard culture conditions; 37 ^{o}C, pH 7 and microaerobic atmosphere (5% O_2/ 10% CO_2/ 85% N_2), this organism has a spiral form. The morphological change from spiral to coccoid form appeared on the third day in aerobic condition (20% O_2 supply) and the second day under microaerobic atmosphere (<0.1% O_2). Furthermore, pH of the culture medium led to the change from spiral to coccoid form at pH 8 and pH 4 on day 5 and on day 6, respectively. Temperature also has an effect on morphology. The culture converted to the coccoid shape on day 4 at the growth temperature of 20 ^{o}C or 42 ^{o}C. These results showed that oxygen concentration, pH and temperature may play a role in the morphology transformation of *H. pylori*. The authors suggested that imbalance of these factors may accelerate *H. pylori* morphological conversion from the spiral to the coccoid form.

The occurrences of straight trichomes of *Spirulina* have been reported. Lewin [31] found that the uncoiled variants were appeared in a pure culture of *S. platensis* which isolated from alkaline salt-flat areas of a coastal lagoon in California. A pure culture was initiated from a single coiled filament. Sub-cultures were grown under constant light generated by warm-white fluorescent lamps (2000 lux) and aerated by constant agitation on a shaker at 32 ^{o}C. From the observation, the straight trichomes showed no tendency in reversion to the helical form. Thus, it was proposed that helicity might not be a generic character for *Spirulina* spp.

The morphological transformation from helical to straight trichomes of *S. fusiformis* was also reported. Jeeji Bai [23] showed that the straight forms appeared in small number in laboratory cultures and then increased to over 50% shortly after transferring to open-air condition. The change from helical to straight form is unidirectional and irreversible during long term observation. It was postulated that the straight trichomes were originated by mutation of a very small percentage of helical trichomes [23].

In addition, two distinct morphological forms in another cyanobacteria were found. Saker *et al*. [49] isolated *Cylindrospermopsis raciborskii* from Solomon Dam in Australia. They compared growth and toxin content of the two morphological forms; straight and coiled trichome under different conditions in the laboratory. Isolates of the two forms were grown in pure culture. It was found that both forms contained the toxin cylindrospermopsin in similar content. The morphological comparison between the two forms showed significant differences in vegetative, heterocyst and akinete cell dimensions. Furthermore, the two morphological forms were maintained under all culture condition investigated in this

study. They observed no straightening of coiled trichomes or curving of straight trichomes throughout the experiment under laboratory condition.

As mentioned above, no information is available about the mechanism(s) of morphological conversion. In the case of irreversible transformation of some species, the conditions inducing the morphological change are not clear. Several investigators have suggested that changes in bacterial shape may link to the cell wall structure [30, 36, 62]. Lazaro *et al*. [30] proposed that the morphological conversion of *Campylobacter jejuni* from spiral to coccoid form might be involved in alterations of the composition of the cell wall and cell membrane, however, it does not affect cellular or DNA integrity. Moreover, morphological change could result from rearrangement of peptidoglycan layer, as suggested by Van Eykelenburg [62].

2.4 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Two-dimensional electrophoresis (2D electrophoresis) was firstly introduced by O'Farrell in 1975 [41].

2.4.1 Principle of 2D electrophoresis

This technique is capable to resolve the complex protein mixtures extracted from cells, organisms, tissues or other biological samples. In the first dimension of 2D-PAGE, proteins are separated by their isoelectric point, pI, where the pH at which their net charge is equal to zero. Then the proteins are separated according to their molecular weights (Mw) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension (Fig. 2.18).

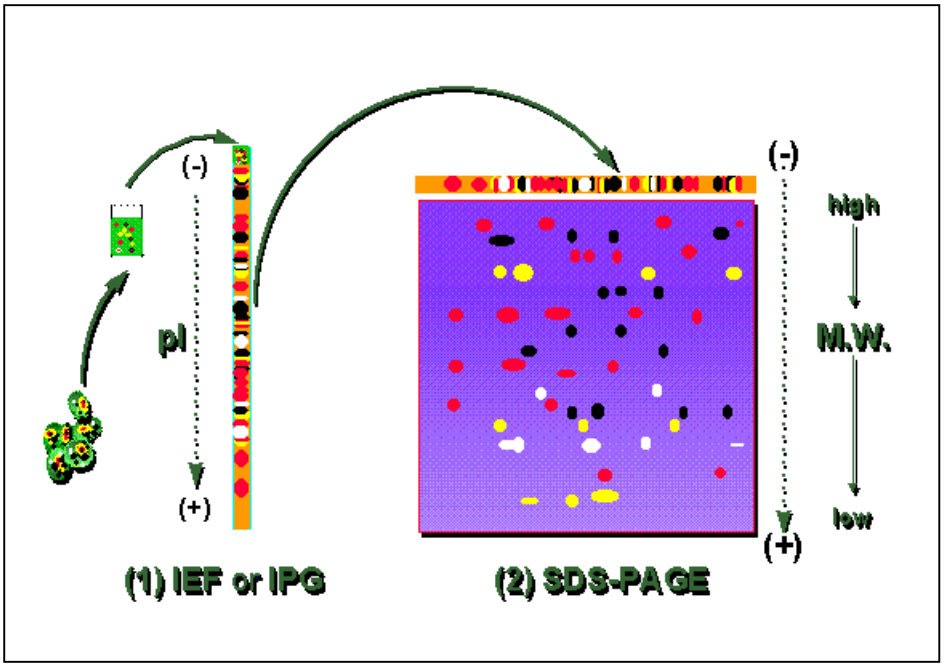


Fig. 2.18 Schematic drawing of the 2D electrophoresis.

The first dimension separation is performed using immobilised pH gradient (IPG) gel. This gradient is built with acrylamide derivatives with buffering groups, the immobilines, by co-polymerization of the acrylamide monomers in a polyacrylamide gel (Fig. 2.19) [68]. Immobiline is a weak acid or base defined by its pK value and the general structure is shown in Fig. 2.20. The pH gradient is obtained by the continuous change in the ratio of immobiline.

Isoelectric focusing (IEF) takes place in a pH gradient and can only used for amphoteric substance such as peptides and proteins. To obtain sharply focused bands, IEF is performed on a flated system at high voltages with temperature control. Running conditions depend on parameters such as interval used of pH, separation distance, additives added to the rehydration solution, sample amount and temperature [15]. In a pH gradient, under the influence of an electric field, the molecules will move to the anode or the cathode until they reach a position where their net charges

is equal to zero (Fig. 2.21). Moreover, all proteins with pI less than lower boundary pH or higher than upper boundary pH of an IPG strip gel, will migrate out of the IPG strip. After IEF terminating, the IPG strip is applied to the second dimension separation.

SDS-PAGE is run as the second dimension. The proteins are separated according to their molecular weights. This step is either performed on horizontal or on vertical system. SDS is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone in ratio of approximately 1.4 grams SDS per gram protein [1]. The tertiary and the secondary structures of protein are eliminated because of the disruption of hydrogen bonds and then the peptide molecules are unfolded. In addition, the molecular weight of proteins can be estimated with standard marker proteins applied in the second dimension.

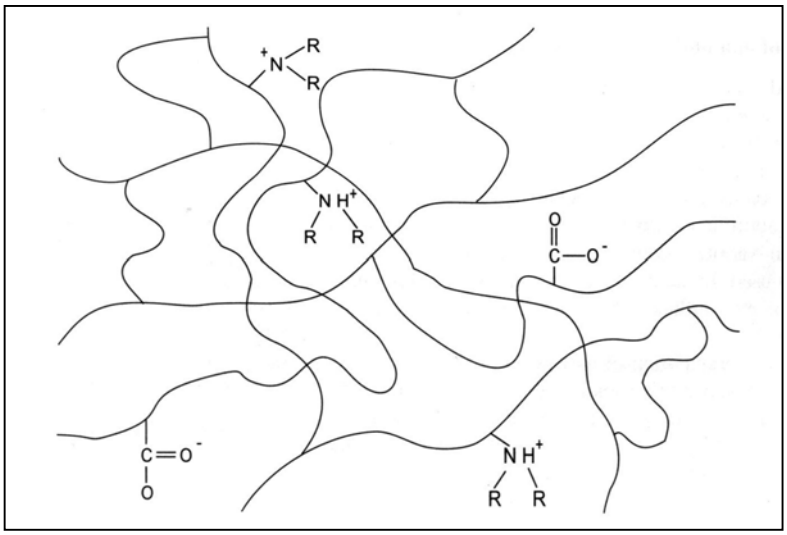


Fig. 2.19 Diagram of a polyacrylamide network with co-polymerized Immobilines [68].

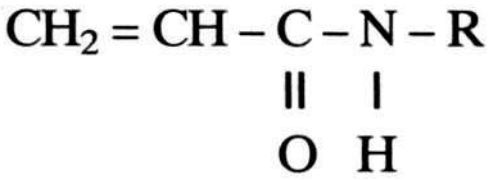


Fig. 2.20 General structure of Immobiline [68].

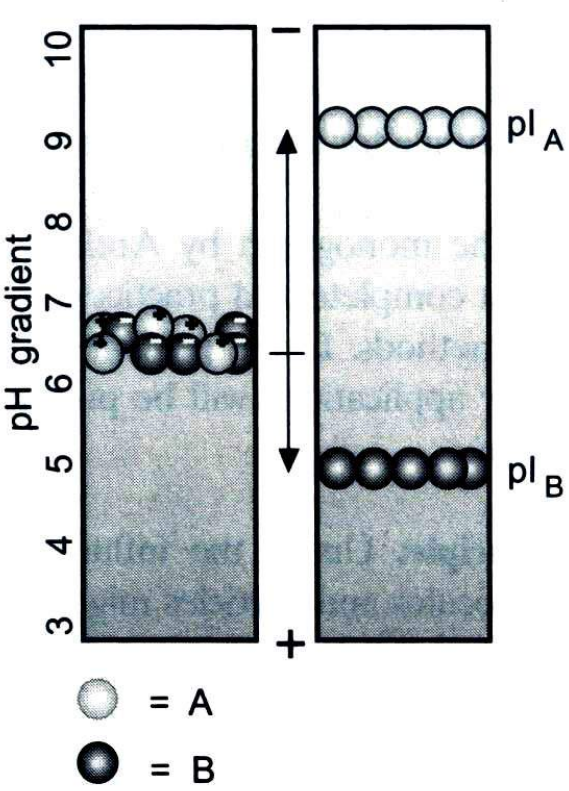


Fig. 2.21 The isoelectric focusing. A and B represent the components of the sample [68].

2.4.2 Application of 2D electrophoresis

The Two-dimensional electrophoresis is widely used as a method for detection and analysis of protein samples. It is a protein separation method that gives higher resolution than one-dimensional electrophoresis. Thus, it is a powerful method to separate a complex mixture of proteins. Furthermore, this technique is available for the rapid and routine identification of the protein spots, and able to perform high-throughput analysis. Therefore, 2D electrophoresis was used as a tool for proteome analysis (or proteomic). The term PROTEOME stands for the total PROTein complement of a genOME.

Proteome analysis was shown in the following steps; (i) sample preparation under maintenance of the protein composition at the actual status of the cell (ii) two-dimensional electrophoresis (iii) detection of protein spots (iv) search for up or down regulated proteins or protein changes with image analysis (v) protein spot identification and (vi) bioinformatics for protein identification and database searching. Chich [5] divided proteomics into two approachs, systematic approach or cell map proteomic, and pragmatic approach or study of changes in protein expression.

2.4.2.1 Systematic approach

The aim of this approach is identification of all proteins in cells or tissues. Characterization of the genome of a wide variety of organisms has been made. The entire genome of some organisms; *Escherichia coli*, *Saccharomycese cerevisiae*, *Haemophilus influenzae*, *Caenorhabditis elegans*, *Staphylococcus aureus* are completely sequenced and the sequences are available in many databases. They can be used as databank.

For example, the whole genomic sequence of cyanobacterium *Synechocystis* sp. strain PCC6803 has been determined. Sazuka *et al*. [52] separated the cyanobacterial proteins into four different fractions; soluble, insoluble, proteins in thylakoid membrane and secretory protein fractions. The results showed that 234 protein spots were found by using 2D electrophoresis coupled with N-terminal sequencing. These spots were also shown in the Cyano2Dbase (http://www.kazusa.or.jp/tech/sazuka/cyano/proteome.html). Because of this cyanobacterial strain carried a complete set of genes for oxygenic photosynthesis, it is a model organism for biochemical and biological studies. This information may help accelerating the molecular study of the cellular response to environmental stresses.

2.4.2.2 Pragmatic approach

The aim of this approach is comparison of two physiological states. In many comparative experiments, a variety of changes in cellular environments such as heat, light, salt and nutrients have been used to induce changes in the existent proteome [13]. This approach is wildely applied in many fields of study including toxicological or medical field where normal and diseased cells are compared to identify molecular markers of the diseased state.

The study by Choi *et al*. [6] is an example of this approach. In this study, the proteome analysis of light-induced proteins in *Synechocystis* sp. PCC6803 was performed. They attempted to identify new light-dependent-express proteins which were not identified by conventional methods. In the first step, the cells were grown axenically at 28 ^{o}C under continuous illumination of white fluorescent lamp (50 μmol• m^{-2}• s^{-1}). The cultures were grown up to mid log

phase ($O.D._{730}$ = 0.8-1.2) and kept in darkness for one day. Then the cultures were divided into two groups; one was kept for 2 days in darkness and the other was illuminated at 100 μmol• m^{-2}• s^{-1} for 12 hours. The soluble proteins extracted from light and dark culture were resolved by 2D electrophoresis. The light-induced protein spots were analyzed by N-terminal sequencing and followed by CyanoBase. They found that 8 proteins were related to photosynthesis and respiration; RbcS/L, CbbA, Gap2, AtpB, CpcB, PsbO and PsbU. Furthermore, four proteins; SodB, DnaK, GroEL2 and Tig were found to be involved in cellular processes, and the functions of another two proteins (rehydrin and membrane protein) were yet unknown.

Munchbach *et al*. [37] demonstrated that heat shock protein expression in *Bradyrhizobium japonicum* which is a nitrogen fixing root nodule symbiont of soya. They compare total soluble proteins of normal cell and heat shocked cell separated by 2D-PAGE. Then all protein spots were analyzed by mass spectrometry and N-terminal sequencing. Under heat shock condition, temperature shift from 28^{o}C to 43^{o}C, nineteen protein spots were clearly up regulated. The results showed that five proteins belong to the small Hsp family (Hsp B, C, D, E, and H) and three proteins were homolog to the sHsp family. Four proteins that have molecular mass of 60 kDa were identified as GroEL2, GroEL4, GroEL5 and DnaK. The other were low molecular weight proteins including GroEL1 and GroEL2 and five novel proteins. It was found that this set of proteins has high similarity to heat shock proteins found in other organisms.

Betts *et al*. [3] compared the proteome of two virulent strains of *Microbactrium tuberculosis*; H37Rv and CDC 1551. The H37Rv was used as laboratory strain that the entire genome has been completely sequenced. For a recent clinical isolated strain, CDC 1551 was a highly virulent strain for an outbreak of tuberculosis. Total cell lysates of the two strains were analyzed by 2D electrophoresis. Approximately 1,750 protein spots were visualized and the protein profiles of both strains were found to be highly similar. A total of 17 protein spots differrences between H37Rv and CDC 1551 strain were observed. Seven protein spots were presented only in CDC 1551 that were identified as alcohol dehydrogenase and transcriptional regulator MoxR. Three spots were corresponded to phosphoribosylformimino-5-aminoimidazole carboxamide ribonucleotide isomerase , which were found only in strain H37Rv. One spot was identified as ribosome recycling factor that showed a marked vertical mobility shift between the two strains. Two spots that showed increased intensity in H37Rv were identified as alkyl hydroperoxide reductase chain C. In the case of four protein spots which increased intensity over time in both strains, they were identified as the heat-shock proteins, Hsp20 and 16 kDa antigen. The authors suggested that the proteome comparison of both strains may aid for design of new drugs and vaccines against tuberculosis.

Hartke *et al*. [16] studied the ability of *Enterococcus faecalis* in adaptation to an oligotrophic environment (tap water). They found that *E. faecalis* were able to survived under complete starvation which introduced by incubation in tap water. The cells had a smooth and spherical appearance and developed to rippled cell surface with irregular shape during

starvation. Comparison of protein synthesis in native and starved cultures were performed by using 2D electrophoresis. The results showed that the incubation of *E. faecalis* in tap water led to an enhanced synthesis of at least 51 proteins. Majority of these proteins induced early during incubation in tap water was low molecular weight proteins. The synthesis of high molecular weight proteins were increased after longer incubation times. The fifty-one proteins were compared with the 42 glucose starvation-induced polypeptides. It was shown that sixteen spots were common between the two different starvation conditions. The authors proposed that these proteins seemed to play a role in observed phenomena of long term survival and development of general stress resistance of *E. faecalis*.

Lazaro *et al*. [30] studied the viability and DNA maintenance in nonculturable *Campylobacter jejuni* cells after long-term exposure to low temperature (4 and 20^{o}C). Under adverse condition, temperature shift from 42 ^{o}C to 4 and 20 ^{o}C, morphological transformation from spiral to coccoid forms was found. Survival of *C. jejini* at low temperature was investigated by using cellular integrity, respiratory activity, protein profiles and intact DNA content as indicators of potential viability of nonculturable cells (coccoid form). Restriction endonuclease digestion and pulsed-field gel electrophoresis (PFGE) were used to determination of the DNA content. They found that DNA content, cellular integrity and respiratory activity of nonculturable cells were detected under incubation at 4 ^{o}C for 116 days. The PFGE banding patterns of coccoid form were the same as those from the spiral form. Furthermore, the protein profiles from crude cell extract of coccoid form were analyzed by 2D electrophoresis and then compared with the profiles of spiral form. Most of protein spots were similarity in two-dimensional profile of both forms.

Major outer membrane protein was relative decreased with increased time of incubation at either temperature. Moreover, differences in the protein profiles of two forms of *C. jejini* grown at 4^{o}C and 20^{o}C were detected, however they required further investigation. The authors proposed that most changes in 2D profiles involved up- or down- regulation.

As described in this section, it illustrated that 2D electrophoresis is a tool for proteomic identification of proteins. This technique allows us to determine proteins which are being expressed in a cell or tissue. Thus, it is useful both as an analytical tool and micropreparative purification tool, consequently, it has become an essential technique in proteome analysis.

Chapter 3

Materials and Methods

3.1 Microorganism and culture medium

S. platensis C1 is a non gas vacuolated and helical trichome form. The strain was kindly provided by Dr. Avigad Vonshak, Ben Gurion University of the Negev, Israel. The culture was maintained and grown in Zarrouk's medium (Appendix A).

3.2 Isolation of a single trichome

In laboratory, the pure cultures initiated from a single coiled trichome were often found to consist of coiled and straight form after many passages of subculturing. To obtain a pure coiled trichome, a single trichome was isolated as described below:

The 0.1 ml of the culture was pipetted and spread onto Zarrouk's agar then incubated at 30 ^{o}C for 24 h. One trichome was picked under stereomicroscope using a sterile Pasture pipette and place into a microfuge tube containing 1 ml of Zarrouk's medium. The tube was incubated under dim light at 30 ^{o}C. The isolates were scaled up for further study.

3.3 Test of bacterial contamination

To check the bacterial contamination, 1 loop of culture was streaked on the Zarrouk's agar containing 0.5% glucose and 0.05% casamino acid. Then it was

incubated at 30 ^{o}C for 24 h. The absence of any bacteria growth on the Zarrouk's agar usually indicates a bacteria free culture.

3.4 Morphological study

The culture samples were observed under light microscope. Diameter of helix and pitch were measured microscopically using a calibrated ocular micrometer.

3.5 Analytical methods

3.5.1 Measurement of growth

Growth was measured by determination of :

A. Cell turbidity at the wavelength of 560 nm.

B. Chlorophyll *a* determination (Appendix B.2).

3.5.2 Carbohydrate determination (Appendix B.4).

3.5.3 Protein determination (Appendix B.5).

3.5.4 Phycocyanin determination (Appendix B.6).

3.5.5 Carotenoid determination (Appendix B.7).

3.5.6 Fatty acid analysis (Appendix B.8).

3.5.7 Photosynthetic activity measurement.

The pure cultures of coiled and straight forms were grown separately under semi-continuous culture condition. The cultures were incubated in a temperature regulated water bath at 35 ^{o}C, gently stirring using magnetic stirrer, under the light intensity of 80 $\mu E \bullet m^{-2} \bullet s^{-1}$. The cells were grown until the optical density at 560 nm reached 0.35.

The cells were harvested by filtration and resuspended with fresh Zarrouk's medium to yield a final concentration of 2.5 μg Chl•mg^{-1}. Then 5 ml of cell suspension were transferred into a double jacket thermoregulated glass vessel with oxygen electrode immersed in the culture. The temperature was kept constant at 35 ^{o}C and the culture was illuminated at light intensity of 160 μE•m^{-2}•s^{-1} provided by a slide projector lamp. The culture was bubbled with O_2-free N_2 to lower the oxygen concentration and to avoid O_2 saturation during the measurement. The signal from the electrode was registered on a chartpen recorder. When stable rates were obtained, photosynthetic activity was calculated as μmol O_2 evolved h^{-1}• mg •chl^{-1} (Appendix B.9).

3.6 Experimental condition

3.6.1 Effect of environmental factors

The effects of nutrient concentration, light intensity, temperature and combined factors on morphology of *S. platensis* C1 strain was studied. The pure helical form was used throughout this study.

The experiments were conducted under batch and semi-continuous mode. Zarrouk's medium was used at normal (1x), high (2x) and low (0.5x) concentration for these experiments. Under semi-continuous condition, the optical density at 560 nm was kept at 0.35 by serial dilution of the culture and replenishment of fresh Zarrouk's medium to keep the constant volume. The cultures in steady state were examined for morphology.

To test the other factors (light intensity, temperature and combined factors), the cultures were grown under semi-continuous mode. The experiments were set up as follows;

Light intensity ($\mu E \bullet m^{-2} \bullet s^{-1}$)	Temperature					
	35 ^{o}C			40 ^{o}C		
80	0.5x	1x	2x	0.5x	1x	2x
300	0.5x	1x	2x			

The effect of UV on morphology of *S. platensis* C1 was performed as followed:

Cultures in helical form were grown in normal Zarrouk's concentration (1x) with constant agitation in the incubator shaker at 35 ^{o}C and illuminated by white fluorescent lamp for 80 $\mu E \bullet m^{-2} \bullet s^{-1}$. The initial inocula had an optical density at 560 nm of 0.1. After 72 h, cultures were filtrated and resuspended in 30 ml of fresh Zarrouk's medium at approximately $2x10^{6}$ trichomes/ml. Then, the culture suspension was placed in opened-Petri dish with constant stirring and irradiated for 5, 7, 10, 12, 15 and 20 min by UV lamp (TUV 15W/G15 UV-C, Philips) at a distance of 15 cm. After incubation in the dark for 10 h, the samples were plated onto Zarrouk's medium and incubated at 30 ^{o}C in an illuminated incubator (Ultra cold, Thailand) under light intensity of 50 $\mu E \bullet m^{-2} \bullet s^{-1}$. Colonies on plate were determined for calculation of percentage survival of cell. The number of colonies on non-irradiated plate was as control (100% survival). Percentage of survival and exposure time were plotted to obtain the optimal UV exposure time that give 10% survival of

cell. The optimal exposure time was used for the UV irradiation experiment. The morphology of cell after UV irradiation were observed.

3.6.2 Comparison of helical and straight form

S. platensis C1 in the helical and straight forms were grown separately in normal concentration (1x) of Zarrouk's medium under semi-continuous condition ($O.D._{560}$ = 0.35). The cultures were incubated at 35 ^{o}C under the light intensity of 80 $\mu E \bullet m^{-2} \bullet s^{-1}$. The steady state cultures were analyzed for biochemical compositions, pigments and photosynthetic characteristics.

To study the protein profiles, the cultures were grown under batch condition for 7 days (stationary phase cell). The cultures were harvested and prepared for cell disruption. The cells were disrupted by French pressing (SLM Instrument, USA) and then the cell debris was removed and the supernatant was kept at –20^{o}C until used.

3.7 Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

3.7.1 First Dimension:

In the first dimension, proteins were separated by their isoelectric focusing properties. Precasted Immobilized pH Gradient (IPG) strips with the pH gradients 4 – 7 L (linear), are available from Amersham Pharmacia Biotech.

3.7.1.1 Sample preparation

The solution containing 1% SDS was added to a sample containing 120 μg of protein to yield 0.1% of SDS final concentration in order to dissolve the membrane-bound and hydrophobic proteins.

3.7.1.2 IPGphor isoelectric focusing system

A.1 The amount of 500 µl of rehydration solution (Appendix C.5) containing DTT (Dithiothreitol) and IPG buffer was placed into an IPGphore strip holder channel.

A.2 The sample solution was slowly loaded onto the sample application well. Air bubbles in the solution must be removed if present.

A.3 Carbonic anhydrase was used as pI marker which has pI range from 4.8 to 6.7 and molecular mass of 30 kDa (Amersham Pharmacia Biotech). The amount of 10 µl of pI marker was loaded onto the sample application well.

A.4 The IPG strip with the gel side facedown was placed into the strip holder and the pointed (nodic) end of the strip directed toward the pointed end of the strip holder. Care must be taken not to trap bubbles under the IPG strip.

A.5 The IPG cover fluid (mineral oil) was dropped into one end of the strip holder until one-half of the IPG strip is covered. Then the fluid was added into the other end of the strip holder until the entire IPG strip is covered.

A.6 The strip holder was covered with a strip holder cover.

A.7 The strip holder was positioned on the IPGphore unit platform and it was ensured that the holder is on a level surface.

A.8 The first dimension was operated by IPGphor Isoelectric Focusing System. The IPG strip was rehydrated for 12 h then it was operated for 3 steps. First, the strip was applied with 500 volts for 500 volt-hour. Second, it was applied with 1,000 volts for 1,000 volt-hour and finally it was applied with 8,000 volts for 16,000 volt-hour in the last step. The total volt-hour is 17,500 volt-hour.

3.7.2 Second dimension:

In the second dimension, the proteins were separated by their molecular weight using a SDS-PAGE.

3.7.2.1 Preparation of 12% separating polyacrylamide gel.

The separating gel solution was prepared by mixing 5.25 ml of distilled water, 6 ml of 30% acrylamide / 0.8% bisacrylamide solution, 3.75 ml of 4X Tris-Cl / SDS pH 8.8, 0.05 ml of 10% ammonium persulfate and 0.01 ml TEMED, then the mixture was gently swirled.

3.7.2.2 The separating gel was immediately pipetted into the glass plate sandwich until the height of solution is 1 cm lower than Teflon comb. Care must be taken not to introduce air bubbles into the gel.

3.7.2.3 Distilled water was added to the glass plate sandwich.

3.7.2.4 The gel was polymerized at room temperature within 30 min.

3.7.2.5 Distilled water was discarded.

3.7.2.6 Equilibration of IPG strip

The amount of 6 ml of equilibration solution containing 60 mg of DTT was put into the tube. The IPG strip from A.7 was placed in the tube and sealed with flexible paraffin film. The IPG strip was equilibrated on a rocker for 15 min.

3.7.2.7 The equilibration solution was decanted.

3.7.2.8 The IPG strip was placed on the polyacrylamide gel. Ensure that no air bubbles are trapped between the IPG strip and the gel surface.

3.7.2.9 The marker solution was dropped onto a filter paper, then picked with forceps and applied to the top surface of the gel next to one end of the IPG strip.

3.7.2.10 The IPG strip was sealed in place with agarose sealing solution.

3.7.2.11 The electrophoresis set was assembled and 1x of SDS electrophoresis buffer was added to the upper and the lower chamber.

3.7.2.12 The power supply was connected to the electrophoresis set and applied 150 volts for 7 h.

3.7.2.13 The power supply was disconnected when bromophenol blue tracking dye has reached the bottom of the separating gel.

3.7.2.14 The gel was carefully removed from the glass plates.

3.7.2.15 The gel was stained by silver staining solution (Amersham Pharmacia Biotech, USA).

3.7.3 Silver staining protocol

3.7.3.1 The gel was soaked in 125 ml of fixing solution (Appendix C.12) then placed on a rocker for 30 min.

3.7.3.2 The fixing solution was decanted and 125 ml of sensitizing solution (Appendix C.13) was poured onto the gel. Then the gel was incubated with rocking motion for 30 min.

3.7.3.3 The sensitizing solution was decanted and the gel was washed with distilled water with rocking motion for 5 min. The washing step was repeated 3 times.

3.7.3.4 The 125 ml of silver solution (Appendix C.14) was poured onto the gel and incubated for 20 min on the rocker.

3.7.3.5 The silver solution was discarded then the gel was washed with distilled water and rocked for 1 min. This step was repeated for 2 times.

3.7.3.6 The 125 ml of developing solution (Appendix C.15) was poured onto the gel and incubated for 10 min with the rocking motion.

3.7.3.7 After the developing solution was decanted, the 125 ml of stopping solution (Appendix C.16) was added onto the gel and incubated for 10 min on the rocker.

3.7.3.8 The gel was washed for 5 min with distilled water. This step was repeated 2 times.

3.7.3.9 The gel was preserved in ethanol containing 13% of glycerol.

3.7.3.10 The gel was air dried with cellophane sheets. Care must be taken not to introduce air bubbles between the two cellophane sheets.

3.7.4 Gel analysis

Protein spots on 2D gel were analyzed by Melanie II 2-D PAGE analysis software (Bio-Rad, USA).

3.8 Electroblotting of protein samples

3.8.1 Three pieces of Whatman filter paper were soaked with blotting buffer (Appendix C.21) then they were lied on electroblotter (Trans-Blot SD, Bio-Rad, USA).

3.8.2 The PVDF membrane which had the same size as the gel was cut. Then the membrane was pre-wetted in absolute methanol for 2-3 sec followed by transferring to the blotting buffer. Afterthat, the membrane was placed on Whatman paper from step one (3.8.1).

3.8.3 Interested protein spot on two-dimensional gel was cut off. Then, it was placed on the PVDF membrane.

3.8.4 Two pieces of Whatman filter paper were pre-wetted with blotting buffer and placed on the top. The assemble of blotting sandwich was performed by following in order; pre-wetted of Whatman paper from step 1 (3.8.1), PVDF membrane from step 2 (3.8.2), the gel and two pieces of pre-wetted Whatman paper.

3.8.5 The lid of the unit was placed and the apparatus was applied by constant electricity of 20 volt for 30 min at room temperature.

3.8.6 The cassette was disassembled. Then, the membrane was carefully removed from the sandwich.

3.8.7 The blotted membrane was stained with membrane staining solution (Appendix C.22) for 30 min. Then, it was destained with membrane destaining solution (Appendix C.23).

3.8.8 After a clear background was obtained, the membrane was picked up and dried at room temperature.

3.8.9 In the case of weakly stained spot, two to four excised spot were combined from multiple membranes which obtained by repetition of 2D electrophoresis.

3.8.10 Protein blotted on PVDF membrane was analyzed by Edman sequencing.

Chapter 4

Results and Discussion

4.1 Effect of environmental factors

The morphological transformation of helical to straight form occurred under laboratory as well as under outdoor condition. The environmental factors not only affect growth and chemical compositions, they may also effect rate of morphological transformation. To determine whether the conditions under outdoor condition enhance the rate of morphological transformation, the effects of nutrient concentration, high temperature, high light intensity and combined factors on morphology of *Spirulina* were examined. Results are as follows:

4.1.1 Effect of nutrient concentrations on morphology of *S. platensis* C1

Mass cultivation under outdoor conditions, daily evaporation or rainfall lead to change in nutrient concentration. It might exert stress to the cells and promote the morphological change from helical to straight trichome. To simulate the condition outdoors, the different nutrient concentrations were used in this study; normal Zarrouk's (1x), low (0.5x) and high (2x) concentration. Cultures of helical form were grown under batch condition at 35 ^{o}C and light intensity of 80 $\mu E \cdot m^{-2} \cdot s^{-1}$. Growth rate and cell morphology were observed. Specific growth rate of cells in 1x, 0.5x and 2x Zarrouk's medium were 0.016, 0.01 and 0.006 h^{-1}, respectively. Morphology of cells under light microscope is shown in Fig. 4.1.

It was found that media concentration affected cell shape. Cultures in high nutrient concentration have more lax helix than that in normal and low concentration. Adversely, decreasing in nutrient concentration initiated the tight helix form. However, batch cultivation has limiting factors such as phase of each cell or density of cultures which concerns light attenuation and nutrient limitation problem. These factors were not constant throughout the experiment. To eliminate the fluctuation of these factors, the next experiments were carried out under semi-continuous condition by which daily dilution was performed to keep a constant of cell concentration at 0.35 of optical density at 560 nm.

Cultures grown in 1x have diameter of helix range from 35 to 37.5 μm and 7.5 to 10 μm for pitch. The helix diameter and pitch declined in cells that grown in low media concentration. The increase in diameter of helix and pitch during growth in higher nutrient was observed (Table 4.1). Cellular morphology was shown in Fig. 4.2.

Table 4.1 Effect of nutrient concentration on growth and morphology of helical form of *S. platensis* C1 under semi-continuous condition; normal (1x), high (2x) and low (0.5x). Value are mean ± SD (n).

Zarrouk's medium	μ (h^{-1})	Number of coil / trichome	Helix diameter (μm)	Pitch of helix (μm)	Color of trichome
0.5x	0.014	1-3	36.2 ± 1.16 (22)	8.4 ± 1.24 (22)	Yellowish green
1x	0.016	1-3	41.02 ± 2.62 (20)	11.5 ± 1.8 (20)	Green (+)
2x	0.013	1-4	47.8 ± 2.46 (20)	13.5 ± 1.2 (20)	Green (++)

+ : intensity of color of trichome

n : number of samples

Statistical analysis (ANOVA, $\alpha = 0.05$) showed that nutrient concentration has an effect on cellular morphology. Moreover, alteration in helicity of trichome is a reversible transformation as shown in Fig. 4.3. Change in morphology was observed when exposed to adverse nutrient conditions. Cultures in loose helix become tighter helix when growth was shifted from high nutrient to low nutrient. Whereas the tight coils at low nutrient converted to lax helix when exposed to high nutrient. This finding was also reported by Jeeji Bai and Seshadri [24]. However, under these study conditions, the straight form of this strain was not found.

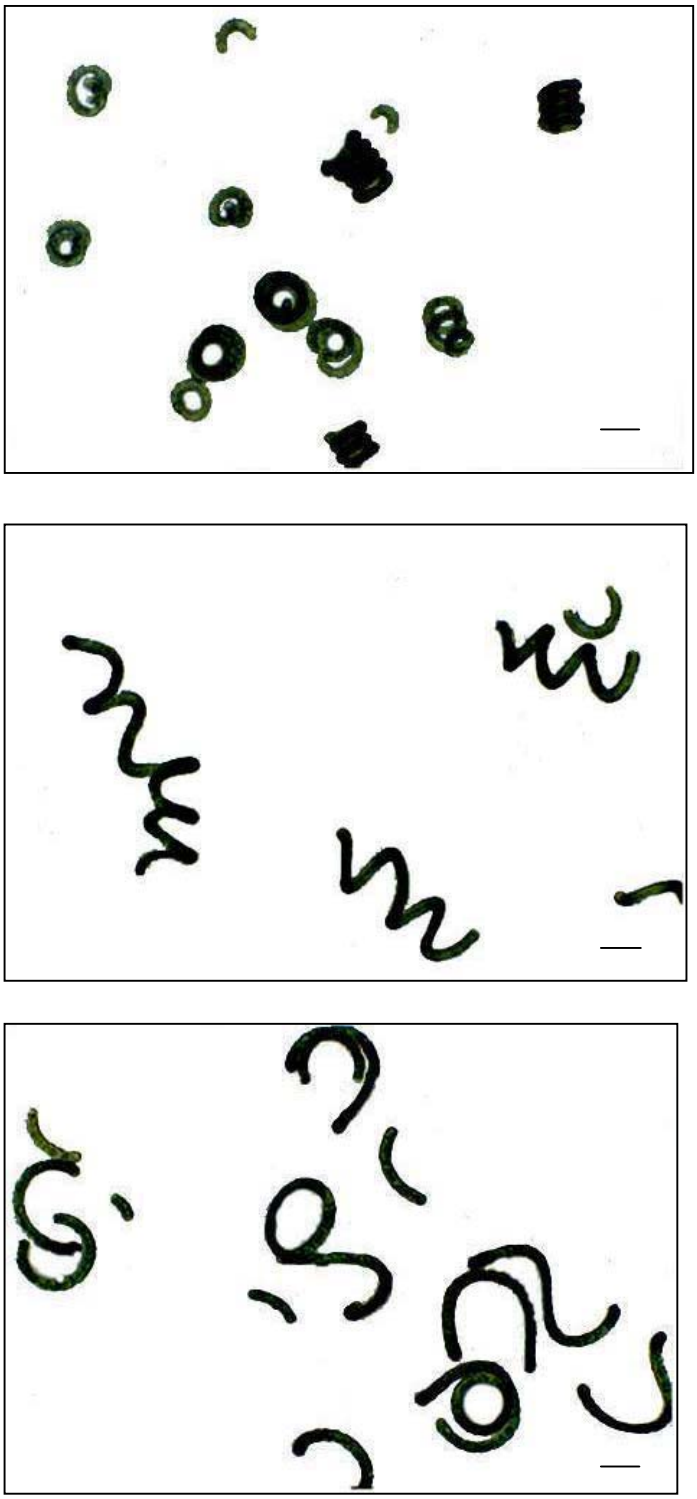


Fig. 4.1 Morphology of *S. platensis* C1 grown at varied Zarrouk's medium concentration; low (0.5x) (A), normal (1x) (B) and high (2x) (C) under batch condition after 56 days. The temperature of 35 ^{o}C and light intensity of 80 $\mu E \bullet m^{-2} \bullet s^{-1}$ were controlled throughout the experiment. Scale bar = 20 μm. Magnification 200x

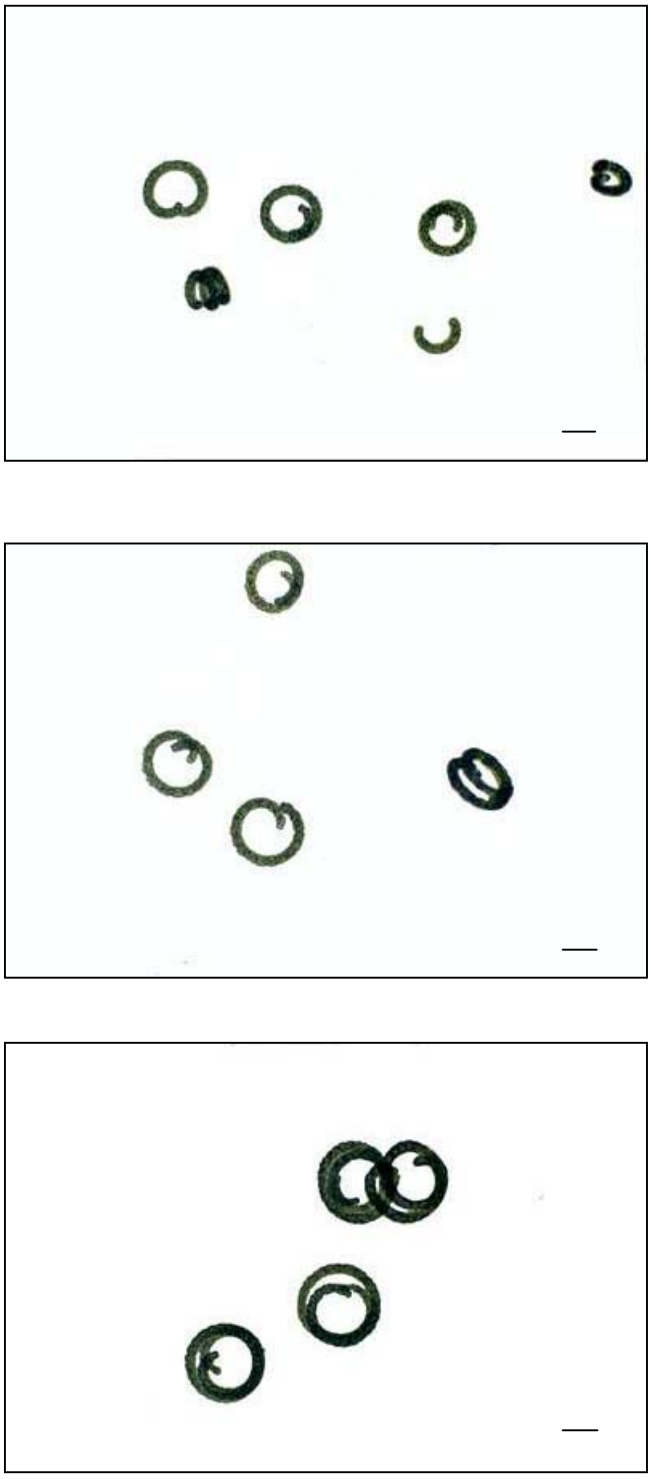


Fig. 4.2 Morphology of *S. platensis* C1 grown under semi-continuous condition at temperature of 35°C and light intensity of 80 μE•m^{-2}•s^{-1}. Zarrouk's medium concentrations; low (0.5x) (A), normal (1x) (B) and high (2x) (C). Scale bar = 20 μm. Magnification 200x

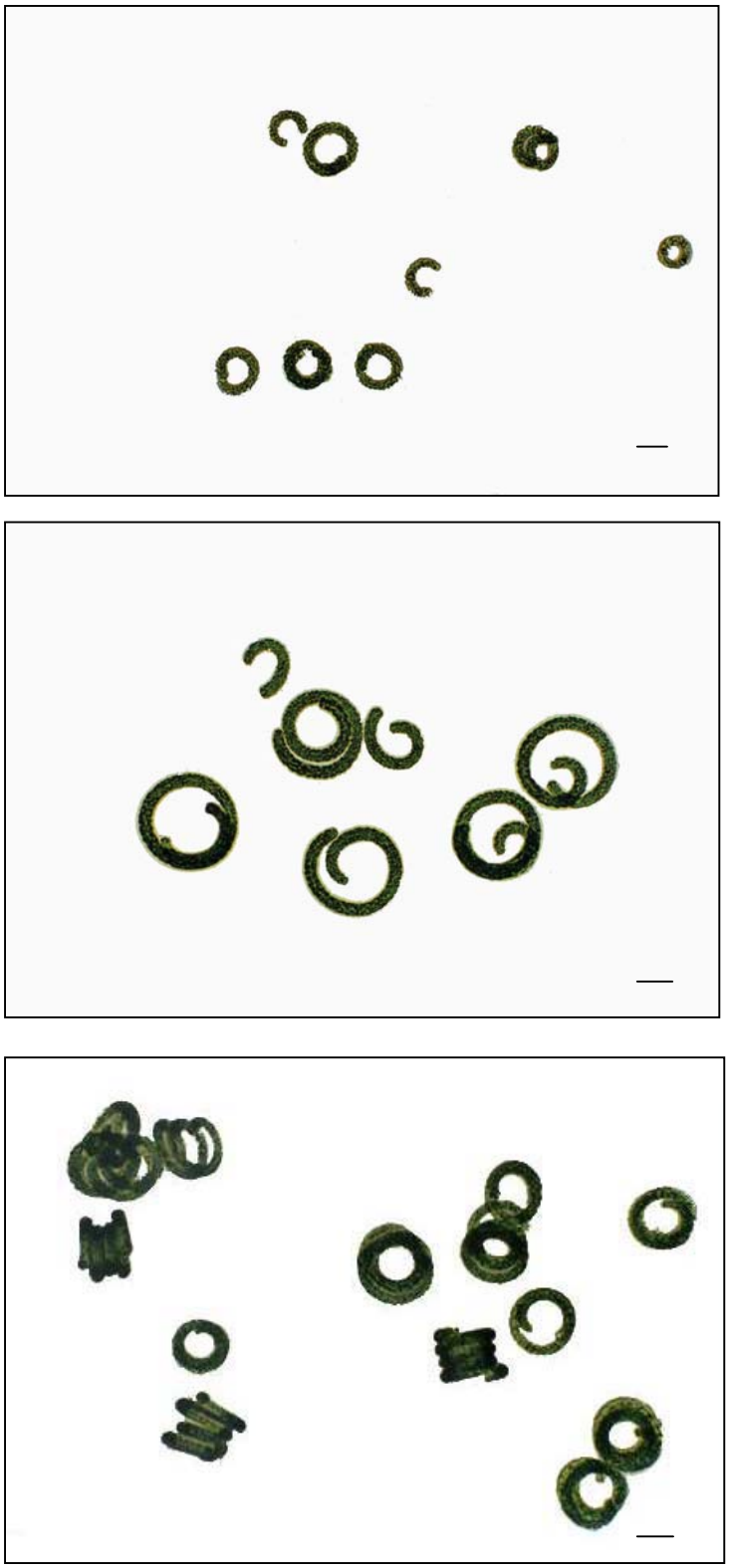


Fig. 4.3 Morphology of *S. platensis* C1 grown under semi-continuous condition for 8 days. The media concentration downshift; 1x to 0.5x (A), 2x to 1x (C) and media concentration upshift; 1x to 2x (B). Temperature of 35 ^{o}C and light intensity of 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ were kept. Scale bar = 20 μm. Magnification 200x

4.1.2 Effect of high temperature on morphology of *S. platensis* C1

Temperature is one of the main factors that affect all metabolic activities. It may have the effect on cell morphology. In this experiment, cells were grown under semi-continuous condition at 40 °C and 35 °C. Morphology of cells were observed under microscope. Experiments were also designed to determine the combined effect of nutrient concentration and temperature. The results are shown in Table 4.2.

Table 4.2 Effect of high temperature and the combined effect of nutrient concentration and high temperature on the morphology of *S. platensis* under semi-continuous condition; optical density at 560 nm = 0.35 and light intensity = 80 $\mu E \cdot m^{-1} \cdot s^{-1}$. Values are mean ± SD (n).

Zarrouk's medium	Temperature (^{o}C)	μ (h^{-1})	Number of coil / trichome	D_{helix} (μm)	Pitch (μm)	Color of trichome
0.5x	35	0.014	1 - 3	36 ± 1.38 (15)	8.5 ± 1.3 (15)	Yellowish green
	40	0.009	1 - 2	38.5 ± 1.4 (15)	11 ± 0.9 (15)	Yellow
1x	35	0.016	1 - 3	42.2 ± 2.5 (15)	11.1 ± 1.2 (15)	Green(+)
	40	0.011	1 - 3	46.4 ± 1.22 (15)	13.6 ± 1.24 (15)	Yellow
2x	35	0.012	1 -4	53.6 ± 2.48 (15)	14.1 ± 1.5 (15)	Green (++)
	40	0.009	1 - 3	58.5 ± 1.6 (15)	16.2 ± 1.26 (15)	Yellowish green

D_{helix} : helix diameter
Pitch : distance between turn
+ : intensity of color of trichome
n : number of samples

The results indicated that high temperature affected cellular morphology by loosening the helix as indicated by the pitch of the helix (distance between coils). Statistical analysis using ANOVA showed that high temperature significantly affected the loosening of coil in *S. platensis* C1 at 95% confidence. However, Van Eykelenburg [62] have shown that *S. platensis* which was isolated from Lake Nakuru in Africa converted to more tightly coiled when growth temperature was increased (13.5 - 40 ^{o}C). Thus, alteration in helicity of cell may not be effected by temperature only, it may also depended on strain of organism.

4.1.3 Effect of high light intensity on morphology of *S. platensis* C1

Outdoor *Spirulina* cultures were exposed to daily fluctuation of light intensity. The fluctuation might lead to the morphological transformation of cells. Cultures were grown under two different light intensities (80 and 300 $\mu E \cdot m^{-2} \cdot s^{-1}$). Combination of light intensity and nutrient concentration were also studied. Growth and cellular morphology were observed during growth under semi-continuous condition.

In normal Zarrouk's medium (1x), cell grown under high light condition (300 $\mu E \cdot m^{-2} \cdot s^{-1}$) had more loosely coiled trichome than cells grown at 80 $\mu E \cdot m^{-2} \cdot s^{-1}$. For the combination of factors, the result showed that high light intensity affected on pitch of cell. Cells grown at high light intensity had more loosely coiled

trichome when compared to cells grown at 80 μE•m^{-2}•s^{-1} at all temperatures. The results are shown in table 4.3.

Table 4.3 Effect of high light intensity and combination of high light intensity and nutrient concentrations on morphology of helical form of *S. platensis* C1 under semi-continuous condition; optical density at 560 nm = 0.35 and temperature = 35 ^{o}C. Values are mean ± SD (n)

Zarrouk's medium	Light intensity (μE•m^{-2}•s^{-1})	μ (h^{-1})	Number of coil / trichome	Helix diameter (μm)	Pitch of helix (μm)	Color of trichome
0.5 x	80	0.014	1 - 3	36.12 ± 1.2 (12)	8.26 ± 1.4 (12)	Yellowish green
	300	0.01	1 - 2	38.5 ± 1.62 (12)	11.3 ± 0.8 (12)	Yellow
1 x	80	0.016	1 - 3	42.5 ± 2.1 (12)	11 ± 1.2 (12)	Green (+)
	300	0.012	1 - 3	44.26 ± 1.95 (12)	13.4 ± 1.4 (12)	Yellow
2 x	80	0.013	1 -3	54.1 ± 2.84 (12)	13.8 ± 1.6 (12)	Green (++)
	300	0.009	1 - 3	61.48 ± 1.2 (12)	16.36 ± 0.94 (12)	Yellowish green

\+ : intensity of color of trichome

n : number of samples

All these results indicated that helicity of trichome can be changed under different environmental conditions. However, these factors did not enhance the transformation to straight trichome under the designed experiments. None of the straight form was detected in these studies.

The cell wall of *Spirulina* is similar to typical gram-negative bacteria cell wall thus the sacculus of *E. coli* of which the property and composition of peptidoglycan have been thoroughly studied could be referred for the discussion. Koch and Woeste [28] studied the elasticity of the peptidoglycan sacculus of *E. coli* by low-angle laser light scattering. They found that the glycan chains were quite inextensible whereas the peptides have conformations varying from a compact to an extended conformation. Hence the sacculus of *E. coli* have shown expanded and contracted reversibly over a fourfold range in area.

It could be proposed that the alteration in coiling of trichome was affected by change in the structure of cell wall. It was known that the peptidoglycan is a shape determining structure covering the cell. Moreover, it has elasticity property that cause by cross-linked peptide bonds in peptidoglycan layer. The peptide chains are highly flexible [19]. They may be unfolded or broken off which led to reformation of chemical bond. Thus, cell wall can expand or contract in response to change in surrounding environment.

4.1.3 Effect of UV light on morphology of *S. platensis* C1

Exposure of bacteria to the natural or artificial ultraviolet (UV) light can led mutation and eventually cause lethal of cells. The morphological conversion of helical form to straight form that is irreversible may cause by UV light during growth under outdoor condition. The effect of UV light on cellular morphology and survival rate was therefore investigated. Cultures were exposed to UV lamp (TUV 15W/G15, UV-C, Philips) at a distance of 15 cm for various exposure times; 5, 7, 10, 12, 15 and 20 min with constant stirring. After incubation in the dark for 10 h the samples were plated onto Zarrouk's medium to determine the number of colonies. In general, the exposure time for obtaining 10% survival of cells was used for the UV irradiation. As seen from Fig. 4.4, the optimal exposure time was 10 min which resulted in 12% survival of cells.

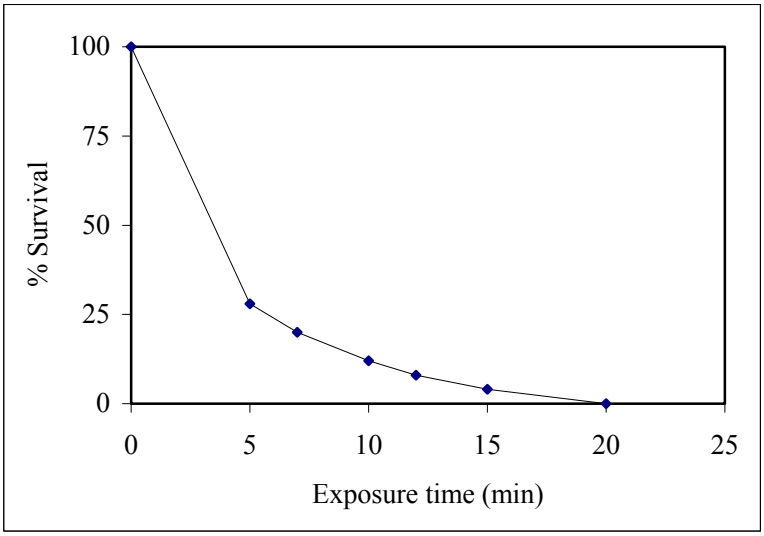


Fig. 4.4 Survival curve of *S. platensis* C1 under various UV exposure times.

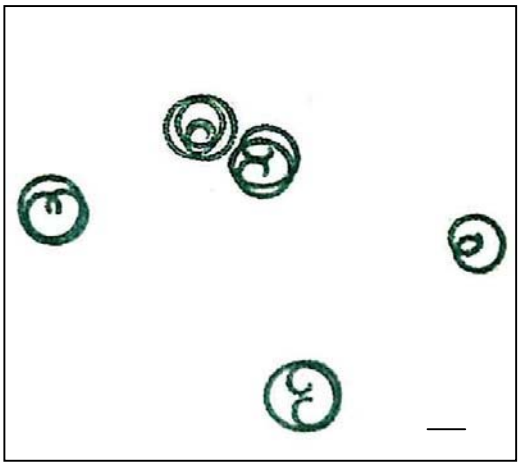
In the mutagenesis with UV irradiation, the morphological transformation of helical to straight trichome was not detected in the survive colonies. This condition results in a level of cell survival which is higher than that recommended to obtain mutants (10%). It may be suggested that the studied condition can not induce the mutagenesis. Therefore, percentage of survival cell may be decreased or down to 1% survival that give rise to the mutant appearance.

The factors or conditions inducing the morphological change to straight trichome of *S. platensis* C1 could not be identified clearly under these studies. This study could only identify the effect of environmental factors on the degree of coiling. However, from the observation in laboratory, under static condition when cultures were placed on the shelf at room temperature with monthly sub-culturing, the straight forms were detected after about 6 months. It may be proposed that the morphological conversion from helical shape to straight form is a spontaneous mutation. In spontaneous mutation, rate of occurrence differs by species that has average rate in prokaryotes between 10^{-8} to 10^{-10} / base pair/ division [21].

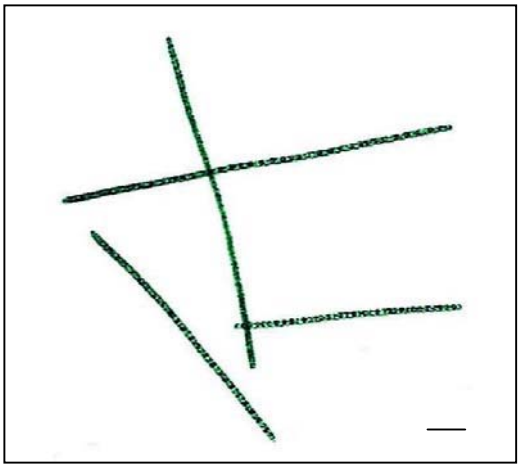
It is of interest to determine other differences between the two forms of *S. platensis* apart from the morphological difference. Two morphologically different forms were isolated as a single trichome and grown in an axenic culture. Then, characteristics of the two forms were compared as shown in the next study.

4.2 Study of helical and straight form

S. platensis C1 occurs as two distinct morphological forms, one with helical trichome (Fig. 4.5A)which was the characteristic of this strain and one with straight trichome (Fig. 4.5B).



(A)



(B)

Fig. 4.5 Morphology of *S. platensis* C1 in different form; helix (A) and straight (B) trichome. Scale bar = 20 μm. Magnification 200x

Two morphones were isolated in pure cultures for comparison in growth, chemical components, photosynthetic activity and protein analysis. The whole protein of both forms was identified by 2D-PAGE.

The growth rate of helical and straight form were 0.017 and 0.016 h^{-1}, respectively. Growth curve was shown in Fig. 4.6. The comparison in pigment, biochemical compounds and photosynthesis of two forms were shown in table 4.4, 4.5 and Fig. 4.7.

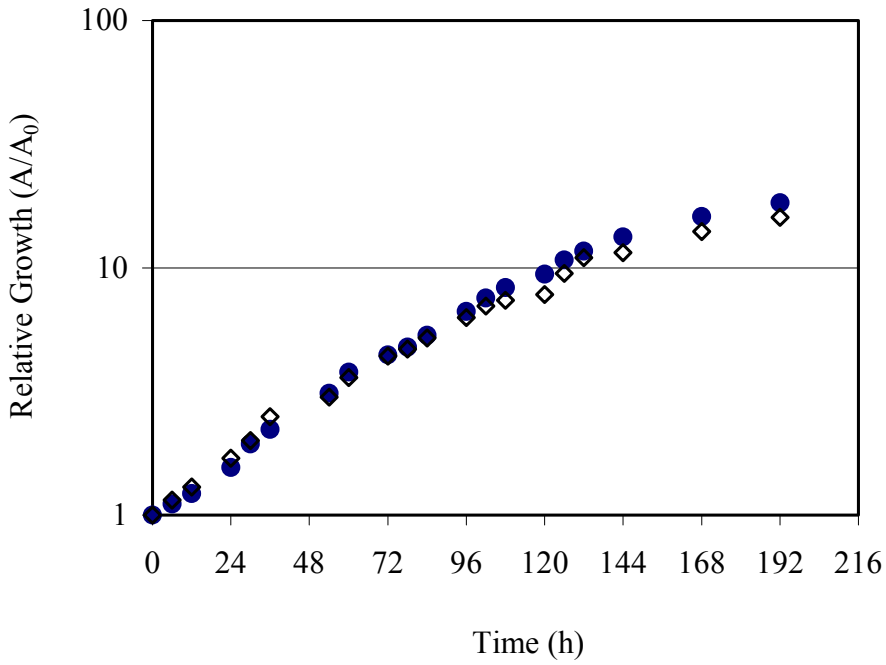


Fig. 4.5 Growth of two forms grown in Zarrouk's medium at 35 ^{o}C 80 $\mu E \bullet m^{-2} \bullet s^{-1}$ (• helix and ◊ straight form).

Table 4.4 Biochemical composition and photosynthesis of helical and straight form grown under semi-continuous condition. Values are mean ± SD (n).

Parameters	**Helical form**	**Straight form**
Chlorophyll a (mg/g cell)	12.5 ± 0.11 (5)	11.75 ± 0.35 (5)
Carotenoid (mg/g cell)	1.6 ± 0.14 (3)	1.5 ± 0.14 (3)
Phycocyanin (mg/g cell)	119.38 ± 2.9 (5)	126.53 ± 1.6 (5)
% Protein	71.03 ± 0.43 (5)	63.58 ± 0.24 (5)
% Carbohydrate	20.09 ± 1.5 (5)	20.6 ± 1.9 (5)
O_2 evolution ($\mu mol\ O_2\ h^{-1} \bullet mg \bullet Chl^{-1}$)	537.52 ± 7.92 (4)	547.6 ± 7.6 (4)

Table 4.5 Comparison of fatty acid composition in helical and straight form under semi-continuous condition. Values are mean ± SD (n).

S.platensis C1	Fatty acid composition (% of Total Fatty Acid)						% Dry weight	
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	TFA	GLA
Helical form	42.17 ± 0.15 (3)	4.63 ± 0.04 (3)	0.59 ± 0.02 (3)	7.09 ± 0.08 (3)	21.11 ± 0.79 (3)	16.97 ± 0.54 (3)	5.08 ± 0.01 (3)	0.87 ± 0.03 (3)
Straight form	42.04 ± 0.04	4.35 ± 0.03	0.6 ± 0.01	6.98 ± 0.18	20.6 ± 0.44	17.05 ± 0.19	4.84 ± 0.04	0.83 ± 0.01

	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)

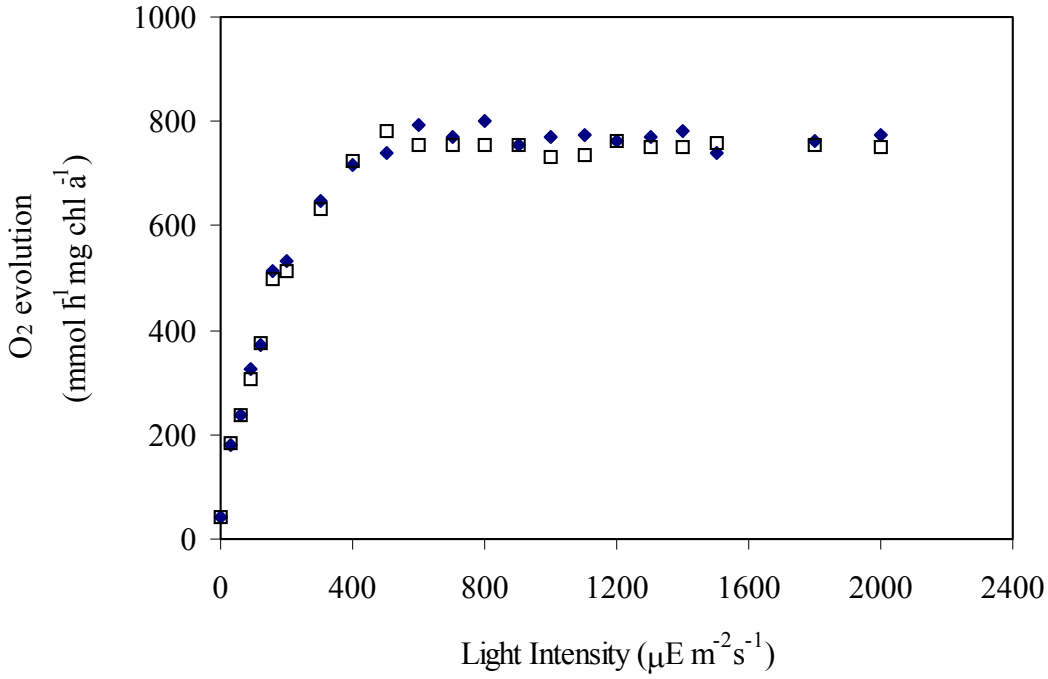


Fig. 4.7 Light response curve of photosynthesis of the two morphological forms; (•) helical and (◊) straight form at 35 ^{o}C, chlorophyll concentration was 2.5 μg ml^{-1}.

The results presented above indicated that no difference in growth, chemical composition and photosynthetic activity of the two morphological forms were found. To further characterize both forms, protein profiles of the whole cell extract were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Membrane-bound proteins were dissolved by SDS solution (Appendix C.4). Then total proteins were separated by their isoelectric point in the first dimension and by molecular weight in second dimension.

In the preliminary experiment, immobilized pH gradient (IPG) with a wide pH range (pH 3 – 10) was used in first dimension. Most of protein spots were appeared at approximate pH of 7 in pH gradient gel. Therefore, the IPG strip gel in a narrow range (pH 4 – 7) was used to improve the resolution in the subsequent experiments.

Each sample contained 120 μg of protein was separated by 2D-PAGE and then stained with Silver stain kit (Amersham Pharmacia, USA). The comparison of protein profiles obtained from 2D-PAGE of the helical and straight form is shown in Fig. 4.8. Some differences in the protein profiles of the two forms were found. A protein at approximate molecular mass of 66.2 kDa (spot 1) was found only in the straight form.

Optical density in all features of the two forms were compared. From Fig. 4.8, the density of protein spots are not equal so they cannot be correctly compared. Then protein concentrations of both forms were adjusted and the experiments were repeated by using IPG strip gel pH 4-7 in the first dimension and 12%SDS-PAGE in the second dimension. Carbonic anhydrase (Amersham Pharmacia, USA) was used as pI marker. It has pI range from 4.8 to 6.7 and molecular mass of 30 kDa which gives twenty spots on 12%SDS-PAGE. The results were shown in Fig. 4.9. The MelanieII software (Bio-Rad, USA) was used to analyze the 2D gel image. All spots were detected and calculated for pI value and molecular mass. In Fig. 4.9, the protein profiles showed 148 and 149 protein spots observed on 2D gels of helix and straight forms, respectively. Both forms have highly similarity in two-dimensional profiles.

Then both gels were aligned and matched for comparison. Optical density values of protein spots were analyzed. It was found that there were 148 spots that were the same in helix and straight forms. Sixty-seven spots among these 148 spots have different spot-density. Fifteen spots of the 67 spots found in the helical form have higher optical density than that of the straight form. While the rest 52 spots of the helical form were observed to have lower spot density than that found in the straight form. These changes in 2D protein profiles of both forms may involve up- and down-regulation. Spot 1 shown in Fig. 4.9 B is the distinct spot found only in the straight form. It has pI value of 5.24 and molecular mass of 66.6 kDa but it is likely to overlap with the nearly spots. The zoom-in of spot one was shown in Fig. 4.10. Thus it necessary to optimize the appropriate condition for the resolution improvement of spot 1.

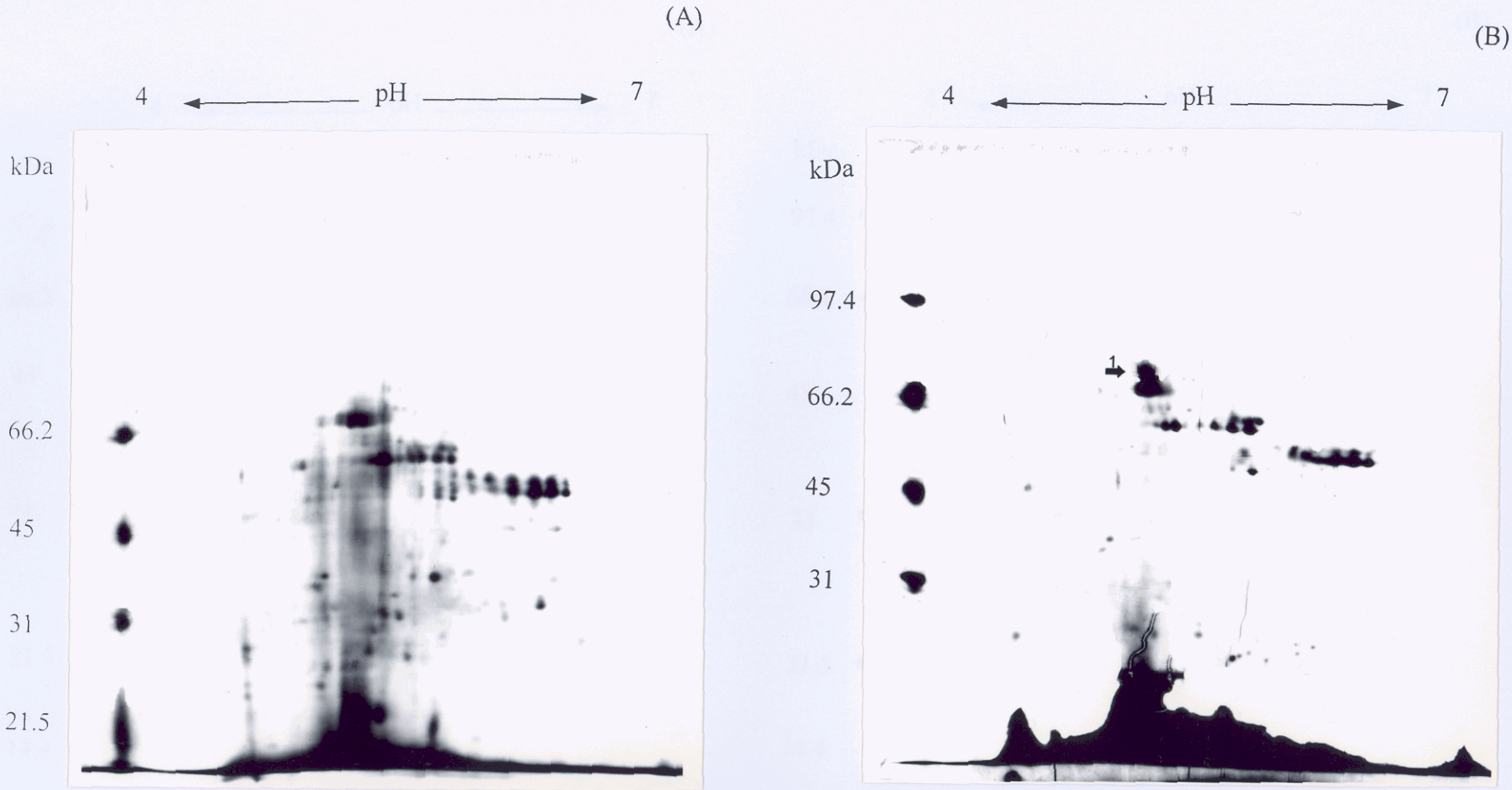


Fig. 4.8 Two-dimensional gels of *S. platensis* C1; helix (A) and straight (B) form from stationary phase (7 days). The first dimension was performed by using an IPG strip gel pH 4 – 7 and 12% SDS-PAGE was employed in the second dimension. A low-range SDS-PAGE protein standard (14.4 to 97.4 kDa ; Bio-Rad, USA) was used in all the gels as molecular weight markers.

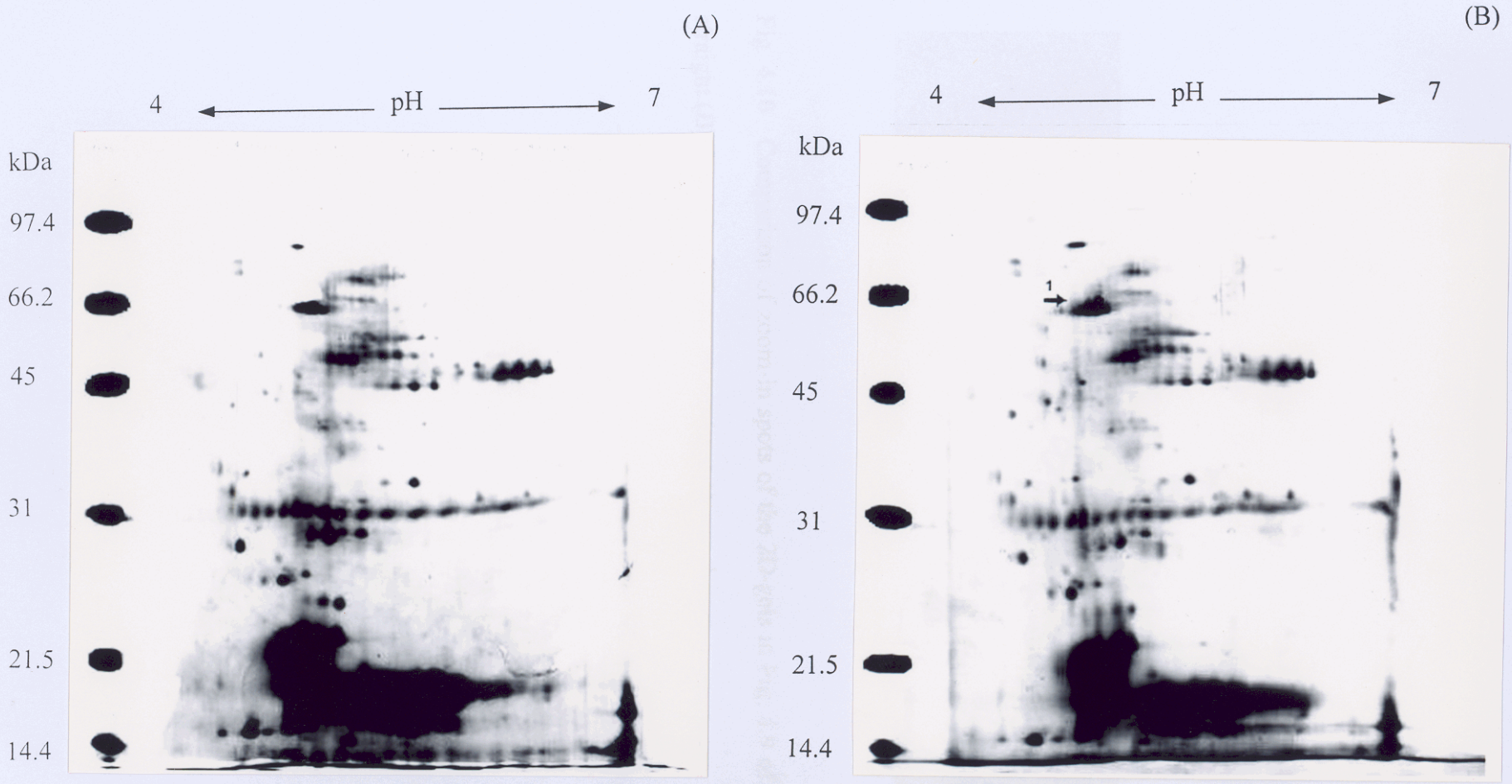


Fig. 4.9 Two-dimensional gels of *S. platensis* C1; helix (A) and straight (B) form from stationary phase (7 days). The first dimension was performed by using an IPG strip gel pH 4 – 7 and 12% SDS-PAGE was employed in the second dimension. The carbonic anhydrase (pI range 4.8 to 6.7 and 31 kDa; Amersham Pharmacia Biotech, USA) was used as pI markers.

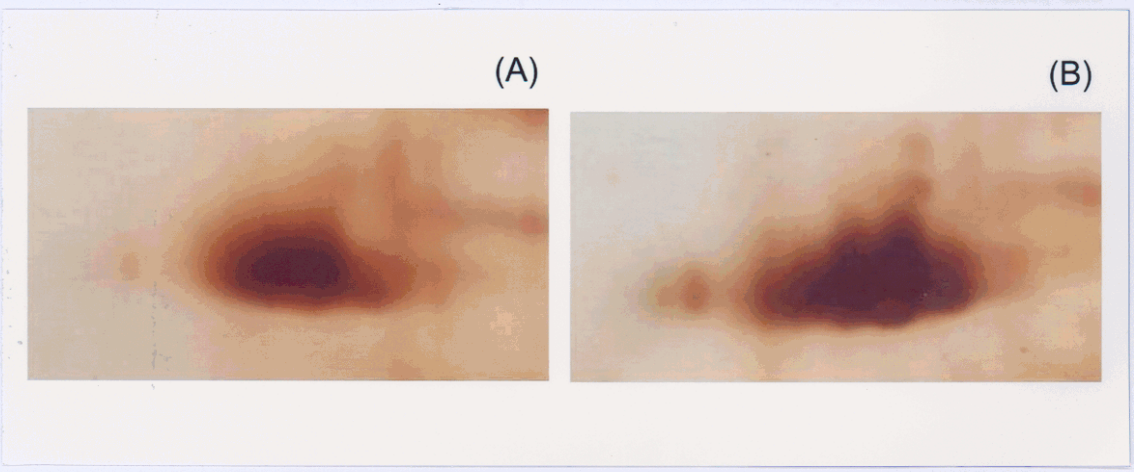


Fig. 4.10 Comparison of zoom-in spots of the 2D-gels in Fig. 4.9 of helix (A) and straight (B) form, the difference spot (spot 1) was shown .

The pI value and molecular mass of protein spots in Fig. 4.9 were used for estimation of these two values of spot 1 in Fig. 4.8B where the pI marker was absent. It was found that spot 1 has an approximate pI of 5.26 and an approximate molecular mass of 73 kDa. Moreover, the group of proteins that has molecular mass less than 21.5 kDa were unresolved, thus these proteins cannot be analyzed. Therefore, the subsequent experiments were performed in order to increase the resolution of the spot 1 and the protein with molecular mass less than 21.5 kDa.

Another electrophoretic condition used in the second dimension that was carried out in order to obtain well-resolved spots on the 2D gel is decreasing pore size of the separating gel. Increasing of percentage of acrylamide led to reducing of gel pore size. Thus, 15% SDS-PAGE was performed and the protein profiles were shown in Fig. 4.11. The spot 1 of the straight form (Fig. 4.11B) was not clearly separated as a single spot. The resolution of the proteins at molecular mass less than 21.5 kDa was not as good as expected; therefore, the analysis of these proteins cannot be done. The results also showed that the decreasing of pore size did not enhance the resolution of proteins of interest. In order to characterize the proteins of interest, the electrophoresis condition must be optimized for separation.

Gradient gel may be an approach to improve the separation of the proteins of interest. This gel provides continuous decrease in pore size from the top to the bottom of the gel. It can be used to separate protein effectively over a wide range of sizes. The 5% to 20% SDS-PAGE was used for the second dimension and the result was shown in Fig. 4.12. It was shown that the pattern of protein molecular mass less than 21.5 kDa of the helical form was the same as that of the straight form. Although, the spot 1 in straight form was not well separated from other spots.

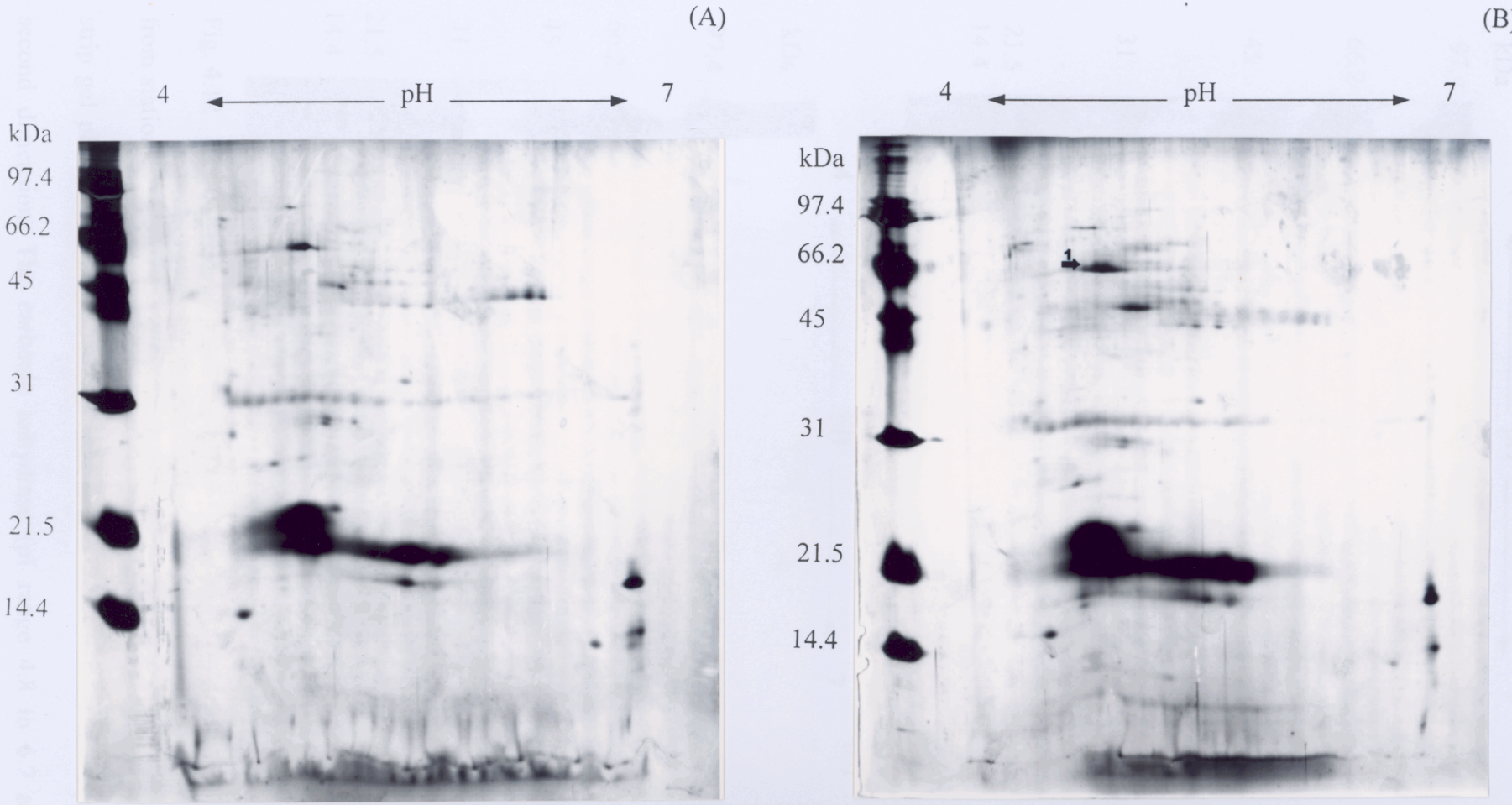


Fig. 4.11 Two-dimensional gels of *S. platensis* C1; helix (A) and straight (B) form from stationary phase (7 days). The first dimension was performed by using an IPG strip gel pH 4 – 7 and 15% SDS-PAGE was employed in the second dimension. The carbonic anhydrase (pI range 4.8 to 6.7 and 31 kDa; Amersham Pharmacia Biotech, USA) was used as pI markers.

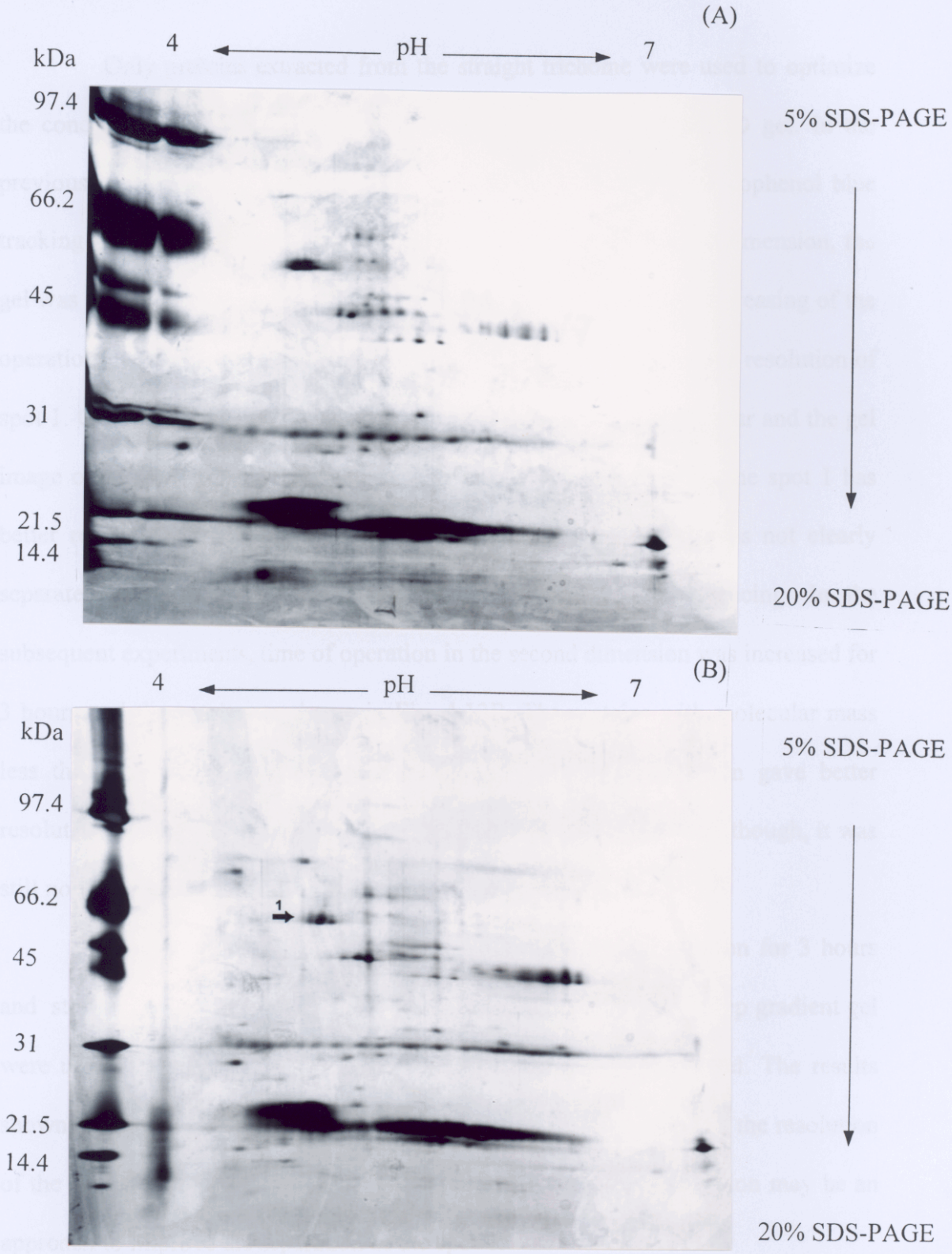


Fig. 4.12 Two-dimensional gels of *S. platensis* C1; helix (A) and straight (B) form from stationary phase (7 days). The first dimension was performed by using an IPG strip gel pH 4 – 7 and gradient gel 5% - 20% SDS-PAGE was employed in the second dimension. The carbonic anhydrase (pI range 4.8 to 6.7 and 31 kDa; Amersham Pharmacia Biotech, USA) was used as pI markers.

Only proteins extracted from the straight trichome were used to optimize the condition that would give a good resolution of the spot 1 on 2D gel. In the previous electrophoresis condition (Fig. 4.8 to Fig. 4.12), when bromophenol blue tracking dye has reached the bottom of separating gel in the second dimension, the gel was removed from glass plates and stained with Silver stain kit. Increasing of the operation time in the second dimension may be the way to improve the resolution of spot 1. Operation time of the second dimension was increased for 1 hour and the gel image of this condition was shown in Fig. 4.13A. It was found that the spot 1 has better resolution than in the previous condition, however, it still was not clearly separated enough for further identification by amino acid sequencing. In the subsequent experiments, time of operation in the second dimension was increased for 3 hours and the result was shown in Fig. 4.13B. The proteins with molecular mass less than 21.5 kDa were fallen from separating gel. This condition gave better resolution of spot1 than the increasing of operation time for 1 hour. Although, it was still not clearly separated as a single spot.

The combination of time increasing in the second dimension for 3 hours and step gradient gel was used to separate the spot 1. Two types of step gradient gel were used; one is 10% to 16% gel, and the other is 12% to 16% gel. The results shown in Fig. 4.14 indicated that the step gradient gel did not improve the resolution of the spot1. Therefore, the modification of the first dimension condition may be an approach to improve the separation of the spot 1.

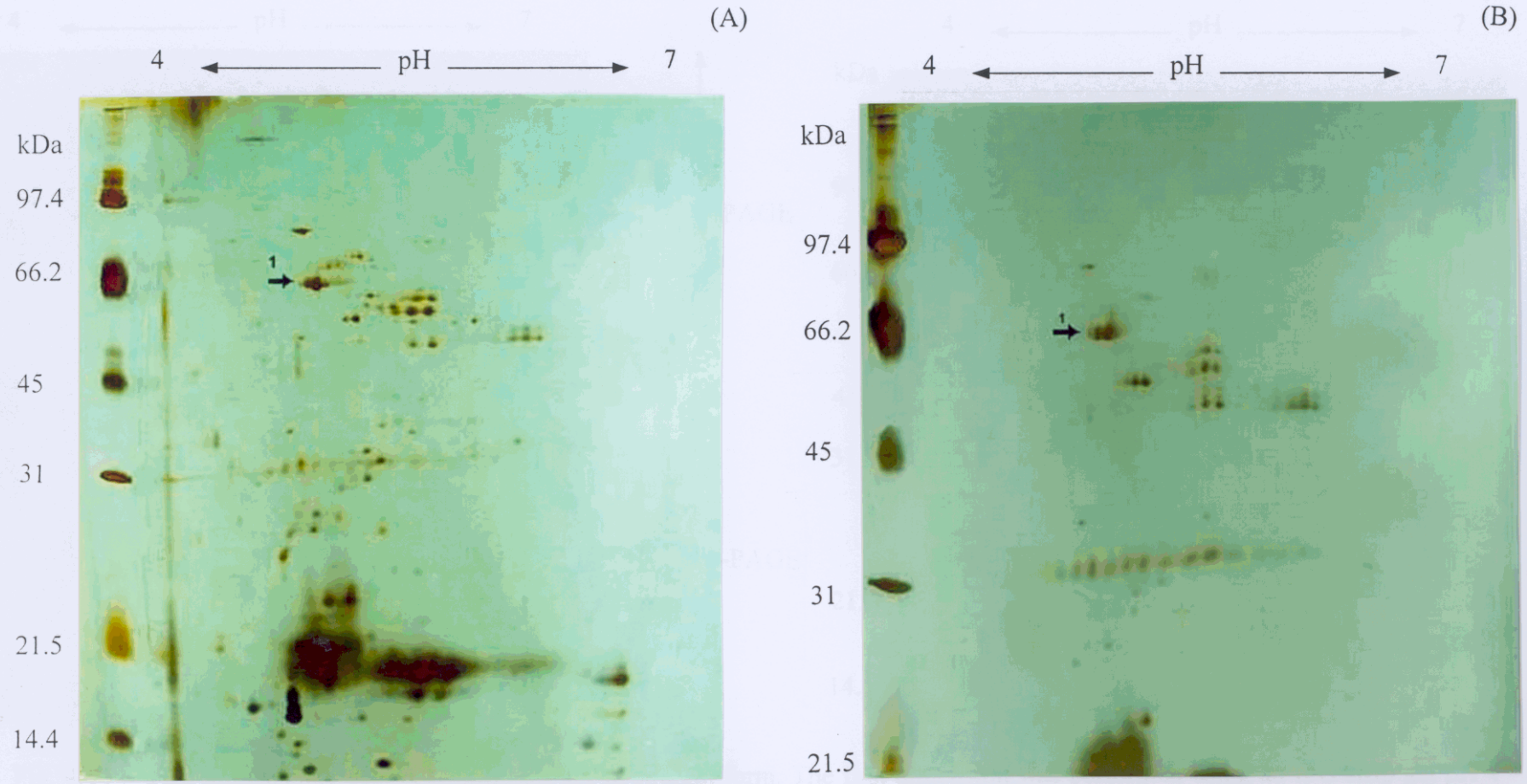


Fig. 4.13 Two-dimensional gels of *S. platensis* C1 in straight form. The first dimension was performed by using an IPG strip gel pH 4 – 7 and 12% SDS-PAGE was employed in the second dimension where the operation time increased for 1 hour (A) and 3 hours (B). The carbonic anhydrase (pI range 4.8 to 6.7 and 31 kDa; Amersham Pharmacia Biotech, USA) was used as pI markers.

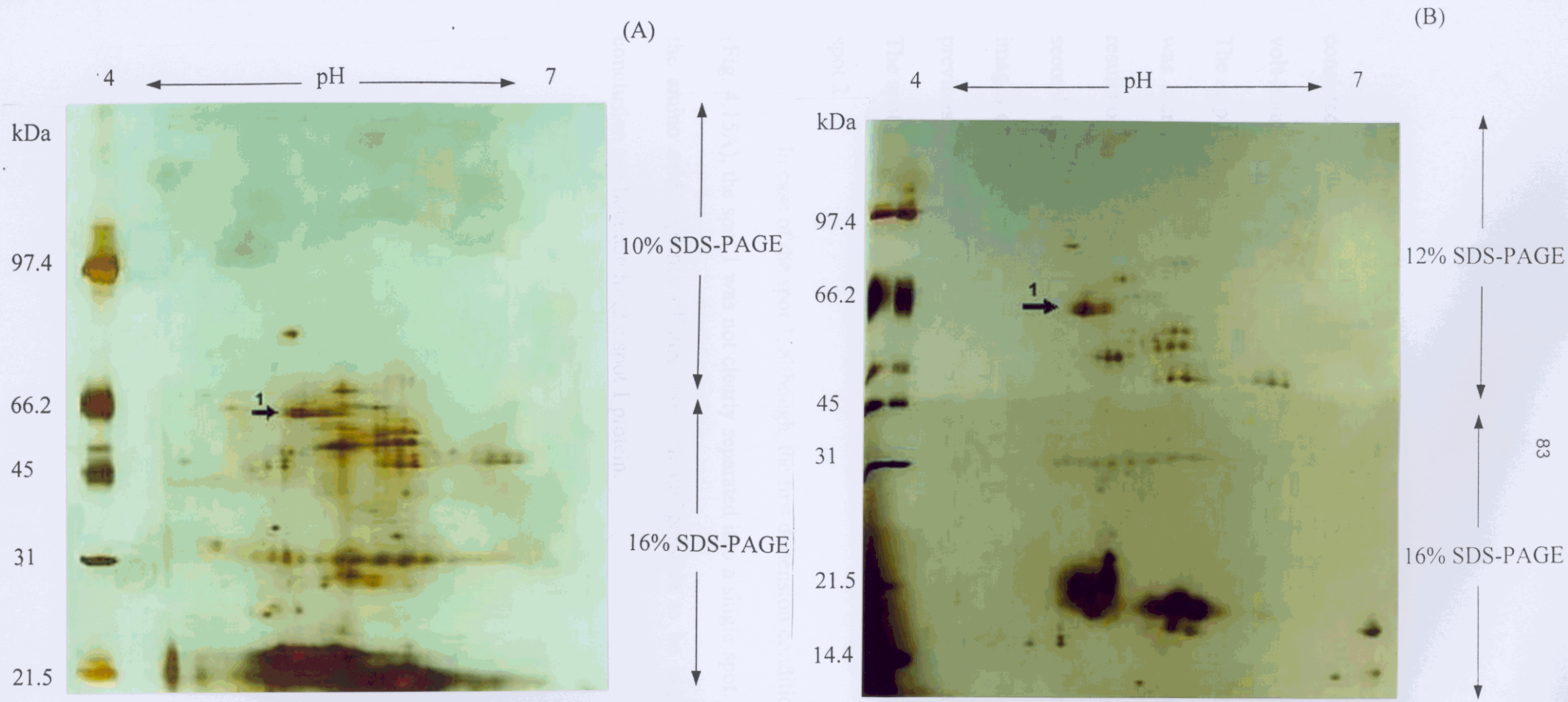


Fig. 4.14 Two-dimensional gels of *S. platensis* C1 in straight form. The first dimension was performed using an IPG strip gel pH 4 – 7. The step gradient gels; 10% to16% SDS-PAGE (A) and 12% to 16% SDS-PAGE (B) was employed in the second dimension where the operation time was increased for 3 hours. The carbonic anhydrase (pI range 4.8 to 6.7 and 31 kDa; Amersham Pharmacia Biotech, USA) was used as pI markers.

In the first dimension, isoelectric focusing (IEF) protocol was followed, it consisted of three steps; (i) 500 volts for 500 volt-hours, (ii) 1,000 volts for 1,000 volt-hours and (iii) 8,000 volts for 16,000 volt-hours. The total volt-hour was 17,500. The step that was modified in the first dimension was step three where the volt-hour was increased from 16,000 to 26,000. Thus, the total volt-hour was 27,500. The results of this first dimension modification combined with the time increasing in the second dimension for 3 hours were shown in Fig. 4.15. After comparison of the images of helical and straight forms, a new spot that has not been found using the previous condition was appeared on the 2D image of the straight form (Fig. 4.15B). The spot has pI value of 4.88 and molecular mass of 66.2 kDa and it is represented as spot 2.

In case of the spot 1, although, the first dimension condition was modified (Fig. 4.15A), the spot 1 was not clearly separated into a single spot. For this reason, the amino acid sequence of this spot still not possible to be identified, thus, no conclusion can be made for the spot 1 protein.

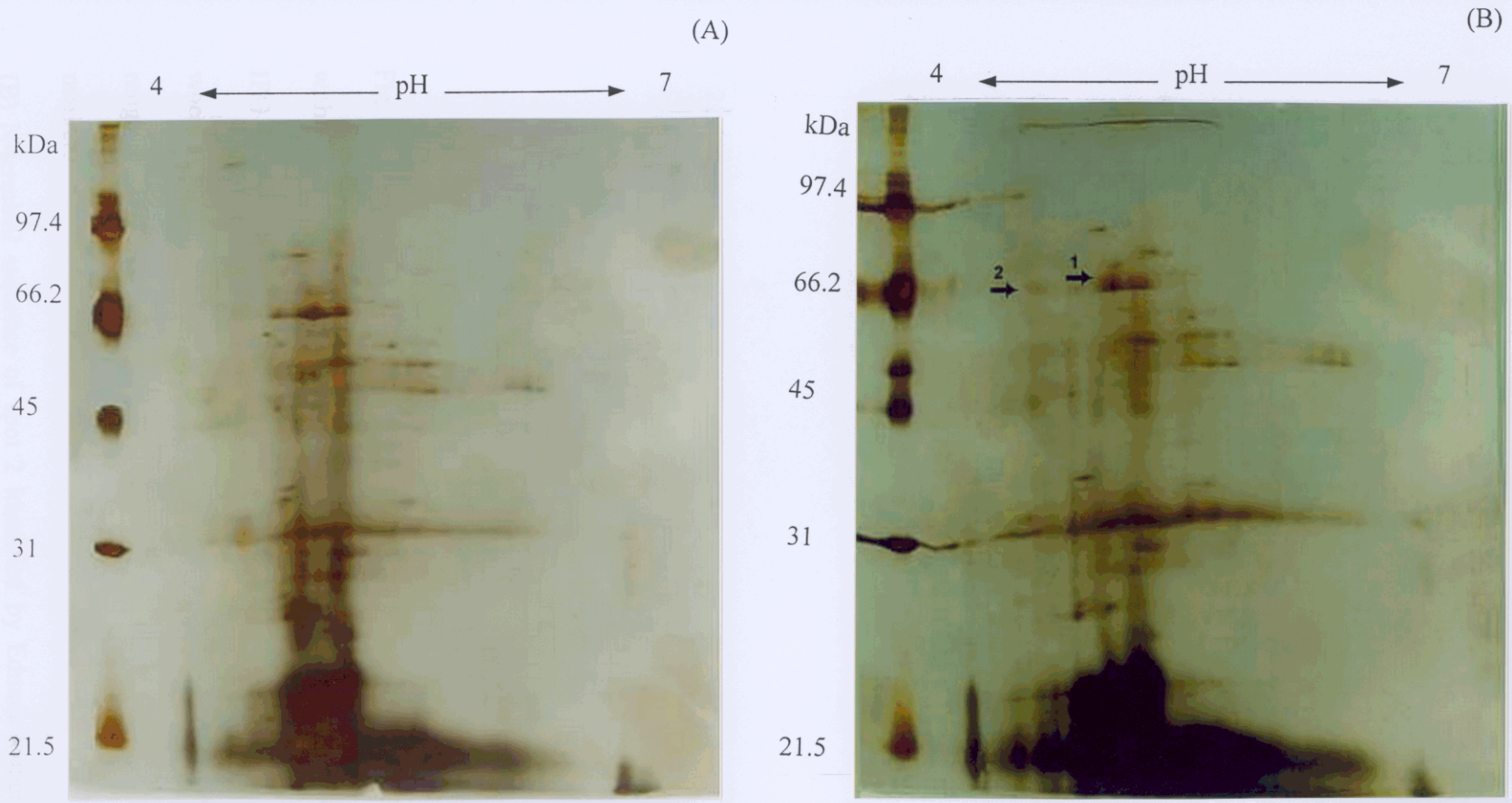
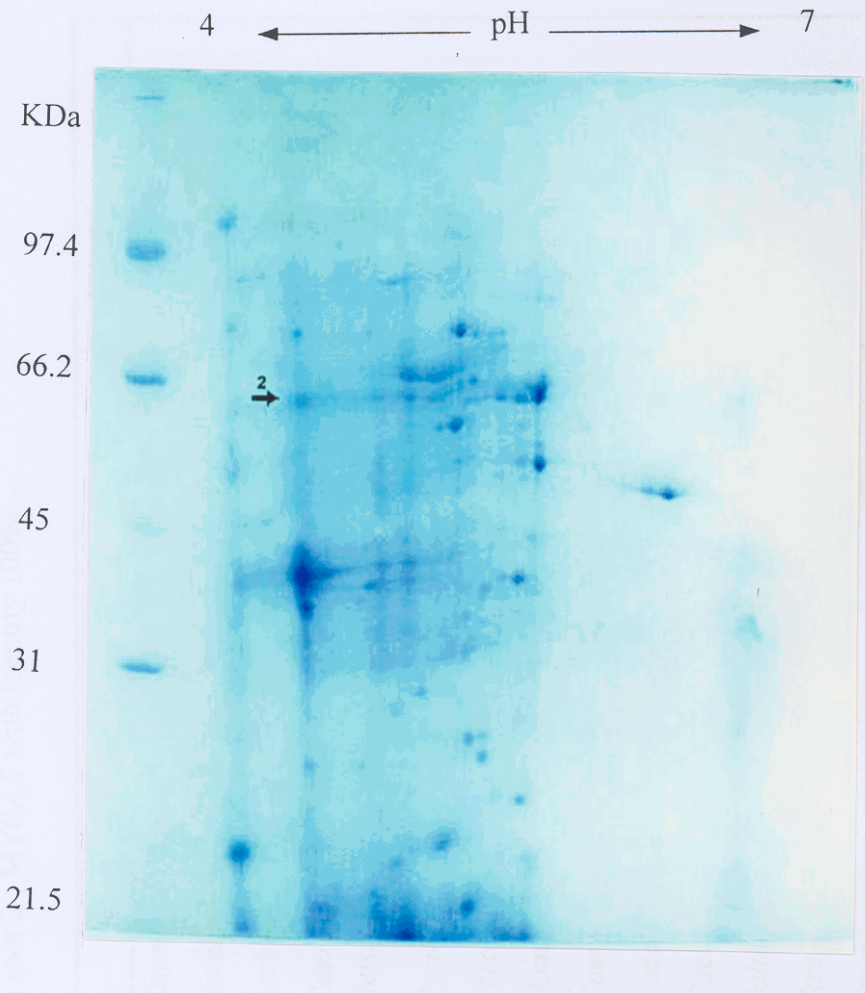


Fig. 4.15 Two-dimensional gels of *S. platensis* C1; helix (A) and straight (B) form. The first dimension was performed by using an IPG strip gel pH 4 – 7 and 12% SDS-PAGE was employed in the second dimension where the operation time was increased for 3 hours. The total volt – hour is 27,500. See the text for a more detail. The carbonic anhydrase (pI range 4.8 to 6.7 and 31 kDa; Amersham Pharmacia Biotech, USA) was used as pI markers.

(A)



(B)

MYSAT

Fig. 4.16 (A) Two-dimensional gels of *S. platensis* C1 in straight form was stained with Coomassie Brilliant Blue R250. The first dimension was performed by using an IPG strip gel pH 4 – 7 and 12% SDS-PAGE was employed in the second dimension where the operation time was increased for 3 hours. The carbonic anhydrase (pI range 4.8 to 6.7 and 31 kDa; Amersham Pharmacia Biotech, USA) was used as pI markers.

(B) N-terminal sequence of spot 2 identified by Edman sequencing. Amino acid residues; M (Met), Y (Tyr), S (Ser), A (Ala) and T (Thr).

Table 4.6 Alignments of N-terminal sequence of protein spot 2 ; MYSAT with identity 100%

Protein	Organism	Length (base)	Matching position
1. Hypothetical protein C36C9.1	*Caenorhabditis elegans*	836	558 - 562
2. Hypothetical protein XP_001834	*Homo sapiens*	405	207 - 211
3. Hypothetical protein	*Archaeoglobus fulgidus*	329	3 - 7
4. Hypothetical protein C34E7.0	*Caenorhabditis elegans*	356	1 - 5
5. Hypothetical DNA-binding protein	*Neisseria meningitidis Z*	559	48 - 52
6. Hypothetical protein	*Pyrococcus horikoshii*	841	37 - 41
7. Hypothetical protein	*Aeropyrum pernix*	1933	1809 - 1813
8. Hypothetical protein XP_032060	*Homo sapiens*	361	163 - 167
9. Hypothetical protein W01C8.6	*Caenorhabditis elegans*	634	627 - 631
10. Hypothetical protein W01C8.6	*Caenorhabditis elegans*	319	312 - 316
11. Hypothetical protein C01F1.3	*Caenorhabditis elegans*	631	421 - 425
12. Putative arylsufate sulfotransferase	*Salmonella typhimurium*	596	540 - 544
13. Putative protease	*Streptomyces coelicolor*	347	310 - 314
14. Putative bifunctional hydroxylase / oxidoreductase	*Streptomyces coelicolor*	801	446 - 450
15. Putative riboflavin kinase	*Mycobacterium leprae*	331	227 - 231
16. ABC transporter	*Arabidopsis thaliana*	1450	1267 - 1271
17. ABC transporter (permease protein)	*Mesorhizobium loti*	298	255 - 259
18. Oligopeptide ABC transporter	*Mesorhizobium loti*	332	124 - 128

Table 4.6 Alignments of N-terminal sequence of protein spot 2 ; MYSAT with identity 100% (continued)

Protein	Organism	Length (base)	Matching position
19. Cystathionase (Cystathionine gamma - lyase)	*Homo sapiens*	405	207 - 211
20. Cystathionine gamma - lyase (EC 4.4.1.1)	*Homo sapiens*	361	163 - 167
21. ATP - dependent RNA Helicase	*Plasmodium falciparum*	433	238 - 242
22. ATP - dependent helicase	*Listeria monocytogenes*	779	385 - 389
23. Cell division protein ftsA	*Rickettsia conorii*	411	369 - 373
24. Polyketide synthase	*Streptomyces coelicolor*	2297	923 - 927
25. CG8728 gene product	*Drosophila melanogaster*	556	404 - 408
26. ESTs	*Arabidopsis thaliana*	306	138 - 142
27. Late expression factor 9	*Spodoptera litura nucleopolyhedrovirus*	498	1 - 5
28. Unknown protein	*Lactococcus lactis*	1649	139 - 143

The protein spot 2 was clearly separated as a single spot. It can be subjected to amino acid sequencing. Therefore, in the subsequent experiment, for the first dimension the proteins extracted from straight trichomes were concentrated and the sample volume was also increased. After the second dimension was completed, the 2D gel was stained with Coomassie Brilliant Blue R250 and the result was shown in Fig. 4.16A. As seen on the 2D-gel image, the protein spot 2 still clearly separated as a single spot. Thus, it was electroblotted onto a PVDF membrane and then analyzed for amino acid sequence. N-terminal sequencing (Edman degradation) was used for sequence determination of this spot. The sequencing was performed by Midwest Analytical, Inc., USA.The amino acid sequence from the N-terminus of the spot 2 (Fig. 4.15B) was subjected to similarity searched by using Blast program (http://www.ncbi.nlm.nih.gov). Because of the short amino acid sequence, five amino acids, there are many groups of protein in the database that similar to the sequence of spot 2.

As seen from table 4.6, majority of the alignment of N-terminal sequence of the protein spot 2 was hypothetical protein. Furthermore, spot 2 was matched with many kinds of protein. The first was arylsulfate sulfotransferase that involved in the metabolic pathway of compound bearing phenolic functional groups. It transfers a sulfate group of phenolic sulfate ester to phenolic compounds [46]. The second was riboflavin kinase that related to riboflavin metabolism. For the third group, the sequence of spot 2 was similar to polyketide synthase which involved in the biosynthesis of natural product such as flavonoids. Moreover, it also similar to cystathionase that was the enzyme for synthesis cysteine. In the similarity of cell

division proteins, these proteins function as the universal prokaryotic division protein. At least nine essential genes were required for cell division and the products of them; ftsA, ftsI, ftsK, ftsL, ftsN, ftsQ, ftsW,ftsZ and ZipA were localized to the division plane during septation [71].

ABC transporter is one of the similarity search results of spot 2. In gram negative bacteria, the ABC transporters are located in the cytoplasmic membrane and periplasm. They are usually highly specific for one substrate transported in one direction, uptake or export [67]. Some of them are involved in transportation of lipids, lipid-like molecules, peptides or peptide-like toxins. The remaining similarity search results are protease, bifunctional hydroxylase/oxidoreductase, ATP-dependent helicase, expressed sequence tags (ESTs) and late expression factor.

From the 2D-electrophoresis results, total number of different protein spots between helical and straight trichome is 69spots which 2 unique spots were only found in straight form. The remaining 67 protein spots were difference in spot density which 15 spots and 52 spots among 69 spots in the helical form have higher and lower of density, respectively, than that of the straight form. Change in protein expression level can effect the density of protein spot on the gel. Thus, increasing or decreasing in spot density refer to up- or down-regulated proteins. It may be proposed that 69 protein spots were involved in the morphological transition of *S. platensis* strain C1.

As mentioned in the results, no occurrence of morphological conversion from helix trichome, which is a typical shape of *S. platensis* C1 to straight trichome during exposure to various study conditions was found. However, the morphological conversion occurred when the culture was grown under static condition. Thus, it can

be proposed that the morphological transformation to atypical shape of this strain is a spontaneous mutation. Furthermore, the morphological transition might be related to 69 protein spots that obtained from comparison of protein profile of both helical and straight form.

Chapter 5

Conclusion and Suggestions

5.1 Conclusion

The effect of environmental factor, nutrient concentration, high temperature, high light intensity and UV, on the morphology of *S. platensis* C1 were studied. The results indicated that these factors led to the change in helicity of trichome. At high nutrient concentration, the cells have loose trichome while the tightening cells appeared in low nutrient concentration medium. Moreover, during exposure to high temperature, the cells also have loose helix which is the same as those found in the high light intensity. No occurrence of morphological conversion from helical to straight form was found under the study conditions. The helical forms were exposed to UV irradiation but the condition can not induce the mutant which differs in morphology. However, the straight forms appeared spontaneously during long-term static cultivation. It might be proposed that the conversion from helical to straight trichomes was a spontaneous mutation.

The comparison between the two forms showed similarity in growth, biochemical compounds and photosynthetic characteristics. The protein profiles of the helical form was compared with the profile of the straight form by using 2D-PAGE. From the 2D-gel, 148 and 150 protein spots were observed in helix and straight form, respectively. The protein profiles of both forms were found to be similar. Sixty-seven spots have different spot intensity. It was found that the 15 spots and the 52 spots of 67 spots found in the helix form have higher and lower spot density than that found in the straight form, respectively.

Furthermore, the result illustrated that there were two protein spots which were unique in the straight trichome. The spot one is the protein that has pI value of 5.24 and molecular mass of 66.6 kDa. The resolution of this spot was not clear enough to be separated into a single spot. Thus, its amino acid sequence cannot be determined. Due to the difficulty in determining amino acid sequence of the spot one (not possible to be identified) thus no conclusion can be made for the spot one. The other is the spot two that has pI value of 4.88 and molecular mass of 66.2 kDa. It was found when the first dimension condition was modified. This spot was analyzed for amino acid sequence using Edman degradation. It consisted of five amino acid residues; M (Methionine), Y (Tyrosine), S (Serine), A (Alanine) and T (Threonine), consecutively from the N-terminus of the polypeptide.

From similarity searches of the spot two, it was similar to many groups of protein in the database. However, the morphological transformation of the cells may involved with these 69 proteins. Furthermore, it was known that cell wall of bacteria is the shape-determining and shape-maintaining structure. The alteration of murein subunits in the peptidoglycan synthesis is responsible for the change in morphology. Thus, the conversion of helical to straight forms may be a result of the alteration of peptidoglycan.

5.2 Suggestions

1. *Spirulina* has cell wall similar to gram-negative bacteria. The transporters in gram-negative bacteria locate in their cytoplasmic membranes. Because the ABC transporters are also located in cytoplasmic membrane; therefore, sample for determination of protein profile by 2D-PAGE should be separately extracted from various cell fractions such as thylakoid membrane, plasma membrane of the two forms. Comparison of the 2D-images of these fractions might be an effective way to find the differences between the two forms.

To investigate the transporters of the two forms, cell membrane should be separated and determined by 2D-PAGE. Differentiation of spots should be analyzed for isoelectric point (pI), molecular mass and N-terminal sequence. Then similarity search with amino acid sequence available in database should be performed. This might be an effective approach to characterize the differences between the two forms of *Spirulina*.

2. It was known that bacterial morphology was affected by the alteration of the cell wall. To investigate the biochemical of the cell wall in the spiral and the straight trichome, the peptidoglycan should be extracted by the method studied by Quintela *et al*. [45] in which cells were boiled in 4% SDS and then broken with glass beads under nitrogen flow in order to keep the suspension chilled. Then, the unbroken cells and glass beads were removed by low speed centrifugation. Determination of muropeptide was performed by enzymatic treatment using muramidase or amidase. The type muropeptides were determined by high performance liquid chromatography (HPLC) or mass spectrometry (MS) technique.

By the technique mentioned, we should be able to pinpoint the difference of cell wall of the two forms.

3. The fluorescence 2D difference gel electrophoresis (2D DIGE) can be used for comparison of protein expression of different samples [72]. In this technique, multiple samples were separated on the same gel and each individual sample was labeled by fluorescent dye, which was different from the dye used for the other sample, prior to 2D electrophoresis. The NHS esters of fluorescent dyes; Cy3 with blue color and Cy5 with red color bind to lysine residues of protein samples [73]. Spots on the gel of each sample were visualized by scanning the gel using excitation and emission wavelengths unique to each dye used. Therefore, 2D DIGE is a high-resolution method that significantly increases throughput, ease-of-use, has accurate quantification of protein expression and reduces the experimental steps in protein processing for identification.

4. Mass spectrometry is an analytical technique that is widely used for characterization of proteins from 2D gels. It is a rapid method for protein identification that relies on the accuracy of molecular weight of proteins. This method can detect low levels of protein at sensitivity below 1 pM and can be operate for automation [13]. The protein of interest was subjected to proteolysis with an enzyme (usually trypsin) and the resulting peptides were analyzed for their mass values, then compared with peptide mass values in database. It is faster and more sensitive than Edman sequencing.

Two common ionization techniques of mass spectrometry for proteins are electrospray (or nanospray for smaller quantities) ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). They are coupled to different mass

analyzers. In general, MALDI-time-of-flight MS is more effective for the analysis of higher molecular weight proteins, whereas ESI-ion trap MS offers better sensitivity of detection, down to the femtomole level of peptides [74]. Therefore, mass spectrometry is a good choice to use for identify and quantify of the 67 protein spots with difference in spot-density that found in both helix and straight form. It is capable of relatively high throughput and rapid for identification of these proteins.

5. Isoelectric focusing of 2D electrophoresis composed of 4 programmable parameters; duration and temperature of rehydration phase, platform temperature and current limit, voltage change pattern and step duration or volt-hours. These parameters can be edited in order to achieve an optimum reproducibility and pattern quality.

As mentioned in the results, it was found that the group of proteins with molecular mass less than 21.5 kDa of both morphological forms were not resolved. It may be possible that the condition of the first dimension was not optimized for these proteins. Thus, modification of the first dimension condition may improve the resolution of these proteins.

6. One of the techniques for comparison of microorganism at the molecular level is nucleotide sequencing of 16s rRNA. Ribosomal RNAs have parts that are highly conserved and parts that are less conserved. Thus, 16s rRNA sequence can be used for studies of bacterial evolution and phylogenic relations whether it is not a widely phylogenetic marker molecules, it provides diagnostic sequence that serve as target sites for taxonomic specific probes [55].

The 16s rRNA sequencing can be used to determine the relationship between helical and straight form of *S. platensis* C1 and also other member of the

Oscillatoriaceae. In comparing sequence, 16s rRNA of both forms were aligned and applied to genetic distance for phylogenetic inference. The results will show the level of evolutionary distance and clustering analysis between both forms and other cyanobacteria. Thus, it is possible to use molecular similarities to infer the relationship of helical and straight forms.

7. Electron microscopy may used for observation of trichome surface particularly the cross wall that divides trichome into cells. This method display the appearance of cells which have cylindrical cell or cylindrical with tapering at the end of cell. The formation of various cells within the trichome may be related to trichome morphology. Thus, observation of helical and straight trichome in cross-section may give some information of differential morphological form of *Spirulina*.

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Appendix A

Zarrouk's medium [65]

Macroelements

	$g \cdot l^{-1}$	
NaCl	1.0	+ 1 ml of microelement solution A_5 and B_6, as listed below
$CaCl_2$	0.04	
$NaNO_3$	2.5	
$FeSO_4 \cdot 7H_2O$	0.01	
NaEDTA	0.08	
K_2SO_4	1.0	
$MgSO_4 \cdot 7H_2O$	0.2	
$NaHCO_3$	16.8	
K_2HPO_4	0.5	

The various salts of the macroelements should be introduced into the solution in the written order. Phosphate should always be added last.

Microelements

A_5	$g \cdot l^{-1}$
$ZnSO_4 \cdot 7H_2O$	0.222
$CuSO_4 \cdot 5H_2O$	0.079
MoO_3	0.015
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81

B_6	$g \cdot l^{-1}$
NH_4VO_3	229.6×10^{-4}
$K_2Cr_2(SO_4)_4 \cdot 24H_2O$	960.0×10^{-4}
$NiSO_4 \cdot 7H_2O$	478.5×10^{-4}
$Na_2WO_4 \cdot 2H_2O$	179.4×10^{-4}
$Co(NO_3)_2 \cdot 6H_2O$	439.8×10^{-4}
$Ti_2(SO_4)_3$	400.0×10^{-4}

Appendix B

B.1 Calculation of algal growth [65]

1. At the logarithemic growth phase, the increase in biomass (dX) is proportional to the amount (X) presented and to the time interval (dt).

$$\text{Thus} \quad dX = \mu X dt$$

$$\mu = \frac{\ln X_2 - \ln X_1}{dt}$$

$$= \frac{\ln X_2 - \ln X_1}{t_2 - t_1}$$

μ (specific growth rate) represents the rate of growth per unit amount of biomass and has the dimension of reciprocal time (1/t)

X_1 , X_2 = biomass concentration at time 1 (t_1), time 2 (t_2) respectively

B.2 Chlorophyll determination [2]

1. Five milliliters of algal suspension was filtered through a Whatman GF/C filter paper (25 mm diameter)

2. The cells and the filter paper was resuspended in 5 ml of absolute methanol and grounded with a glass tissue homoginizer. The sample was heated a water bath at 70 °C for 2 min then it was centrifuged at 2000g (3500 rpm) for 5 min.

3. The supernatant was measured at wavelength 665 nm by spectrophotometer. (Spectronic 21, Milton Roy Company)

Calculations :

Optical density at 665 nm multiply (X) factor (derived from the absorption coefficient) = chlorophyll concentration in μg/ml or mg/l

The factor for *Spirulina* and *Isochrysis* is 13.9

for *Anabaena* 12.65

for *Porphyridium* 13.2

B.3 Dry weight determination in microalgae [64]

1. The sample of 25 or 50 ml algal suspension was filtered through a Whatman GF/C filter paper that was pre-dried in the oven for 24 h at 70 °C or 2 h at 105 °C and weighed prior to the filtration.

2. While the sample is being filtered it is washed with 20 ml acidified water pH 4 in order to free the algae from insoluble salts.

3. Then the filter paper was put in a glass petri dish in the oven under the above condition.

4. After cooling the filter paper in a desiccator (20 min) it is weighed again, this time with the sample on it. The samples should be dried to a constant weight.

Calculation :

$$\frac{(\text{weight of filter + algae}) - (\text{weight of filter alone}) \times 1000 \text{ ml}}{\text{volume of algae in ml}} = \text{algal dry weight in mg/l}$$

B.4 Carbohydrate determination [11]

1. The amount of algal suspension 1 ml was added with 1 ml of 5% (w/v) phenol solution and mixed well on a vortex stirrer.

2. The 5 ml of concentrated H_2SO_4 was rapidly added (should be finished within 20 seconds).

3. The tube was cooled down at room temperature for approximately 30 min.

4. The solution was measured at wavelength 485 nm and calculate carbohydrate content from the standard curve.

5. The standard curve was prepared as followed:

One mg/ml of glucose was diluted to 0, 10, 20, 40, 60, 80 and 100 μg of glucose then made up to 1 ml total volume by distilled water. Then the set of tubes were determined of carbohydrate as the above condition.

Note : (A) A fresh standard curve must be prepared for each batch of samples.

(B) Step addition of 5% (w/v) phenol solution and concentrated H_2SO_4 should be carried out in a fume hood while wearing gloves and safety glasses.

B.5 Protein determination by Lowry's method [32]

1. The 0.5 ml of algal suspension was added with 0.5 ml of 1N NaOH. Then it was boiled for 20 min and cooled down to room temperature.

2. The 2.5 ml of reagent D was filled in the solution and mixed well.

3. After 10 min, the amount of 0.5 ml Folin-Ciocalteus reagent (diluted 1:1 with water) was added and mixed well. Then wait for 30 min.

4. The solution was read the absorbance at 750 nm and calculate protein content from the standard curve.

5. Preparation of the standard curve:

From a stock solution of bovine serum albumin (BSA) at a concentration of 2 μg/ml, stock solution was diluted to 0, 10, 20, 40, 100, 200, 400, 600 and 800 μg of BSA. Each concentration was determined protein following method described above.

Note : The reagent D was made in the following order:

Forty eight milliliters of 5% Na_2CO_3, 1 ml of 1% $CuSO_4 \cdot 5H_2O$ and 1 ml of 2% $NaKC_4H_6O_6 \cdot 4H_2O$ (sodium potassium tartrate) were mixed together. This solution should be freshly prepared.

B.6 Phycocyanin determination [35]

1. Freeze-dried cell at the amount of 5 – 10 mg was weighed into tube.

2. The sample was added with 5 ml of 0.1M sodium phosphate buffer and mixed well. Then it was incubated at 35 °C for 18 – 24 h.

3. The mixture was centrifuged at 2000g (3500 rpm) for 10 min.

4. The supernatant was measured for absorbance at wavelength 618 nm.

Calculation :

$$\text{mg phycocyanin} = \frac{\text{Optical density at 618 nm X 1000 X 5}}{6500}$$

$$\text{mg phycocyanin / g dry weight} = \frac{\text{mg phycocyanin X 1000}}{\text{mg sample}}$$

Note : Number 6500 is the value from phycocyanin standard (Sigma Co.).

B.7 Carotenoid determination [17]

1. The 15 mg of freeze-dried cell was added with 10 ml of C_2H_5OH and 1 ml of 60% KOH into the centrifuge tube and mixed well. Then it was extracted in water bath at 45 – 50 °C for 5 min.

2. Afterthat, the sample was centrifuged at 2000g (3500 rpm) for 5 – 10 min.

3. The green clear supernatant was separated by decanting into the separatory funnel.

4. The 2 ml of C_2H_5OH was filled on the pellet for washing and repeated step 2 – 3 for fully extraction and combine the supernatant.

5. The amount of 15 – 20 ml dimethyl ether and 20 ml of 1 N NaCl were added then mixed well.

6. The mixture was separated into two phases; green color at the bottom and yellow color on the top. The green phase was slowly released to erlenmeyer flask.

7. 20 ml of 1 N NaCl was added into the yellow phase and repeat step 6 few times until the mixture was separated into two phase of yellow color and clear white color.

8. The yellow phase was separated then it was added with some Na_2SO_4 for water absorption.

9. The orange-yellow phase was adjusted to 25 ml total volume with diethyl ether and mixed well. Then it was measured at wavelength of 450 nm.

Calculation :

$$\text{mg carotenoid / g cell} = \frac{\text{Optical density at 450 nm X 25 X 1000}}{\text{260 X mg dry weight}}$$

B.7 Carotenoid determination [17]

1. The dried biomass powder cell was added with 10 ml of C_2H_5OH and 1 ml of 60% KOH into the centrifuge tube and mixed well. Then it was extracted in water bath at 45 – 50 °C for 5 min.

2. Afterthat, the sample was centrifuged at 2000g (3500 rpm) for 5 – 10 min.

3. The green clear supernatant was separated by decanting into the separatory funnel.

4. The 2 ml of C_2H_5OH was filled on the pellet for washing and repeated step 2 – 3 for fully extraction and combine the supernatant.

5. The amount of 15 – 20 ml dimethyl ether and 20 ml of 1 N NaCl were added then mixed well.

6. The mixture was separated into two phases; green color at the bottom and yellow color on the top. The green phase was slowly released to erlenmeyer flask.

7. 20 ml of 1 N NaCl was added into the yellow phase and repeat step 6 few times until the mixture was separated into two phase of yellow color and clear white color.

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9. The orange-yellow phase was adjusted to 25 ml total volume with diethyl ether and mixed well. Then it was measured at wavelength of 450 nm.

Calculation :

$$\text{mg carotenoid / g cell} = \frac{\text{Optical density at 450 nm X 25 X 1000}}{\text{260 X mg dry weight}}$$

B.8 Fatty acid analysis [29]

1. Freeze-dried sample of algal biomass 100 mg was weighted into vial.

2. 1 ml of Heptadecanoic acid (C17 :0) from stock solution was added into sample. Then the mixture was added with 2 ml of methanol – hydrochloric (95 : 5) solution.

3. The vial was sealed with flexible paraffin film and heated under argon atmosphere at 80 °C for 1 h.

4. After cooling, it was diluted with 1 ml of distilled water and extracted with 1 ml of hexane containing 0.01% BHT.

5. The sample was mixed well and left for separation.

6. The upper layer was separated and filtered through a few of Na_2SO_4.

7. Then the solution was injected into the Gas Chromatography.

Condition of Gas Chromatography

Gas Chromatographic analysis was performed on a 15% DEGS-column (3m X 3mm) at 190 °C (FID, injector and flame ionization detector temperature at 220 °C and integrated with a Shimadzu CR-3A integrator). Fatty acid methyl esters were identified by co-chromatography with authentic standard (Sigma co.) and by calculation of the equivalent chain length. Fatty acid contents were determined by comparing their peak with that of the internal standard.

B.9 Calculation of oxygen evolution

Photosynthesis activity was assayed by measuring the rate of oxygen evolution using a Clark – type oxygen electrode. The signal from the recorder could be calculated from the following formula.

$$\text{O}_2 \text{ evolution } (\mu \text{ mol } O_2\ h^{-1}\ mg\ chl^{-1}) = \frac{\text{Slope x A x60 x 1000}}{\text{B x 32 x C}}$$

Slope = slope of each sample (unit/min)

A = solubility of oxygen in water at 35 °C (mg/l)

B = the difference between the N_2 saturated line and the air line (unit)

C = chlorophyll concentration (mg/l)

Table of solubility of oxygen in water under varied temperature [66]

Temperature (^{0}C)	O_2 (ppm)	O_2 (μmol ml^{-1})
0	14.16	0.442
5	12.37	0.386
10	10.92	0.341
15	9.76	0.305
20	8.84	0.276
25	8.11	0.253
30	7.52	0.230
35	7.02	0.210

The values in column 2 (O_2, ppm) may also be derived from the empirical formula

$$C_s = 14.16 - 0.3943\ T + 0.007714\ T^2 - 0.0000646\ T^3$$

Where

C_s = saturation concentration (ppm)

T = Temperature (^oC)

Appendix C

Reagents and solutions

C.1 Standard heptadecanoic acid (C17 : 0) solution

The solution was prepared as followed: 100 mg of $C_{17}H_{34}O_2$ was dissolved in 10 ml petroleum ether. Then the suitable volume of the solution 100 µl or 1 mg of C17:0, was taken.

C.2 10 mM HEPES containing 3% PMSF

The solution was made in the following order: 0.2383 g of HEPES was dissolved in 70 ml of distilled water and adjusted to pH 7 with 1N NaOH. Then the solution was made to total volume of 100 ml with distilled water. To prepare 3% PMSF solution, 0.03 g PMSF was dissolved in 1 ml methanol. Then 16.6 µl of 3% PMSF was added to 10 mM HEPES and the volume was adjusted to 5 ml.

Caution : PMSF (Phenylmethyl sulphonyl fluoride) is very toxic.

C.3 1.5M Tris-Cl pH 8.8

The solution was prepared as followed: 9.07 g of Tris base was dissolved in 40 ml of distilled water and adjusted to pH 8.8 with 1N HCl. The solution was made up to 50 ml total volume by distilled water.

C.4 1% SDS

The solution was prepared as followed :

0.01 g of SDS was dissolved in 1 ml of distilled water and mixed well.

C.5 Rehydration stock solution without IPG buffer

(8M urea, 2% (w/v) CHAPS, bromophenol blue, 1 ml)

The solution was made in the following order: 0.48 g of urea, 0.02 g of CHAPS and a few grains of bromophenol blue were dissolved in distilled water and made up to 1 ml total volume. The solution was stored at –20°C.

DTT and IPG buffer were added just prior to use: Add 1.4 mg DTT and 2.5 µl IPG buffer per 0.5 ml of rehydration stock solution.

C.6 SDS equilibration buffer

(50mM Tris-Cl pH8.8, 6M urea, 30% glycerol, 2% SDS, bromophenol blue, 30 ml)

The solution was made in the following order: 10.81 g of urea, 0.6 g of SDS and a few grains of bromophenol blue were dissolved in 20 ml distilled water and 1 ml of 1.5M Tris-Cl pH 8.8 and 10.35 ml of 87% (w/w) glycerol were added. Then it was made up to 30 ml total volume with distilled water and stored at –20 °C.

60 mg of DTT was added to 6 ml of SDS equilibration buffer prior to use.

C.7 30% acrylamide / 0.8% bisacrylamide

The solution was made in the following order: 30 g of acrylanide and 0.8 g N/N' – methylene- bisacrylamide were dissolved and made up to 100 ml total volume by distilled water. The solution was filtrated through a 0.45 µm filter and stored at 4 °C in the dark.

Caution : Acrylamide monomer is a neurotoxic substance. Gloves should be worn while handling the solution, and the solution should not be pipetted by mouth.

C.8 4x Tris-Cl / SDS, pH 8.8

(1.5M Tris-Cl containing 0.4% SDS)

The solution was made in the following order: 91 g of Tris base and 2 g of SDS were dissolved in distilled water. The solution was adjusted to pH 8.8 with 1N

HCl and made up to 500 ml total volume with distilled water. The solution was filtrated through a 0.45 μm filter and stored at 4 °C.

C.9 10% ammonium persulphate

The solution was prepared as followed: 0.1 g of ammonium persulphate was dissolved in distilled water and made up to 1 ml total volume. This solution should be freshly made.

C.10 5x SDS electrophoresis buffer

The solution was prepared as followed: 72 g of glycine, 15.1 g of Tris base and 5 g of SDS were dissolved in distilled water and made up to 1000 ml total volume.

Do not adjust the pH of the stock solution, since the pH is 8.3 when the solution is diluted to 1x for use in the protocol.

C.11 Agarose sealing solution

The solution was prepared as followed: 0.5 g of agarose and a few grains of bromophenol blue were added in 100 ml of 1x SDS electrophoresis buffer. Then it was swirled and heated in a microwave oven on low heat level until the agarose is completely dissolved. Do not allow the solution to boil over. Store at room temperature.

C.12 Solution for fixation step

The solution was prepared as followed: 100 ml of ethanol and 25 ml of acetic acid glacial were mixed in a 500 ml erlenmeyer flask. Then it was made up to 250 ml with distilled water.

C.13 Solution for sensitizing step

The solution was prepared as followed: 75 ml of ethanol, 1.25 ml of 25% (w/v) glutaraldehyde, 10 ml of 5% (w/v) sodium thiosulphate and 17 g of sodium acetate were mixed and made up to 250 ml with distilled water.

C.14 Solution for silver reaction step

The solution was prepared as followed: 25 ml of 2.5% (w/v) silver nitrate solution and 0.1 ml of 37% (w/v) formaldehyde were added in a 500 ml erlenmeyer flask. Then it was made up to 250 ml with distilled water.

C.15 Solution for developing step

The solution was prepared as followed: 6.25 g of sodium carbonate was added in distilled water and it was vigorously stirred to dissolve. Then 0.05 ml of 37% (w/v) formaldehyde was added and the solution was made up to 250 ml with distilled water.

C.16 Solution for stopping step

The solution was prepared as followed: 3.65 g of EDTA-$Na_2 2H_2O$ was dissolved and made up to 250 ml with distilled water.

C.17 Solution for preserving step

The solution was prepared as followed: 75 ml of ethanol and 11.5 ml of 87% (w/w) glycerol were mixed and made up to 250 ml with distilled water.

C.18 Solution for Coomassie staining

The solution was made in the following order: 114 ml of absolute methanol and 22 ml of glacial acetic acid were mixed. Then 0.613 g of Coomassie blue R250 was dissolved and the solution was made up to 250 ml with distilled water.

C.19 Solution for destaining

The solution was prepared as followed: 300 ml of absolute methanol and 100 ml of glacial acetic acid were mixed and made up to 1000 ml with distilled water.

C.20 Preparation of 0.1M sodium phosphate buffer at 25 °C [50].

pH	Volume of 1M Na_2HPO_4 (ml)	Volume of 1M NaH_2PO_4 (ml)
5.8	7.9	92.1
6.0	12.0	88.0
6.2	17.8	82.2
6.4	25.5	74.5
6.6	35.2	64.8
6.8	46.3	53.7
7.0	57.7	42.3
7.2	68.4	31.6
7.4	77.4	22.6
7.6	84.5	15.5
7.8	89.6	10.4
8.0	93.2	6.8

The 0.1M sodium phosphate buffer pH 7 was made in the following order: 57.7 ml of 1M Na_2HPO_4 and 42.3 ml of 1M NaH_2PO_4 were mixed and made up to 1000 ml with distilled water.

C.21 Blotting buffer

The solution was made in the following order: 0.3 g of Tris and 1.44 g of glycine were dissolved in 20 ml of absolute methanol. Then, the solution was adjusted to pH 8.3 with 1N HCl and made up to 100 ml final volume with distilled water.

C.22 Membrane staining solution

The solution was prepared as followed: 0.08 g of Coomassie blue R-250 was dissolved in 18 ml of absolute methanol. Then 4 ml of gracial acetic acid was added and made up to 40 ml with distilled water.

C.23 Membrane destaining solution

The solution was prepared as followed: 90 ml of absolute methanol and 7 ml of gracial acetic acid were mixed. Then, the solution was made up to 100 ml final volume with distilled water.