ลักษณะสมบัติของกลูตาเมทดีคาร์บอกซิเลสใน *Synechocystis* sp. PCC 6803 และบทบาทในการตอบสนองต่อความเครียดอะไบโอติก

นางสาวไซแมป คอนวอร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมีและชีววิทยาโมเลกุล ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2556
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

CHARACTERIZATION OF GLUTAMATE DECARBOXYLASE IN *Synechocystis* sp. PCC 6803 AND ITS ROLES IN RESPONSE TO ABIOTIC STRESS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biochemistry and Molecular Biology
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2013

Thesis Title	CHARACTERIZATION OF GLUTAMATE DECARBOXYLASE IN *Synechocystis* sp. PCC 6803 AND ITS ROLES IN RESPONSE TO ABIOTIC STRESS
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Field of Study	Biochemistry and Molecular Biology
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ไชแมป คอนวอร์ : ลักษณะสมบัติของกลูตาเมทดีคาร์บอกซิเลสใน *Synechocystis* sp. PCC 6803 และบทบาทในการตอบสนองต่อความเครียดอะไบโอติก. (CHARACTERIZATION OF GLUTAMATE DECARBOXYLASE IN *Synechocystis* sp. PCC 6803 AND ITS ROLES IN RESPONSE TO ABIOTIC STRESS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. อรัญ อินเจริญศักดิ์ , 101 หน้า.

Glutamate decarboxylase (GAD) คือเอนไซม์ที่ต้องใช้ pyridoxal-5′-phosphate ในการทำงาน ทำหน้าที่เร่งปฏิกิริยาการเปลี่ยน กลูตาเมต เป็น gamma-aminobutyric acid (GABA) ผ่านปฏิกิริยา decarboxylation ที่ผันกลับไม่ได้ เอนไซม์ GAD มีการกระจายตัวอย่างกว้างขวางในธรรมชาติ และถูกพบใน แบคทีเรีย เชื้อรา พืชชั้นสูง และระบบประสาทส่วนกลางของสัตว์เลี้ยงลูกด้วยนม ผลของปัจจัยทางกายภาพที่ต่างกัน เช่น แสง (แสงขาวและยูวี) อุณหภูมิ ค่าความเป็นกรดด่าง แหล่งไนโตรเจนและคาร์บอนทางเลือก ความเครียดออสโมติก การเติมกลูตาเมต และโพลีเอมีนต่อการทำงานของเอนไซม์ GAD ของ *Synechocystis* ได้รับการศึกษาแบบแยกทีละปัจจัย เอนไซม์ GAD ของ *Synechocystis* แสดงการทำงานที่ดีขึ้นเมื่อเซลล์อยู่ในช่วงสุดท้ายของ log phase ได้รับอุณหภูมิ 40 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง การชักนำให้เกิดความเครียดออสโมติกทำได้โดยการใช้ 50 มิลลิโมลาร์โซเดียมคลอไรด์ ร่วมกับ 100 มิลลิโมลาร์ซอร์บิทอล และการฉายรังสียูวีเป็นเวลา 60 นาที แหล่งคาร์บอนที่เหมาะสมต่อการทำงานของ GAD คือ กลูโคส 0.2 % โดยน้ำหนักต่อปริมาตร การสะสม GABA ในเซลล์ของ *Synechocystis* เพิ่มขึ้นเมื่อใช้กลูโคส 0.1 % เป็นแหล่งคาร์บอน การฉายรังสียูวี การเติม โซเดียมคลอไรด์ และ ซอร์บิทอลชักนำให้เกิดความเครียดออสโมติกที่ความเข้มข้นสูง การเติม 0.5 มิลลิโมลาร์สเปอร์มิดีน และ10 มิลลิโมลาร์กลูตาเมต ส่งผลให้การทำงานของ GAD เพิ่มขึ้น 2.2 และ 3.5 เท่าตามลำดับ ซึ่งสอดคล้องกับปริมาณ GABA ภายในเซลล์ ใน *Synechocystis* สายพันธุ์กลายที่ไม่มีการทำงานของ GAD มีปริมาณ GABA ภายในเซลล์น้อยมาก และมีปริมาณ กลูตาเมต สูงเมื่อเทียบกับสายพันธุ์ปกติ แต่อย่างไรก็ตามการเติมโพลีเอมีนและการฉายรังสียูวีทำให้ปริมาณ GABA ในสายพันธุ์กลายเพิ่มมากขึ้น แสดงให้เห็นทางเลือกอื่นในการสังเคราะห์ GABA ใน *Synechocystis* ระดับการแสดงออกของยีนที่สร้าง GAD (*gad*) ได้รับผลกระทบจากทางเลือกของแหล่งคาร์บอนและภาวะเครียดออสโมติกระยะสั้น ความเครียดที่ชักนำโดยการฉายรังสียูวีส่งผลให้การแสดงออกของยีน *gad* ลดลง แสดงให้เห็นว่าน่าจะมีความเกี่ยวข้องกับกระบวนการดัดแปลงหลังการแปลรหัสยีนของเอนไซม์ GAD ยีน *gad* (*sll*1641)ของ *Synechocystis* ได้รับการโคลนและใส่เข้าไปใน *E. coli* เพื่อสร้าง GAD ลูกผสม การทำงานของ GAD ลูกผสมสูงสุดที่ค่าความเป็นกรดด่าง 5.8 ความเข้มข้นที่เหมาะสมของสารตั้งต้นในการเปลี่ยนไปเป็น GABA โดย GAD ลูกผสม คือ กลูตาเมต 30 มิลลิโมลาร์ เอนไซม์ GAD ลูกผสมได้รับการยืนยันว่าเป็น โมโนเมอร์ มีขนาดมวลโมเลกุลเท่ากับ 53 กิโลดาลตัน และเป็นเอนไซม์ที่ต้องใช้ pyridoxal-5′-phosphate ในการทำงาน ค่า K_m และความเร็วสูงสุด (V_{max}) คือ 19.6 มิลลิโมลาร์ และ 21.5 นาโนโมลต่อนาทีต่อมิลลิกรัมโปรตีนตามลำดับ สรุปโดยรวมผลการทดลองแสดงให้เห็นว่าการเปลี่ยนแปลงระดับการทำงานของ GAD และปริมาณ GABA ภายใต้ภาวะเครียดทางกายภาพที่แตกต่างกันช่วยยืนยันบทบาทการบรรเทาภาวะเครียดของ *Synechocystis* และ GAD มีบทบาทสำคัญในการเชื่อมโยง คาร์บอนและหรือไนโตรเจน เมตาบอลิซึ่มใน *Synechocystis*

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2556

5373921723 : MAJOR BIOCHEMISTRY AND MOLECULAR BIOLOGY
KEYWORDS: ABIOTIC STRESS / GABA ACCUMULATION / GAD ACTIVITY / SYNECHOCYSTIS SP. PCC 6803

SIMAB KANWAL: CHARACTERIZATION OF GLUTAMATE DECARBOXYLASE IN *Synechocystis* sp. PCC 6803 AND ITS ROLES IN RESPONSE TO ABIOTIC STRESS. ADVISOR: PROF. ARAN INCHAROENSAKDI, Ph.D., 101 pp.

Glutamate decarboxylase (GAD) is a pyridoxal-5′-phosphate dependent enzyme that catalyzes the conversion of glutamate to gamma-aminobutyric acid (GABA) through irreversible decarboxylation reaction. GAD is widely distributed in nature and found to be occurring in bacteria, fungi, higher plants and mammalian central nervous system. Effect of various abiotic factors such as light (visible and UV), temperature, pH, alternative nitrogen and carbon source, osmotic stress, glutamate and polyamine supplementation on glutamate decarboxylase (GAD) activity of *Synechocystis* was studied by using a one-variable-at-a time approach. *Synechocystis* GAD showed higher activity when late log phase cells were exposed to 24 h of 40 ℃ temperature, osmotic stress induced by 50 mM NaCl and 100 mM sorbitol, and 60 min of UV-B irradiation. 0.2 % w/v glucose was considered as an optimum carbon source for GAD activity. GABA accumulation inside the cells of *Synechocystis* was observed to be increasing in response to 0.1 % glucose as carbon source, UV-B irradiation, and NaCl and sorbitol induced osmotic stress at higher concentrations. 0.5 mM spermidine and 10 mM glutamate supplementation resulted in an increase in GAD activity by 2.2 and 3.5- fold respectively, with a concomitant increase in intracellular GABA levels. *Synechocystis* mutant strain deficient in GAD activity, showed very low intracellular GABA and higher glutamate levels when compared to wild type strain. However increase in GABA content was observed in mutant under polyamine supplementation and UV-B irradiation, suggesting the alternative routes of GABA production in *Synechocystis.* Transcript levels of gene encoding GAD (*gad*) were observed to be affected by alternative carbon sources and short term osmotic stress conditions. Stress induced by UV-B radiation resulted in down-regulation of *gad* transcripts, suggesting the involvement of post translational modification of GAD enzyme. *Synechocystis gad* gene (sll1641) was cloned and transformed into *E. coli* to obtain a recombinant GAD. Activity of recombinant GAD was optimal at pH 5.8. Optimum concentration of substrate for conversion into GABA by recombinant GAD was 30 mM glutamate. Recombinant GAD was confirmed as a monomer with apparent molecular mass of 53 kDa and found to be a pyridoxal-5′-phosphate dependent enzyme. Km and V_{max} values were 19.6 mM and 21.5 nmol min^{-1} mg^{-1} respectively. Altogether the results suggested that alteration in GAD activity and GABA levels under abiotic stress conditions confer a stress relieving role to *Synechocystis* and GAD plays an important role in connecting the C/N metabolism in *Synechocystis.*

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature	.....
Academic Year:	2013		

ACKNOWLEDGEMENTS

My past four years of completion of this study comprised a journey of learning, dedication and hard work that let me involved in the fascinating world of biochemistry. The time devoted to this study was full of challenges and new experiences, that was difficult to pass through without the assistance and understanding of these people whom I would like to thank with the core of my heart. First and foremost, thank God for the wisdom and perseverance that He has been bestowed upon me during this study, and indeed, throughout my life. I would like to thank my supervisor, Prof. Dr. Aran Incharoensakdi for accepting me as a Ph. D student and showing great trust, expectation and conviction in me being a foreigner. His guidance, instructions, support, endless patience and dedicated involvement in every step throughout the process of my Ph. D thesis led me to make improvements in my scientific way of thinking and made me to accomplish this task straightforwardly.

I would also like to show gratitude to my thesis committee, including Prof. Dr. Anchalee Tassanakajon, Assoc. Prof. Dr. Jarunya Narangajavana, Assoc. Prof. Dr. Teerapong Buaboocha and Assist. Prof. Dr. Tanakarn Monshupanee for lending me their precious time for providing useful comments and suggestions.

My appreciation is also extended to Dr. Saowarath Jantaro, Dr. Rajesh P. Rastogi, Dr. Wanthanee khetkorn, Dr. Wipawee Baebprasert, Dr. Panutda Yodsang, Dr. Kanteera Soontharapirakkul, Dr. Cherdsak Maneeruttanarungroj, Miss. Bongkoj, Miss. Aporn, Miss. Khanittha, Mr. Piriya, Miss Pichanan and Miss Apiradee for brilliant comments and suggestions and helping me to excel throughout my laboratory training. Sincere thanks to all members of cyanobacterial lab and cyclodextrin group for creating a nice working place and lending a helping hand whenever I needed. They were always very patient with my knowledge gaps in the area and their efforts in developing understanding in me about thai culture are greatly appreciated. Special thanks to Dr. Mitti, Dr. Thitiya poonpet, Mr. Baldur Kristjansson and Mr. Thomas Mabey for their wonderful friendship and technical assistance during process of thesis preparation. A special thanks to my friend Dr. Nazif ullah for his kind friendship, continual support and encouragement throughout this study.

Thanks to the Department of Biochemistry, Faculty of Science and the 90th Anniversary of Chulalongkorn University Ratchadaphiseksomphot Endowment Fund for granting me a Ph. D scholarship.

Most importantly, this task could never have accomplished without support of my family. I am for ever grateful to my parents, brothers, sister, and my family friend Mr. Syed Faiz M. for all the sacrifices they made on my behalf. Their prayers helped to sustain me thus far and provided a driving force to make me strive towards my goal. This dissertation stands as a testament for unconditional love and encouragement of my family.

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LIST OF ABBREVIATIONS

bp	base pair
BSA	bovine serum albumin
C	carbon
°C	degree Celsius
EDTA	ethylenediamine tetraacetic acid
GAD	glutamate decarboxylase
GABA	gamma-aminobutyric acid
h	hour
HEPES	hydroxyethyl piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
kb	kilo base
LL	late log
Mb	mega base
min	minute
µg	microgram
µl	microliter
mM	millimolar
MSG	monosodium glutamate
$mWcm^{-2}$	milli watt per centimeter square
N	nitrogen
ng	nanogram
nm	nanometer
OD	optical density
OPA	o-phthaldialdehyde
PCR	polymerase Chain Reaction
PLP	pyridoxal-5'-phosphate

Put	putrescine
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
s	second
Spd	spermidine
UV	ultraviolet
w/v	weight/volume

CHAPTER I INTRODUCTION

1.1 Glutamate decarboxylase

Glutamate that is commonly present in cyanobacteria, is an important amino acid in nitrogen assimilation pathway (Riccardi *et al.* 1989). Glutamate degradation follows various routes catalyzed by different kind of enzymes as shown in Figure 1.1. Among the enzymes involved in glutamate degradation, glutamate decarboxylase (GAD, EC:4.1.1.15), is an important enzyme that catalyzes the single step conversion of L-glutamate into gamma-aminobutyric acid (GABA) and CO_2 by an irreversible α-decarboxylation reaction (Figure 1.2). Enzyme uses PLP (Pyridoxal-5′-phosphate) as a coenzyme for activity. The GAD enzyme is characterized as a Ca^{2+}-dependent calmodulin (CaM)-binding protein in plants (Baum *et al.* 1993). And there are reports that the activity of GAD enzyme is induced under various biotic and abiotic stresses, increasing the efficiency of GABA shunt pathway. Other than plants, GAD is also present in a variety of organisms ranging from prokaryotes to eukaryotes (Satyanarayan and Nair 1985) existing either as monomeric or dimeric form. Some examples of organisms in which GAD is thoroughly characterized are, *Escherichia coli* (Shukuya and Schwert 1960), *Clostridium perfringens* (Cozzani *et al.* 1970), *Lactococcus lactis* (Nomura *et al.* 1999), *Streptococcus salivarius* ssp. thermophilus Y2 (Yang *et al.* 2008) and *Oryza sativa* L. (Oh *et al.* 2005).

1.2 GABA: Formation, degradation and roles

GABA is a four-Carbon, non-protein amino acid with the general formula, $C_4H_9NO_2$, abundantly exists in a variety of organisms including prokaryotes and eukaryotes (Shelp *et al.* 1999). GABA was synthesized in 1883 for the first time, afterward it was known to be existing in plants and other microorganisms as well. But GABA gained more consideration when in 1953 Ernst Florey (Florey 1953) isolated GABA as an unknown compound from horse brain for exogenous application on Cray fish and got startling results.

GABA is formed by the action of GAD using glutamate as a substrate and catabolized by the action of GABA transaminase and succinic semialdehyde

dehydrogenase consecutively (Bouche and Fromm 2004). The final product enters into the TCA cycle. The overall metabolic pathway of GABA is also known as GABA shunt pathway as shown in Figure 1.2. Apart from glutamate decarboxylation, GABA formation from polyamine degradation is also reported in plants by the action of diamine or polyamine oxidases (Flores and Filner 1985). Bioinformatic studies have shown that *Synechocystis* has the genes that could encode the enzymes involved in GABA formation via polyamine catabolism (Figure 1.1). However the pathway needs experimental validation.

In mammals, GABA is known to be acting as an inhibitory neurotransmitter (Kaupmann *et al.* 1997) and also plays diuretic, tranquilizing and antidiabatic functions (Krnjević 1974). Various inferences about the possible role of GABA under biotic and abiotic stress conditions are made on the basis of experimental studies conducted in bacteria and plants. GABA is known to play various roles in organisms such as stress signaling, osmo-regulation, cytosolic pH regulation and maintenance of C:N balance (Kinnersley and Turano 2000, Bouche and Fromm 2004). Not only GABA, the overall GABA shunt pathway is considered imperative to play a protective role for organism under stress circumstances (Renault *et al.* 2010).

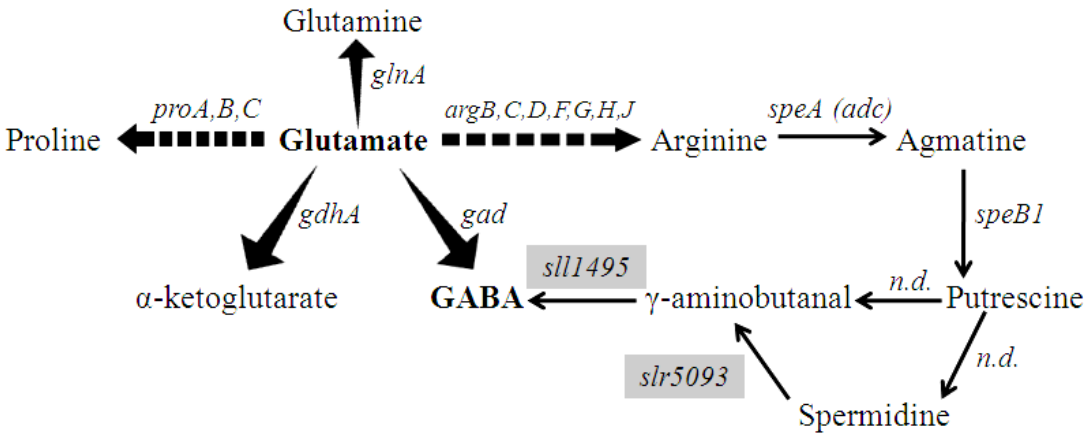


Figure 1.1 Glutamate catabolism and possible route of GABA formation via polyamine degradation in *Synechocystis*, with related genes indicated in italic letters based on cyanobase. Italic and grey highlighted letters are considered as putative genes. Broken arrows denote cascade of intermediate products. N.d= not detected.

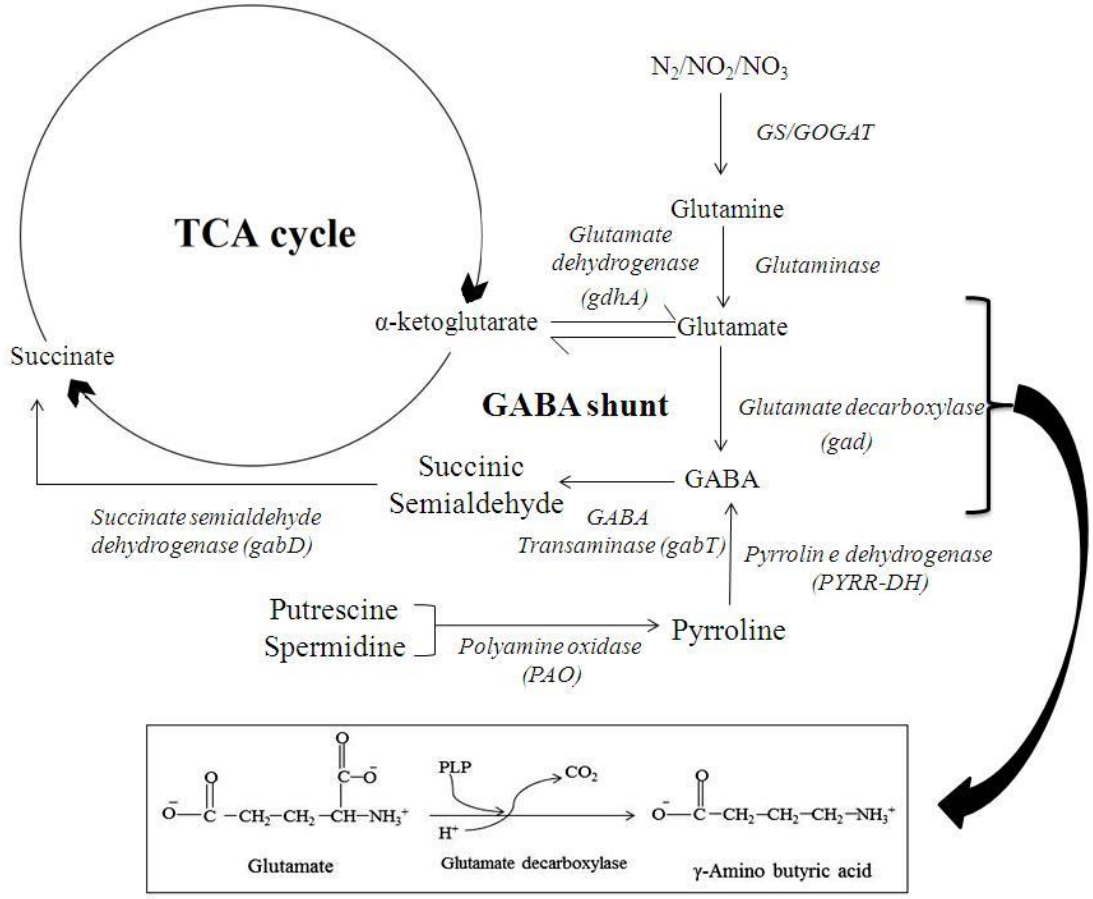


Figure 1.2 Diagrammatic representation of GABA shunt pathway comprising three enzymes encoded by *gad*, *gabT* and *gabD* genes. The GAD enzyme encoded by gad gene catalyzes the conversion of glutamate to GABA. The overall decarboxylation reaction is shown in the bottom box. Further catabolism of GABA is done by GABA transaminase and succinate semialdehyde dehydrogenase consecutively (Kanwal et al. 2014).

1.3 GAD in different organisms and its roles

GAD is known to be present in almost all kind of organisms including bacteria, cyanobacteria, fungi, plants and mammals. The enzymatic properties and expressions of GAD are largely studied in various organisms, some of them are summarized in table 1.1.

GAD is considered as the key enzyme in GABA shunt pathway that provides an alternative route for connecting glutamate to TCA cycle. GAD confers important physiological roles to these organisms such as involvement in the germination of seed, conidia, and bacterial endospores (Foerster 1972, Hao and Schmit 1991, Matsuyama *et al.* 2009). GAD is also known to play important role in acid resistance in bacteria by accommodating H^+ ions (Karatzas *et al.* 2012). There are reports that anaerobic stress conditions also affect the transcription of *gad* genes in certain bacteria (Blankenhorn *et al.* 1999, Jydegaard-Axelsen *et al.* 2004). GAD in mammals received remarkable attention for showing altered levels under various neurological disorders, particularly because the substrate (L-glutamate) and the product (GABA) acts as an excitatory and inhibitory neurotransmitters respectively (Battaglioli *et al.* 2003). It is suggested that under oxidative stresses, activity of GAD has overall impact on GAD pathway and GABA shunt by altering the expression of genes involved in these pathways (Coleman *et al.* 2001). Intracellular glutamate levels are maintained by GAD activity by converting glutamate into GABA (Shelp *et al.* 1999). It is evident from literature review that properties of GAD vary a lot among various organisms depending on the kind of environment in which the organism is adapted.

Table 1.1 Comparison of glutamate decarboxylase from different sources. NR= not reported.

Organism		Molecular weight	Km Glutamate (mM)	pH optimum	Reference
Bacteria	*Escherichia coli*	300, 000	1.0	3.8	(Strausbauch and Fischer 1970)
	Clostridium Perfringens	290,000	NR	4.5	(Cozzani *et al.* 1970)
	Lactobacillus brevis IFO 2005	180,000	9.3	4.2	(Ueno *et al.* 1997)
Cyanobacteria	*Synechocystis*	53,050	19.6	5.8	Present work
Fungi	*Neurospora crassa*	33,200	2.2	5.0	(Hao and Schmit 1993)
	Claviceps purpurea	NR	17.4	5.3	(Anderson *et al.* 1961)
Plants	Barley roots	310,000	3.1	6.2	(Inatomi and Slaughter 1975)
	Sunflower cotyledons	NR	9.1	5.7	(Smith and Waygood 1961)
	Solanum tuberosum	91,000	5.6	5.8	(Satyanarayan and Nair 1985)
Mammals	Rat brain	110,000	1.6	7.3	(Denner *et al.* 1987)
	Mouse brain	85,000	0.7	7.0	(Wu *et al.* 1973)

1.4 Cyanobacteria

Cyanobacteria, also known as blue green algae are prokaryotic organisms, having the photosynthetic apparatus like plants. They are considered as the ancestors of plant chloroplast, hence making a clear evolutionary link to plants (Rodriguez-Ezpeleta *et al.* 2005). Due to their abundance in fossil fuels, the term "the age of cyanobacteria" was used for Proterozoic Era (2500 - 570 Ma) by Schopf and Walter (1982) (Whitton and Potts 2002). Cyanobacteria are reported to be occurring in a variety of habitats including oceans, deserts, fresh water bodies and soils (Rippka *et al.* 1979, Whitton and Potts 2001). They are the organisms able to tolerate severe environmental stresses such as desiccation, cold temperatures, low oxygen conditions and UV stress (Whitton and Potts 2001, Meeks and Elhai 2002). There are both halophilic and halotolerant species in cyanobacteria.

Cyanobacteria are very diverse organisms morphologically. They include species having unicellular to filamentous forms that may occur singly or may form colonies. Some cyanobacteria have the ability to fix atmospheric nitrogen. Cyanobacteria are among the important microorganisms that are considered valuable source of bioenergy and bioactive secondary compounds of medicinal and industrial value (Rastogi and Sinha 2009, Lindberg *et al.* 2010).

1.5 *Synechocystis* sp. PCC 6803

Synechocystis sp. PCC 6803 (hereafter *Synechocystis*) is a well-known and one of the widely investigated cyanobacterial strain. *Synechocystis* is unicellular, gram negative, non-toxic, non-nitrogen fixing, fresh-water cyanobacterium. The organism is capable to grow both phototrophically and heterotrophically by utilizing different types of carbon sources (Labarre *et al.* 1987) and reproduces by binary fission by dividing into two or three successive planes. *Synechocystis* it is one of the microorganisms who exhibit a 24 hours circadian rhythms in their physiology and gene expression (Kondo *et al.* 1993, Lakin-Thomas 2006). *Synechocystis* is considered as a good choice for making biochemical investigations regarding enzymatic reactions or stress responses. There are various reasons for choosing this organism for such kind of studies such as, the complete genome sequence of *Synechocystis* is available and freely accessible, and another reason is that the organism is easily transformable by exogenous DNA that

makes it a suitable choice for conducting molecular studies. *Synechocystis* provides a good model for investigating stress mechanisms and long term adaptation in variable environmental conditions.

Genome sequence of *Synechocystis* shows one gene i.e. *gad* (Gene ID: sll1641) coding for GAD enzyme (http://www.kazusa.or.jp/cyano/cyano.html). Computational studies regarding the gene regulation and activity of GAD with possible functions of GABA shunt pathway in *Synechocystis* are conducted previously (Schriek *et al.* 2007). Yet the accurate role of GAD and GABA in this organism to acclimate stress conditions is unclear.

Synechocystis is selected in this project to investigate the GAD enzyme with the aim to learn its properties and possible roles under abiotic stresses. The project comprises three main steps. In the first step, the optimum conditions for the activity of GAD enzyme and GABA production are investigated along with the factors affecting GAD at transcriptional level. In the second step, GAD deficient mutant is analyzed for GABA production and the third step deals with the over-expression and characterization of recombinant GAD. The results are expected to provide additional basic knowledge in the regulation of GAD enzyme and GABA production and their important roles in response to abiotic stresses.

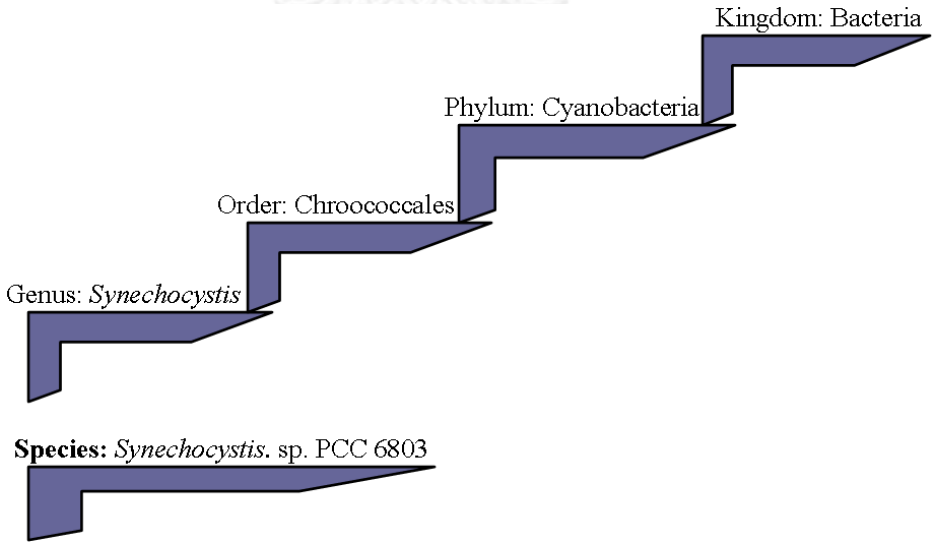
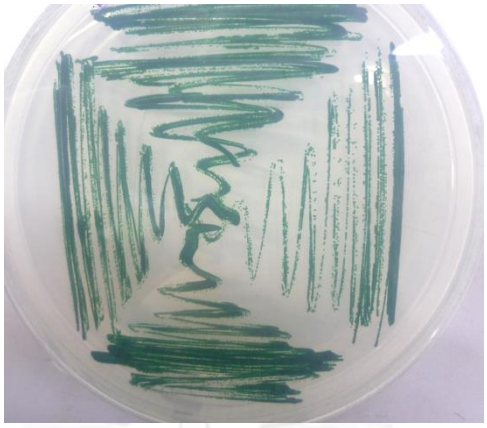


Figure 1.3 Scientific classification of *Synechocystis.*

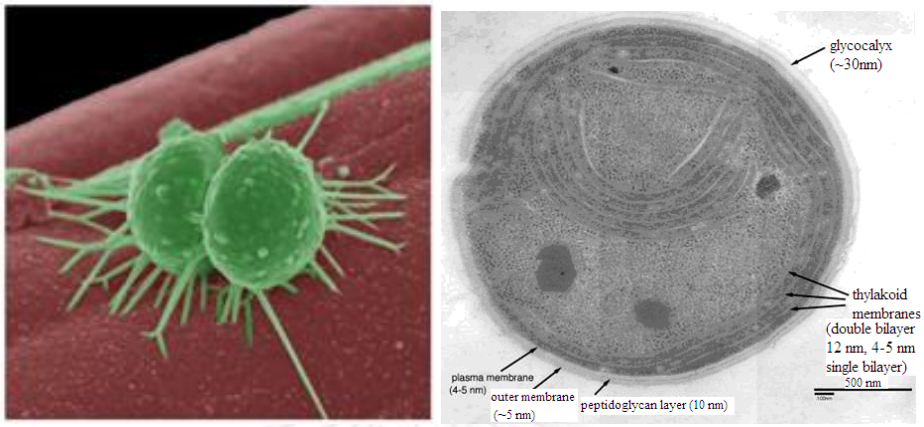
A



B

Figure 1.4 Unicellular cyanobacterium *Synechocystis* cells grown in **A)** BG-11 agar medium, and **B)** BG-11 liquid medium by bubbling method.

A B



C

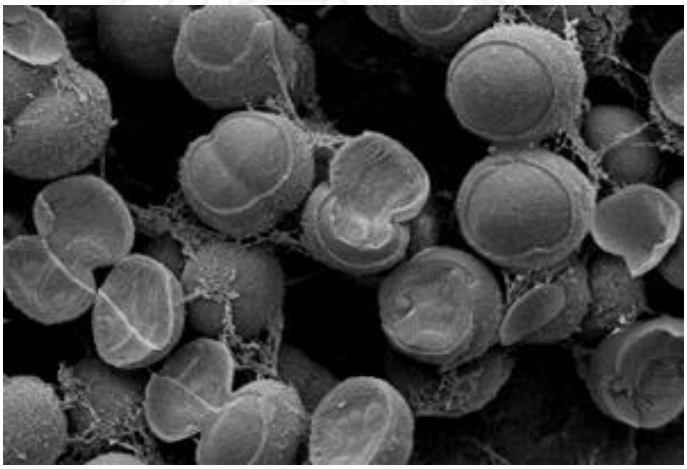


Figure 1.5 A) Diagram showing the cell of *Synechocystis*. **B)** Transmission electron micrograph of a thin section of *Synechocystis* cell. **C)** Cryo scanning electron micrograph of *Synechocystis* cell imaged at -120 °C. Cells are broken opened to reveal layers of thylakoid membranes and other membranes.

(http://bioenergy.asu.edu/faculty/vermaas/biofactories.html)

(http://newunderthesunblog.files.wordpress.com/2013/09/cyanobacterial-cell biology.jpg)

(http://www.nature.com/nrmicro/journal/v4/n9/images/nrmicro1502-i1.jpg)

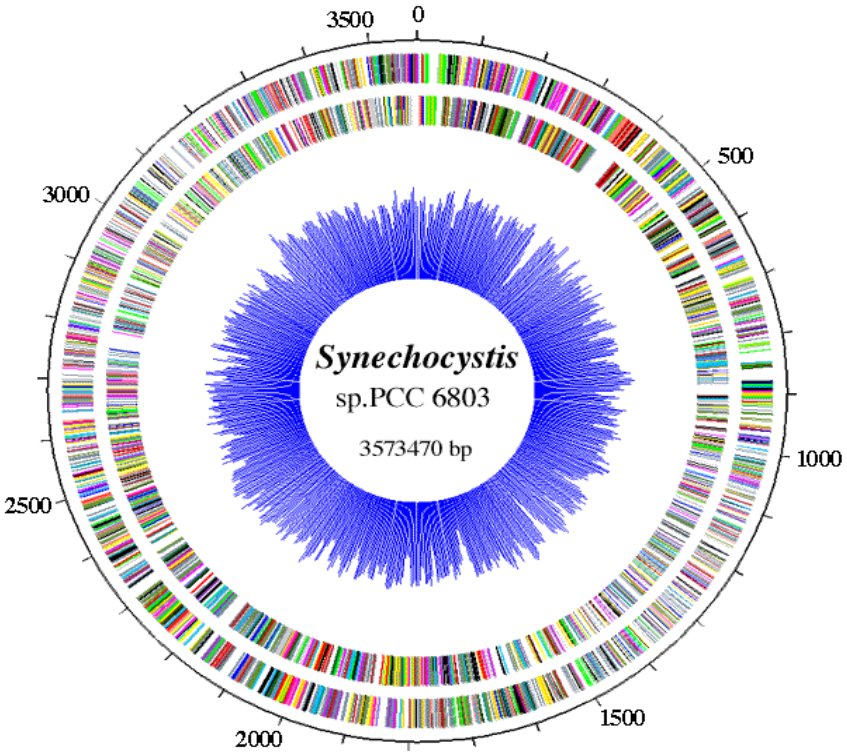


Figure 1.6 The circular genome (3.6 Mb) of *Synechocystis.*

(http://www.kazusa.or.jp/cyano/Synechocystis).

OBJECTIVES OF THIS RESEARCH

- To determine the effect of various physiological factors and abiotic stress conditions on GAD activity, transcription and GABA accumulation in *Synechocystis*.
- To examine the function of GAD and GABA production in detail through *Synechocystis gad* deficient mutant strain.
- To study the properties of *Synechocystis gad* by characterization of recombinant enzyme.

CHAPTER II MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

AAA analytical column	ZORBAX Eclipse Agilent, USA
Autoclave	Model HA-30, Hirayama Manufactering Corporation, Japan
Balances	METTLER PJ360 DeltaRange® GWB, USA
Centrifuge	MIKRO220*/220R* Germany
Centrivap concentrator	Labconco, USA
Degasser	Shimadzu, DGU-14A
Digital Lux meter	Model FT-710, Taiwan
Electrophoresis unit	BIO-RAD PROTEIN® II xi Cell, USA
Geldoc® enabled	DNA visualisation UV light, USA
Gel documentation	Syngene ® Gel documentation
HPLC auto-injector	Shimadzu, SIL-10AD VP
HPLC column oven	CTO-10A VP
Laminar flow	BVT-124 International Scientific Supply, Thailand
Light source unit	Prekeo S250 Zeiss IKON, Japan
Microcentrifuge	Model 5417C, Eppendorf, Germany
PCR apparatus	PERKIN ELMER DNA Thermal Cycler, Japan
pH meter	METTLER TOLEDO, Switzerland
Power supply	BIO-RAD POWER PAC 1000, USA
Shaker	InnovaTM 2100 PLATFORM SHAKER, USA
Spectrophotometer	SPECTRONIC®GENESYSTM2, USA
	Jenway UV/VIS 6400, USA
Sonicator	Sonuplus UW2200, Germany
UV-B tube	UVP34-0039-01, 15 W, Japan

Vortex	Model K-550-GE, Scientific Industries, USA
Water bath	THERMOMIX® B B.BRAUN, USA
Western blot Unit	Model HEP-1/3, Owl separation systems, USA

2.1.2 Chemicals

5-Bromo-4-chloro-3-indolyl phosphate (BCIP)	Sigma, USA
Acetic acid	BDH, England
Acetonitrile	SK Chemicals, Korea
Acrylamide	Merck, Germany
Agarose	Vivantis, USA
Ammonium ferric citrate	Ajax Finechem, Australia
Ammonium persulfate (APS)	Merck, Germany
Ampicillin	Sigma, USA
Brilliant blue	Sigma, USA
Bromophenol blue	Sigma, USA
BSA (Fraction V)	Sigma, USA
Chloroform	Merck, Germany
Citric acid	Ajax Finechem, Australia
Coomassies blue R-250	Sigma, USA
Dithiothreitol (DTT)	Sigma, USA
Dimethylformamide (DMF)	Sigma, USA
Di-Sodium hydrogen phosphate	Carlo Erba, Italy
EDTA	Ajax Finechem, Australia
Ethanol	Merck, Germany
Ethidium bromide	Sigma, USA
Fructose	Ajax Finechem
GABA	Sigma Aldrich, UK
Glucose	Sigma, USA

Glutamate	BDH, England
Glycerol	Ajax Finechem, Australia
Isopropanol	Sigma, USA
Lactose	BDH, England
Mercaptoethanol	Sigma, USA
Methanol	SK Chemicals, Korea
Methylene-bis-acrylamide	Amersham Bioscience, Sweden
N´-2-ethanesulfonic acid (HEPES)	Sigma, USA
O-phthaldialdehyde (OPA)	Agilent, USA
p-Nitro blue tetrazolium chloride (NBT)	Sigma, USA
Phenol	Merck, Germany
Putrescine	Sigma, USA
Pyridoxal-5´-phosphate	Sigma, USA
Sodium bicarbonate	BDH, England
Sodium chloride	Ajax Finechem, Australia
Sodium dodecyl sulfate	Ajax Finechem, Australia
Sodium hydroxide	Ajax Finechem, Australia
Sodium nitrate	Ajax Finechem, Australia
Sodium phosphate monobasic	Carlo Erba, Italy
Sodium thiosulfate	Ajax Finechem, Australia
Sorbitol	Ajax Finechem, Australia
Spectinomycin	Sigma, USA
Spermidine	Sigma, USA
Streptomycin	Sigma, USA
Sucrose	Ajax Finechem, Australia
TEMED	BIO-RAD, USA
Trizol® reagent	Invitrogen, USA
Tween-20	BIO-RAD, USA

Urea	Sigma, USA
Xylene cyanol FF	Sigma, USA

2.1.3 Kits and suppliers

GeneRuler™ 1 kb DNA Ladder	Fermentas, Canada
His Trap FF	GE Health care, Sweden
Nylon membrane filters	0.45 and 0.22 μm Sartorius, Germany
PCR amplification kit	Invitrogen, USA
PCR purification kit	Machery-Nagel, USA
Plasmid extraction Kit	Fermentas, Canada
Prestained Protein Marker	Fermentas, Canada
Quick ligation™ Kit	BioLabs
SuperScript™ III First-Strand Synthesis Kit	Invitrogen, USA
Whatman 3MM paper	Whatman International, England

2.1.4 Enzymes

DNAase	Fermentas, Canada
Pfu DNA polymerase	Promega, USA
Restriction enzymes	Fermentas, Canada
RNase-free DNase	Invitrogen, USA
Taq DNA polymerase	Invitrogen, USA

2.1.5 Organisms

Two organisms, namely *Escherichia coli* and *Synechocystis* sp. PCC 6803 wild type strains were used in this study.

2.1.5.1 *Escherichia coli* strains were obtained from the Laboratory of Biochemistry, Department of Biochemistry, Chulalongkorn University, Thailand.

1. Strain DH5α (F-φ80lacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA supE44, λ-, thi-1, gyrA96, relA1) was used for DNA manipulation.
2. Strain BL21 (DE3) (F- ompT hsdS(rBmB) gal dcm(DE3)) was used for gad expression.

2.1.5.2 *Synechocystis* sp. PCC 6803 wild type strain was obtained from the Laboratory of Plant Physiology and Molecular Biology, Department of Biochemistry and Food Chemistry, University of Turku, Finland.

2.1.6 Plasmids

The three plasmids, namely pT&A (RBC), pGEM-T easy (Promega) and pET-22b+ (Novagen) vector were used in this study. Circle maps are shown in APPENDIX J.

2.1.7 Oligonucleotides

Table 2.1 Oligonucleotides used in this study.

Primers	Sequence 5′ to 3′	Purpose of primer	Amplified fragment length (bp)
16SF	AGTTCTGACGGTACCTGATGA	PCR for *16S* rRNA	521
16SR	GTCAAGCCTTGGTAAGGTTCT		
gadF	GGAATTCCATATGGTGCATAAAA AAATTGAC	PCR for *gad*	1423
gadR	CCGCTCGAGATGGCTAAAGTGGGA		
RTgadF	CAGGTGCCTCTGCACATAGA	RT-PCR for *gad*	320
RT gadR	TGGATTTTGCGGTAACCTTC		

2.2 Methods for *Synechocystis*

2.2.1 Culture growth

Synechocystis wild type (WT) strain was grown in BG-11 medium (Rippka *et al.* 1979) containing 17.6 mM $NaNO_3$ (17.6 mM) as nitrogen source and Na_2CO_3 (0.19 mM) as carbon source buffered with 20 mM HEPES-NaOH (pH 7.5) at 28 ± 2 ˚C, with a total volume of 200 ml in cotton-plugged 500 ml conical flasks at 160 rpm using a rotatory shaker under continuous illumination (warm white fluorescent tubes) of 50 µmol/m^2/s. The liquid cultures for the experiments were started at OD_{730} ~0.2. In addition, the mutant strain *Δgad* (*Synechocystis* strain with *gad* knockout gene) was grown in BG-11 medium supplemented with streptomycin and spectinomycin antibiotics at concentration of 10 µgml^{-1} and 20 µgml^{-1}, respectively. Growth rate of culture was determined by measuring the optical density at 730 nm with a Spectronic® Genesys™2 spectrophotometer.

2.2.2 *Synechocystis* cell adaptation under various conditions

Synechocystis wild type (WT) cells in the late log (LL) growth phase (OD_{730} ~0.7-0.8) were harvested by centrifugation at 2,500x*g* for 10 min at room temperature. The cell pellet was suspended in 200 ml of modified BG-11 media for 24 h (or conditions unless otherwise stated) to investigate the effect of abiotic stresses on GAD activity, *gad* transcript levels and GABA content in *Synechocystis*. Conditions used for cell adaptation are described below:

2.2.2.1 pH variation: Cells at LL phase were adapted in modified BG-11 medium and adjusted pH to different values by using universal buffer solution (Britton and Robinson 1931), ranging from 5.5 to 9.5 (with an interval of 10) and at the pH 7.5 which was adjusted with HEPES at concentration of 10 mM to study effect of pH on GAD activity.

2.2.2.2 Light: Cells at LL phase were adapted in fresh BG-11 medium incubated under various light intensities at 1,000, 2,000 and 3,500 lux, and dark to study GAD activity. Effect of UV-B was done by exposing 200 ml of cell suspension in an open, flat container exposed to UV-B radiation (280–320 nm) with 30 mWcm^{-2} intensity for 60 and 120 min to study GAD activity and GABA content.

2.2.2.3 Temperature variation: Cells at LL phase were adapted in BG-11 medium and incubated at various temperatures ranging from 20 to 50 ˚C (with an interval of 10 ˚C) to investigate effect of temperature on GAD activity.

2.2.2.4 Variable nitrogen sources: Cells at LL phase were washed with N-deprived BG-11 medium and later adapted in BG-11 medium containing various N-sources having equimolar concentration of N i.e., $NaNO_3$, $NaNO_2$, Urea, $(NH_4)_2SO_4$, and N-deprived medium to study effect of alternative N source on GAD activity and GABA content.

2.2.2.5 Variable carbon sources and concentrations: Cells at LL phase were washed with C-deprived BG-11 medium and later adapted in BG-11 medium supplemented with 0.1 % (w/v) of various types of sugars such as glucose, fructose, sucrose and lactose, and C-deprived BG-11 medium to investigate the effect of alternative carbon sources on GAD activity, *gad* transcript levels and GABA content. BG-11 medium containing Na_2CO_3 as carbon source was used as a control. Effect of glucose at variable concentrations ranging from 0 to 0.4 % (w/v) was also tested.

2.2.2.6 Osmotic stress: Cells at LL phase were adapted in BG-11 medium by either supplementation with various NaCl concentrations, i.e. 2, 50, 150, 350 and 550 mM, or with various concentrations of sorbitol, i.e. 4, 100 and 300 mM to investigate GAD activity, *gad* transcript levels and GABA content under osmotic stress.

2.2.2.7 Polyamine supplementation: Cells at LL phase were adapted in BG-11 medium supplemented with 0.5 mM of putrescine (Put) and spermidine (Spd) to study the effect of polyamines on GAD activity and GABA content.

2.2.2.8 Exogenous glutamate supplementation: Cells at LL phase were adapted in BG-11 medium supplemented with various concentrations (i.e., 5, 10, 20, 30 and 40 mM) of monosodium glutamate (MSG) to study the effect of glutamate on GAD activity, intracellular glutamate levels and GABA content.

2.2.3 Mutant cell adaptation under various conditions

Synechocystis Δgad mutant cells in the LL growth phase (OD_{730} ~0.7–0.8) were harvested by centrifugation at 5,000 rpm for 10 min at room temperature. The cell pellet was suspended in 200 ml of modified BG-11 media containing streptomycin and spectinomycin antibiotics at concentration of 10 $\mu g ml^{-1}$ and 20 $\mu g ml^{-1}$, respectively, for 24 h (or conditions unless otherwise stated) to investigate the effect of variable N-source, UV-B radiation, polyamine supplementation and exogenous glutamate

supplementation on glutamate and GABA contents. Conditions used for cell adaptation were similar as described earlier (section 2.2.2).

2.3 GABA and glutamate determination

Synechocystis wild type and mutant cells were harvested after adaptation to various conditions by centrifugation (2,500x*g*, 10 min) at room temperature. GABA and glutamate was extracted in 500 µl of 70 % ethanol by 2 h incubation at room temperature. After extraction, the aliquots were centrifuged (9,800x*g*, 10 min) at 4 ˚C and supernatants were transferred into new Eppendorf tubes and evaporated to dryness at 40 ˚C in a vacuum evaporator. The residues left after evaporation were redissolved in 250 µl 0f ultra-pure water. 50 µl of chloroform was added to this solution and vortexed gently. After centrifugation (6,200x*g* for 10 min), the uppermost pigment free water phase was transferred into new Eppendorf tubes and filtered through a 0.45 µm Millipore filter. The filtrate was derivatized with o-phthaldialdehyde (OPA) according to the manufacturer's instruction (Agilent technologies) and subsequently analyzed with the HPLC system. Pre-column OPA derivatized samples (50 µl) were injected into the HPLC column through an auto-injector. The column oven temperature was set at 40 ˚C. The mobile phase A was acetonitrile and B was 0.1 % (v/v) acetic acid in ultra-pure water with a flow rate of 1 ml/min. The elution gradient program was used as described earlier (Jannoey *et al.* 2010). GABA and glutamate contents were analyzed by comparing the peak area with standards. Chromatograms of GABA and glutamate, and standard curve of GABA are shown in the appendix K.

2.4 GAD activity assay

Synechocystis cells were harvested after adaptation to various conditions by centrifugation (2,500x*g*, 10 min) at room temperature. After washing with 50 mM sodium phosphate buffer (pH 7), cells were suspended in the same buffer (pH 5.8). 0.1 mm glass beads were added to the cells to break by sonication to release crude protein extract followed by centrifugation at 12,000x*g* (30 min, 4 ˚C). The supernatant was then collected and protein concentrations were determined by Bradford method (Bradford 1976) using BSA as a standard protein. GAD enzymatic assay was done by the method of Zhang *et al.* (2006) with some modifications and was performed by directly measuring GABA production resulting from the GAD reaction. Enough amount of protein was incubated in 300 µl of reaction mixture containing 50 mM sodium phosphate (pH

5.8), 30 mM L-glutamate, and 20 μM pyridoxal-5′-phosphate. Reactions were incubated at 25 ˚C for 30 min, and terminated by boiling in water for 10 min. The suspension was filtered through a 0.45 micron filter and derivatized with OPA solution. The filtrate was analyzed for GABA content by HPLC. GAD activity was defined as the amount of GABA (nmol) produced per min per mg of protein.

2.5 Study of *gad* transcript levels

2.5.1 Genomic DNA extraction

Genomic DNA was extracted from 2 ml of exponentially growing cultures of *Synechocystis* (OD_{730} ~0.8). Cells were collected by centrifugation (2,500x*g*, 10 min) and resuspended in 400 μl of TE-Buffer pH 7.5. Sterile glass beads up to half of the volume, 8 μl of 10 % SDS and 400 μl of absolute phenol were added into the mixture. Cells were broken by vortexing 1 min for 3 times, with cooling on ice between intervals. Cell lysate was centrifuged (10,000x*g*, 10 min) at room temperature and supernatant (containing total DNA) was transferred to a new Eppendorf tube. After that, one volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, vortexed and centrifuged (12,000x*g*, 10 min) at room temperature. Viscous supernatant was transferred to a new Eppendorf tube followed by adding 1:1 volume of chloroform:isoamyl alcohol (24:1) and then centrifuged (12,000x*g*, 10 min) at room temperature. Supernatant was transferred into another tube, added 2 volume of cold absolute ethanol followed by incubation on ice for 30 min and then centrifugation (12,000x*g*, 4 ˚C, 10 min). 70 % cold ethanol (v/v) was added to the pellet and gently mixed by inversion. The mixture was centrifuged (12,000x*g*, 4 ˚C, 10 min). Washed pellet was air dried at room temperature. 100 μl of TE-Buffer pH 8.0 was added and DNA concentration was monitored by checking absorbance at 260 nm (OD 1.0 = 50 μl/ml). DNA sample was run in 0.8 % agarose gel electrophoresis with 0.8X TAE buffer pH 8.0.

2.5.2 Total RNA extraction and analysis of *gad* transcript levels

Synechocystis cells at late log phase were adapted to 24 h osmotic stress and variable C-sources, and UV-B radiation for 60 and 120 min as described earlier (section 2.2.2.2, 2.2.2.5 and 2.2.2.6). TRI Reagent was used to extract total RNA. Following the manufacturer's instruction, single stranded cDNA was synthesized after DNase

treatment from 1 µg of total RNA with the SuperScript™ III First-Strand Synthesis Kit. Reverse transcription-polymerase chain reaction (RT-PCR) amplifications using cDNA of the *gad* and 16S as a reference gene were performed using primer sequences for *gad* (cyanobase. Gene ID: sll1641), RTgadF and RT gadR giving a PCR product of 320 bp, and using primer sequences for 16S rRNA, 16SF and 16SR giving a PCR product of 521 bp. Negative controls for the RT reaction and PCR reactions were RT-PCR on DNaseI treated RNA without RT-enzyme and PCR amplification without cDNA added, respectively. Whereas genomic DNA using the corresponding forward and reverse primers was used as positive control. The PCR conditions consisted of: denaturation at 94 ˚C for 5 min, followed by 28 cycles of 94 ˚C for 30 s, annealing temperature of 55 ˚C for 1 min and 72 ˚C for 20 s, followed by a final extension at 72 ˚C for 3 min. The PCR product was analyzed using a 1.0 % (w/v) agarose gel electrophoresis system using 1X SB buffer.

2.6 Methods for *Escherichia coli*

2.6.1 Culture growth

E. coli strains DH5α and BL21 (DE3) were grown in LB medium at 37 ˚C with shaking at 250 rpm using a rotary shaker.

2.6.2 Preparation of competent cells

A single colony of *E. coli* strain DH5α or BL21 (DE3) was inoculated in 3 ml of LB medium and incubated at 37 ˚C overnight with shaking at 250 rpm on a rotary shaker. From overnight grown cell culture, 1 ml was introduced into 100 ml of fresh LB medium and grown until the OD_{600} reached 0.3-0.4. Cells were collected by centrifugation (2,500x*g*, 4 ˚C, 10 min) and cell pellet was gently resuspended in 15 ml of cold TFBI buffer followed by centrifugation (2,500x*g*, 4 ˚C, 10 min). Supernatant was removed and cell pellet was resuspended gently in 2 ml of cold TFBII buffer. After that, 100 µl of cells suspension were aliquoted into pre-chilled sterile Eppendorf tubes and stored at -80 ºC immediately.

2.7 Methods for gene manipulation

2.7.1 Primers design

A *gad* gene of *Synechocystis* was amplified by using primers designed from the genome database for cyanobacteria (http://genome.kazusa.or.jp/cyanobase/).

2.7.2 Amplification of *Synechocystis gad* gene

The *gad* sequence (gene ID: sll1641) encoding the GAD protein of *Synechocystis* (Kaneko *et al.* 1996) was amplified from the genomic DNA (section 2.5.1) using *pfu* DNA polymerase and gene specific primers. For the convenience of cloning, an *Nde*I site was designed to the 5′-end of the forward- gadF primer and a *Xho*I site was added to 5′-end of the reverse- gadR primer after the stop codon. *pfu*-based PCR amplification was performed in a 1X PCR mix (1X *pfu* buffer, 2 mM $MgSO_4$, 0.2 mM dNTPs mixture, 0.5 pmol of each forward and reverse primer) using 1 µg of template DNA and 0.05 U of *pfu* polymerase, making a total volume of 20 µl with sterile DI water. The PCR conditions consisted of: denaturation at 94 ˚C for 5 min, followed by 30 cycles of 94 ˚C for 30 s, annealing temperature of 55 ˚C for 1 min and 72 ˚C for 20 s, followed by a final extension at 72 ˚C for 3 min. The PCR product was analyzed using a 0.8 % (w/v) agarose gel electrophoresis system in TAE buffer system.

2.7.3 Plasmid construction of pTAGAD

A *gad* gene was purified by gel extraction kit. The gene was tailed with an adenine nucleotide and ligated to the cloning pTA vector, called pTAGAD.

2.7.3.1 TA Ligation reaction

TA ligation was done by mixing vector and inserted DNA in a ratio of 1:3. The following equation was used to calculate the appropriate amount of insert (PCR product) for ligation reaction:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}^*$$

A 20 µl of ligation reaction was made by adding suitable concentration of vector and insert (as described above) in nuclease free water with 2X quick ligation reaction buffer

and quick T4 DNA ligase (3U/ µl). The reaction was incubated for 10 min at 22 ˚C. 2 µl of ligation mixture was transformed into competent cells of *E. coli* DH5α.

2.7.4 Heat-shock transformation

An aliquot of competent *E.* coli DH5α was gently thawed on ice for 10 min. 2 µl of ligation reaction was mixed with the competent cells and kept on ice for 30 min. The cells were transformed by heat-shock at 42 ˚C for 45 s, then placed on ice for 2 min followed by adding 900 µl of LB medium and incubated at 37 ˚C with shaking at 250 rpm for 1 h. The mixture was spread on LB agar plates containing appropriate antibiotic and incubated overnight at 37 ˚C. The next day, transformants were selected using the blue white screening on the ampicillin agar plates. The selected clones were sequenced with an automated sequencer by a commercial service (1st base, Malaysia) to confirm correct vector and insert.

2.7.5 Plasmid construction of pETGAD

A single white colony of *E. coli* DH5α harboring a pTAGAD was grown in 1.5 ml LB broth containing 100 µg/ml of ampicillin and incubated at 37 ˚C overnight. The pTAGAD was extracted by a high speed plasmid mini-kit. The plasmid was resuspended in TE-buffer.

2.7.5.1 Restriction Digestion

Double restriction digestions of pTAGAD and pET 22b+ vectors with fast digest *Nde*I and *Xho*I were incubated at 37 ˚C for 10-15 min. The total volume of the reactions was 10 µl containing 1X fast digest restriction buffer, 1-2 U of restriction enzymes and the DNA sample. The digested plasmids were analyzed by 0.8 % agarose gel electrophoresis in TAE buffer system.

2.7.6 Transformation of BL21 (DE3) strain

The *gad* gene fragment and liner pET 22b+ vector were purified by plasmid purification kit and subsequently ligated together by the method described previously (section). The ligation mixture was transformed by heat shock method into competent cells of *E. coli* BL21 (DE3) for *gad* gene expression. A single white colony of transformants was selected on ampicillin agar plates and plasmid was confirmed by sequencing.

2.7.7 Construction of pGADSpSm

pGADSpSm was constructed and transformed to *Synechocystis* previously by Dr. Wanthanee Khetkorn by the method described below.

A 1.4-kb of *gad* gene of *Synechocystis* was amplified by PCR with specific primers as described in section 2.7.2. The PCR product was transferred into pGEM-T Easy vector (Promega) by TA cloning to create pGgad plasmid harbouring *gad* gene. The *gad* gene in pGgad was inactivated by cloning 1.9-kb KpnI streptomycin and spectinomycin resistance cassette from pRL 5801 vector into KpnI site of pGgad resulting in pGADSpSm plasmid. Gene knockout was confirmed by restriction digestion and analyzed by 0.8 % agarose gel electrophoresis in TAE buffer system.

2.7.8 Confirmation of complete segregation of *Δgad* mutant

pGADSpSm plasmid was transformed to *Synechocystis* wild type cells by natural transformation to generate mutant strains disrupted in glutamate decarboxylase (*Δgad*). Transformants were selected on BG-11 plate containing streptomycin and spectinomycin antibiotics at concentration of 10 µg/ml and 20 µg/ml, respectively and were transferred to BG-11 broth containing antibiotics at the same concentration. Genomic DNA extraction of recombinant colonies was done and amplified by using a primer pair specific to *gad*. The results were analyzed by 0.8 % agarose gel electrophoresis by comparing to wild type strain to check the complete segregation of *Δgad* mutant.

2.8 Methods for recombinant protein analysis

2.8.1 Induction and expression of rGAD protein

A single colony of BL21 (DE3) strain transformed with pETGAD vector was picked from the spread plate and inoculated in 100 ml of LB containing 100 µg/ml of ampicillin in 500 ml of conical flask. The culture was incubated at 37 °C with shaking until OD_{600} reached 0.4-0.5. Induction of rGAD was achieved by adding 1 mM of isopropyl-D-thiogalactoside (IPTG). One ml of culture medium was collected after 0, 1, 2, 3, 6 hours of induction by centrifugation (2,500x*g*, 10 min, 4 °C). Cell pellets were suspended in cold extraction buffer (20 mM sodium phosphate, pH 7.0, 3 mM EDTA) and broken by

sonication to release protein. Both supernatant and pellet fractions were analyzed by Sodium Dodecyl Sulphate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE).

2.8.2 Sodium Dodecyl Sulphate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE)

Samples of both supernatant and pellet fractions were treated with the sample buffer for SDS-PAGE and boiled for 5 min. After boiling, samples were cooled for few minutes at room temperature and loaded to a slap gel (12 % of separating gel and 5 % of stacking gel, Appendix D). After gel polymerization, electrophoresis run was performed at a constant current of 25 mA per gel for 50 min followed by gel staining with coomassie blue.

2.8.3 Western blot analysis

SDS-polyacrylamide gel was transferred to immobilon™ PVDF transfer membrane by blotting solution (Appendix E). Polyvinylidene fluoride (PVDF) membrane was cut equal to the size of the gel and activated with 100 % methanol for 5 min. Six pieces of Whatman filter papers were cut equal to the size of the gel and were soaked completely in blotting buffer along with activated PVDF membrane. Gel sandwich was prepared by making subsequent layers of filter papers, gel, membrane and filter papers again and transferred by following the instructions provided by the transfer system manufacturer. Membrane was immediately washed with PBS-T buffer and placed in blocking buffer at room temperature for 1 h. Membrane was washed again with PBS-T buffer, 3-4 times and incubated with anti-His antibody in 10 ml of PBS-T buffer containing 5 % of Anlene® milk at room temperature for 3-4 h. Membrane was washed with PBS-T buffer again and incubated with goat anti mouse IgG in 10 ml of PBS -T buffer containing 5 % of Anlene® milk at room temperature for 3-4 h. Membrane was washed again with PBS-T buffer and developed with developing buffer for 3 min followed by washing with DI water for 1 min.

2.8.4 Ni-Sepharose column purification

The rGAD protein extract was suspended in working buffer (20 mM Na-phosphate pH 7.4, 20 mM Imidazole) and purified with His-Trap FF column kit according to the manufacturer's protocol. Briefly, sample was loaded onto precharged Ni-sepharose

column previously equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4). The protein was eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). 3 ml of fractions were collected and analyzed by SDS-PAGE to identify the fraction containing rGAD. The selected fractions were dialyzed extensively against phosphate buffer, pH 7.4 to remove imidazole. The purified protein was stored at -20 °C for further analysis.

2.8.5 Effect of substrate concentration on recombinant protein activity

Effect of substrate concentration on recombinant enzyme activity was determined by using various concentrations of glutamate ranging from 0 mM to 30 mM. 10 µg of purified enzyme was incubated with various concentrations of glutamate (0, 2, 4, 8, 10, 15, 20 and 30 mM) with 20 µM PLP in 50 mM sodium phosphate buffer at 37 °C for 30 min. The reaction was terminated by boiling in water for 10 min. The suspension was derivatized with OPA solution and analyzed for GABA content by HPLC.

2.8.6 Effect of PLP on recombinant protein activity

Effect of different concentrations of PLP on recombinant GAD activity was determined by varying PLP concentration from 0 µM – 28 µM. 10 µg of purified rGAD was incubated with 30 mM glutamate and various concentrations (0, 4, 12, 20 and 28 µM) of PLP in 50 mM sodium phosphate buffer, pH 5.8. The reaction was incubated for 30 min at 37 °C prior to the activity determination of recombinant enzyme.

2.8.7 Effect of pH on recombinant protein activity

The effect of pH on recombinant GAD activity was determined within a range of pH 4.5 to 7.5. 10 µg of purified GAD was treated with 50 mM citrate-phosphate buffer (pH 4.5) and 50 mM sodium phosphate buffer (pH 5.8, 6.5 and 7.5) supplemented with 30 mM of glutamate and 20 µM PLP at 37 °C. The reaction was incubated for 30 min followed by activity determination of enzyme.

CHAPTER III RESULTS

3.1 Characterization of GAD activity in *Synechocystis* under various conditions

3.1.1 Dependence of GAD activity on stage of cell growth

The unicellular, photosynthetic cyanobacterium *Synechocystis* was grown in BG-11 medium up to three weeks. During the growth of cells, samples were taken from the flask growing culture, every two days of cultivation to check growth rate by measuring optical density (OD) at 730 nm. OD of cells was found to be increasing with the increase of cultivation time. From the Figure 3.1A, the growth rate of *Synechocystis* could be divided in to three stages, mid log phase (2-4 days of cultivation), late log phase (6-10 days of cultivation) and stationary phase (more than 10 days of cultivation). Stationary phase was persistent even at day 21.

To determine the GAD activity at three growth stages, cells were collected at day 4, day 8 and day 12. Cells grown till late log phase showed highest GAD activity as compared to those grown till mid log phase and stationary phase (Figure 3.1B).

3.1.2 Time course assay for GAD activity

Synechocystis cells were grown till late log (LL) phase of growth and GAD activity from crude enzyme extract was determined by varying the incubation time of reaction. The reaction mixture was incubated at 25 ˚C for various time periods i.e., 0, 10, 20, 30, 45 and 60 min. After the reaction was terminated at the set time periods, GAD activity was determined. An increase in GAD activity was observed when reaction time was increased from 10 to 20 min (Figure 3.2). The rate of reaction was found to be increasing linearly until the highest GAD activity was observed at 45 min. Afterward GAD activity was found to be stable and not increasing till 60 min of incubation time.

3.1.3 Effect of pH, temperature and light illumination

Cells grown till LL phase were further adapted for another 24 h in modified BG-11 media at various pH ranging from 5.5 to 9.5. Whereas effect of 24 h of temperature variation and light illumination on GAD activity was also studied by varying the

temperature from 20 to 50 ˚C (with an interval of 10 ˚C) and various light illuminations at 0, 1,000, 2,000 and 3,500 lux respectively. The results revealed that GAD activity was highest at neutral pH, suggesting that both acidic and basic pH ranges were not found suitable for GAD activity in *Synechocystis* (Figure 3.3A). Whereas an increase in GAD activity was found at 40 ˚C in comparison to normal temperature condition i.e. 30 ˚C followed by a drastic decline in activity at 50 ˚C (Figure 3.3B). Cells grown at light intensity of 2,000 lux depicted higher GAD activity as compared to dark, low light intensity (1,000 lux) and higher light intensity (3,000 lux) (Figure 3.3C).

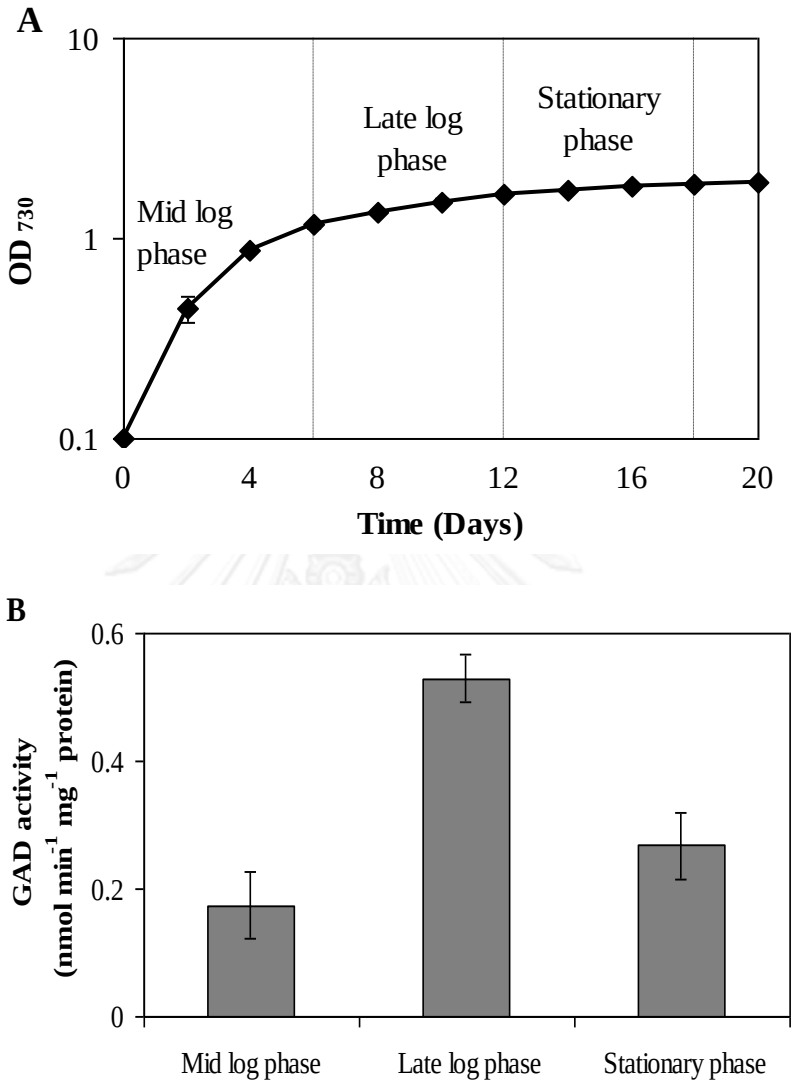


Figure 3.1 Effect of *Synechocystis* growth stage on GAD activity. **A)** Growth of *Synechocystis* in BG-11 medium by measuring the optical density of culture at 730 nm. **B)** GAD activity of *Synechocystis* cells grown and collected at different cell growth stages. Means ± S.D. (n=3).

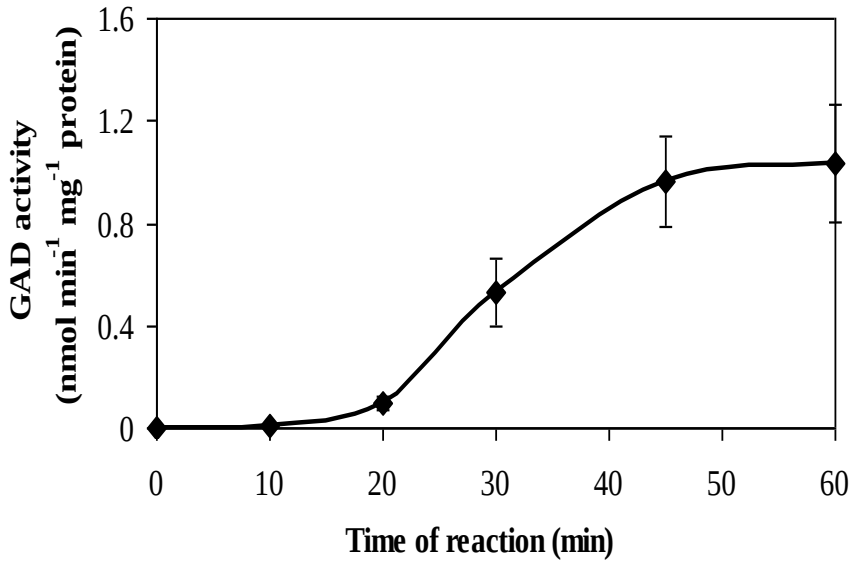


Figure 3.2 Effect of various incubation time of enzyme assay on GAD activity of *Synechocystis.* Means ± S.D. (n=3).

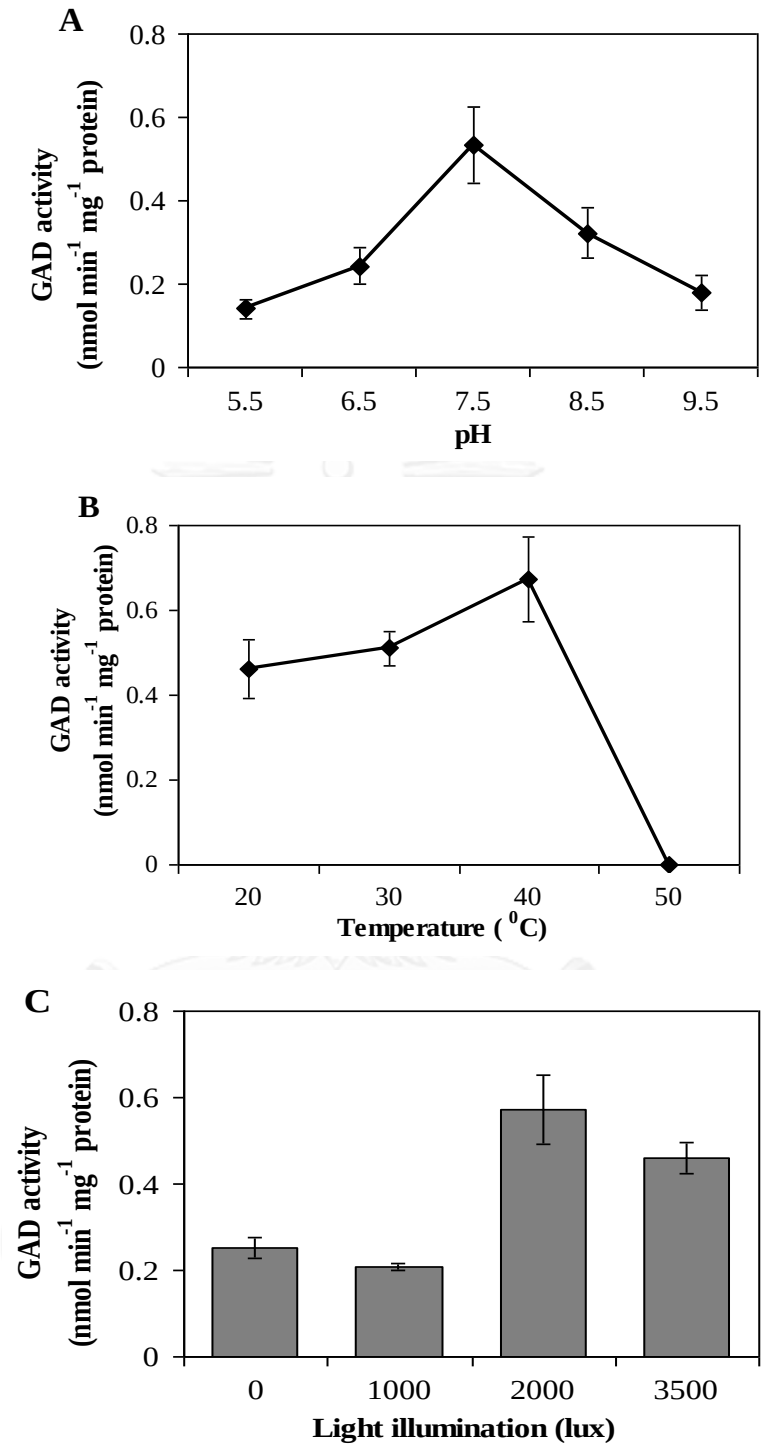


Figure 3.3 Effect of different physical factors **A)** pH, **B)** temperature, and **C)** light on GAD activity of *Synechocystis*. Means ± S.D. (n=3).

3.1.4 Effect of UV-B irradiation

Synechocystis cells were harvested at LL phase and exposed to UV-B radiation for 60 and 120 min. GAD activity in 60 min of UV-B irradiated cells was found 3 times higher as compared with control with no UV treatment (Figure 3.4). Increase in UV-B irradiation time to 120 min resulted in a decline in GAD activity. GAD Activity was lost when cells were exposed to UV-B radiation for 24 h.

3.1.5 Effect of alternative nitrogen sources

LL phase cells of *Synechocystis* were grown for another 24 h in BG-11 medium without nitrogen (N-deprived) and BG-11 media containing various nitrogen sources, $NaNO_3$, $NaNO_2$, Urea and $(NH_4)_2SO_4$ having equimolar concentration of N. Figure 3.5 shows that cells grown in nitrate exhibited the highest GAD activity followed by nitrite, ammonium and nitrogen deprived condition. Urea as N source was not found to be contributing much in elevating the GAD activity.

3.1.6 Effect of alternative carbon sources and glucose concentration

LL phase *Synechocystis* cells were further adapted for 24 h in modified BG-11 medium with no carbon source (C-deprived) or containing 0.1 % (w/v) of various carbon sources, glucose, fructose, sucrose and lactose, replacing Na_2CO_3 (carbon source in BG-11 medium). Highest GAD activity was attained by cells with 0.l % (w/v) glucose as carbon source (Figure 3.6A).

Furthermore, in order to optimize the glucose concentration for GAD activity in *Synechocystis*, cells were grown for 24 h in BG-11 media containing various w/v concentrations of glucose, 0.05 %, 0.1 %, 0.2 %, 0.3 % and 0.4 % (Figure 3.6B). GAD activity was found to be elevating till 0.2 % (w/v) of glucose and was observed to be declining at concentrations higher than 0.2 %.

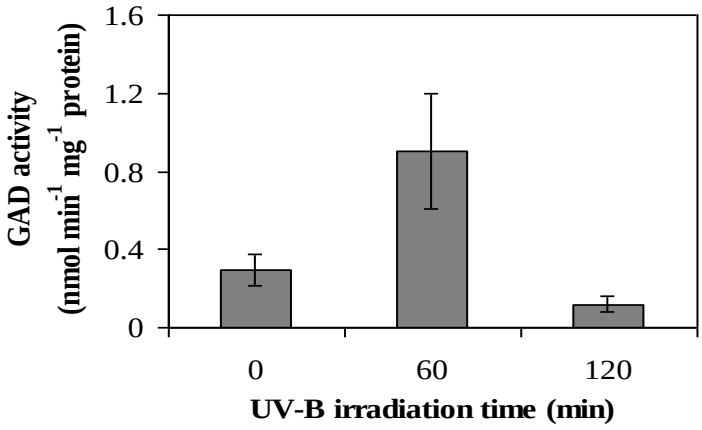


Figure 3.4 GAD activity in *Synechocystis* in response to UV-B irradiation. Means ± S.D. (n=3).

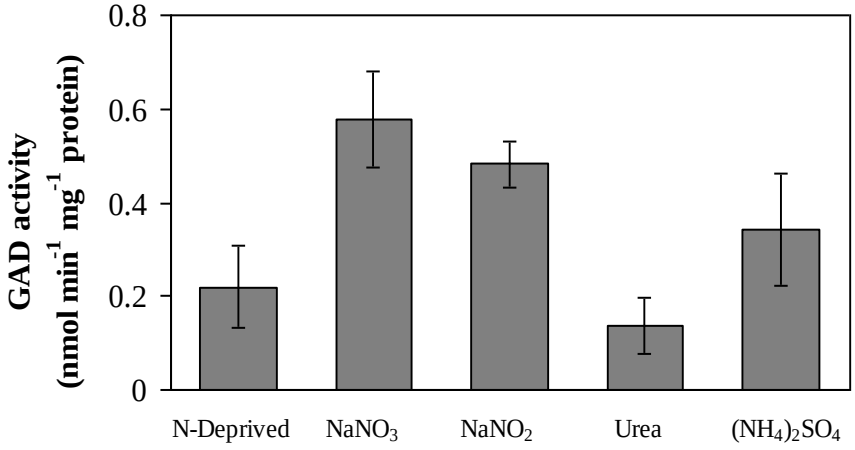


Figure 3.5 GAD activity in *Synechocystis* grown for 24 h in modified BG-11 media containing no N-source (N-deprived) or various N-sources, $NaNO_3$, $NaNO_2$, urea and $(NH_4)_2SO_4$. Means ± S.D. (n=3).

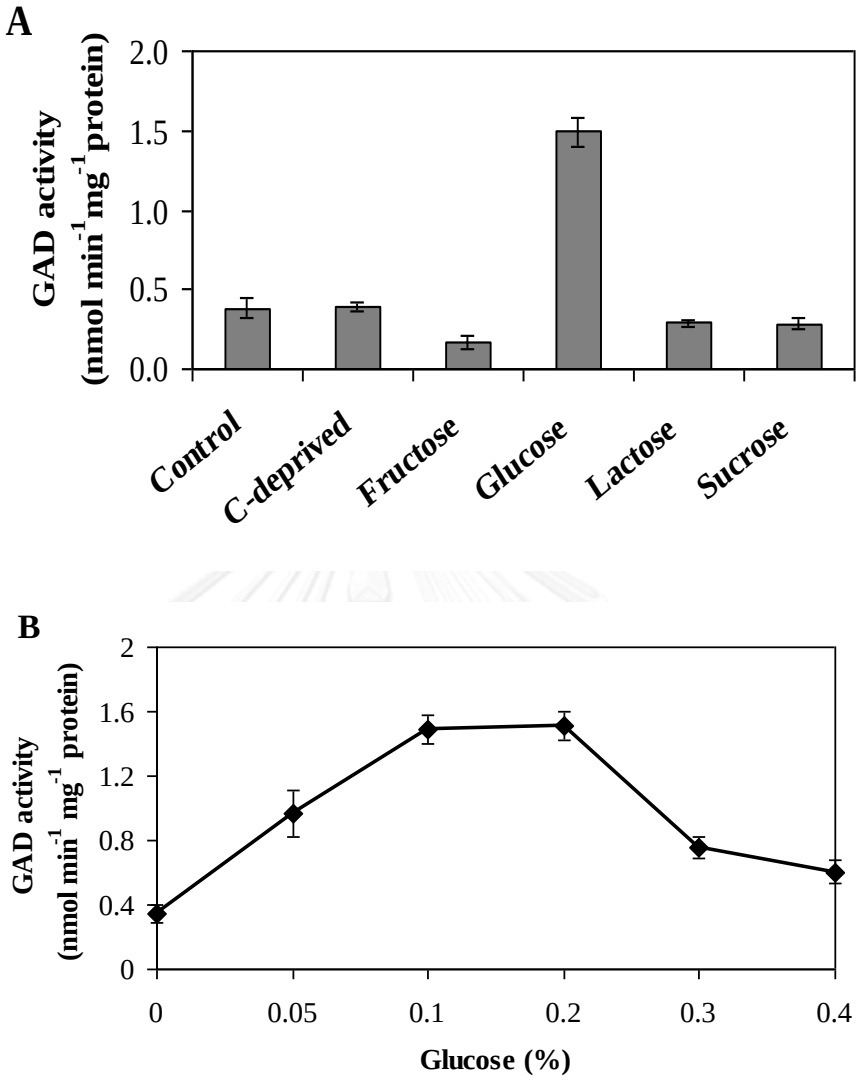


Figure 3.6 A) GAD activity in *Synechocystis* grown for 24 h in modified BG-11 media containing no C-source (C-deprived) or five different C-sources, Na_2CO_3 (control), fructose, glucose, lactose and sucrose at 0.1 % (w/v). **B)** Effect of glucose concentration on GAD activity in *Synechocystis*. Means ± S.D. (n=3).

3.1.7 Effect of osmotic stress

Effect of osmotic stress induced by NaCl and sorbitol on GAD activity of *Synechocystis* was studied. Cells were adapted for 24 h in BG-11 media containing NaCl at various concentrations, 2, 50, 150, 350 and 550 mM, or sorbitol at various concentrations. 4, 100 and 300 mM. Results indicated that NaCl induced osmotic stress led to a gradual increase in GAD activity up to 50 mM followed by a decline in GAD activity at NaCl concentrations higher than 50 mM (Figure 3.7A). Osmotic stress induced by sorbitol resulted in increase in GAD activity with the highest activity obtained at 100 mM that was about 5.5- fold in comparison to the control (0 mM), whereas further increase of sorbitol concentration inducing higher osmotic stress resulted in the decline of GAD activity (Figure 3.7B).

3.1.8 Effect of polyamine supplementation

Effect of exogenous polyamine supplementation on *Synechocystis* GAD activity was studied by adapting cells for 24 h in BG-11 media containing 0.5 mM Put and Spd individually. Both Put and Spd resulted in an increased GAD activity (Figure 3.8). Cells adapted in media supplemented with Spd exhibited twice as much activity as compared to the control.

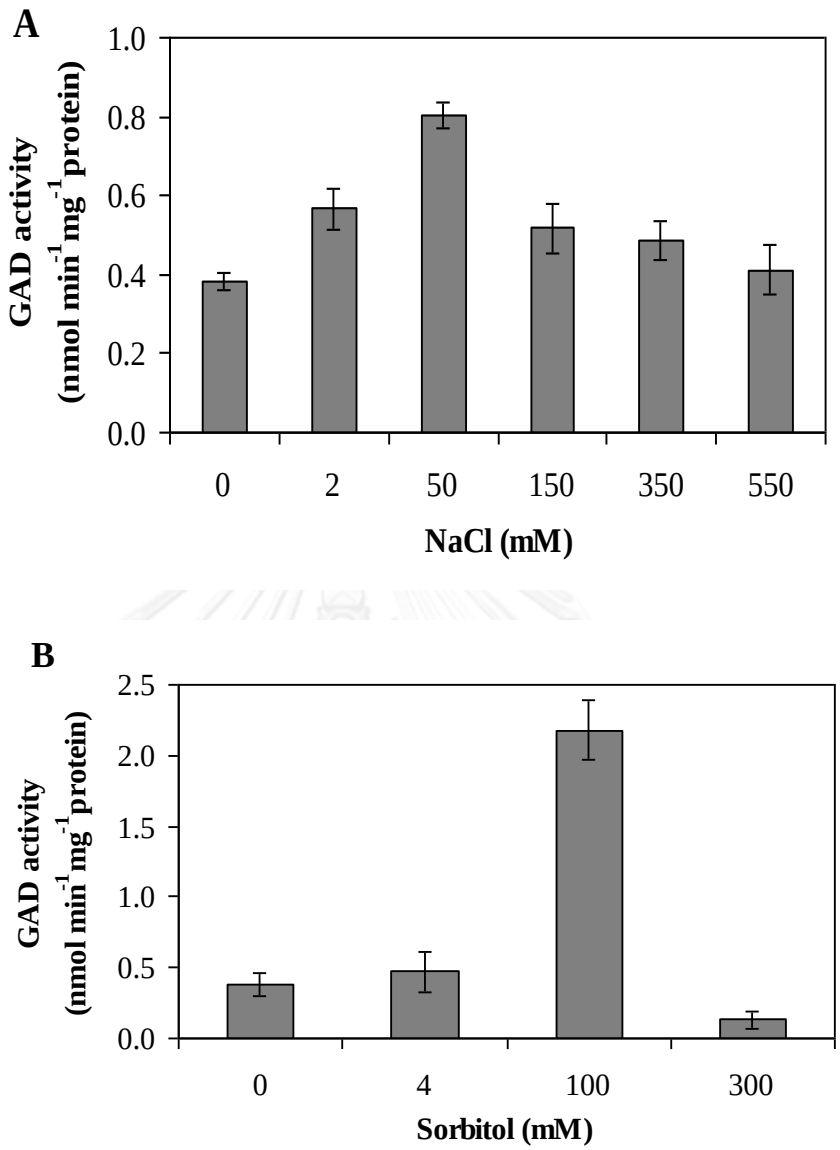


Figure 3.7 GAD activity of *Synechocystis* in response to osmotic stress induced by **A)** NaCl and **B)** sorbitol at various concentrations. Means ± S.D. (n=3).

3.1.9 Effect of exogenous glutamate supplementation

The experiment was performed by adapting *Synechocystis* cells for 24 h in BG-11 media supplemented with various concentrations of glutamate, 0, 5, 10, 20, 30 and 40 mM. Exogenous glutamate supplementation at lower concentration (5 mM) was found to be positively affecting the GAD activity, but glutamate at higher concentrations could not increase the GAD activity resulting in a drastic decline in activity at 30 and 40 mM (Figure 3.9).

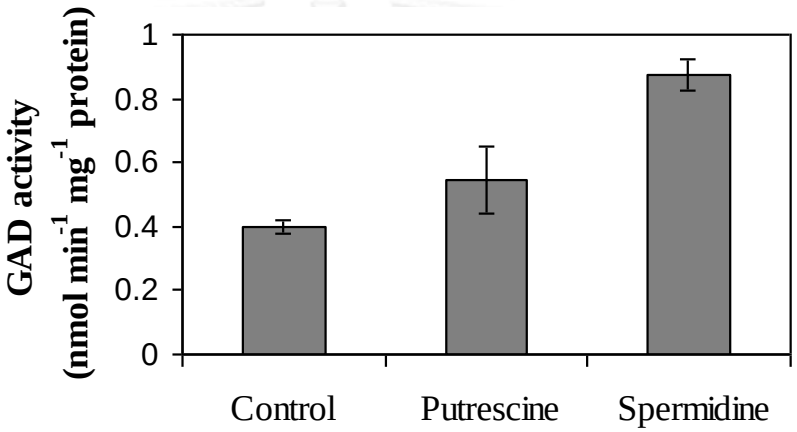


Figure 3.8 Effect of polyamine supplementation on GAD activity of *Synechocystis.* Cell suspensions in BG-11 medium were supplemented with Put and Spd at concentration of 0.5 mM for 24 h before determination of GAD activity. Means ± S.D. (n=3).

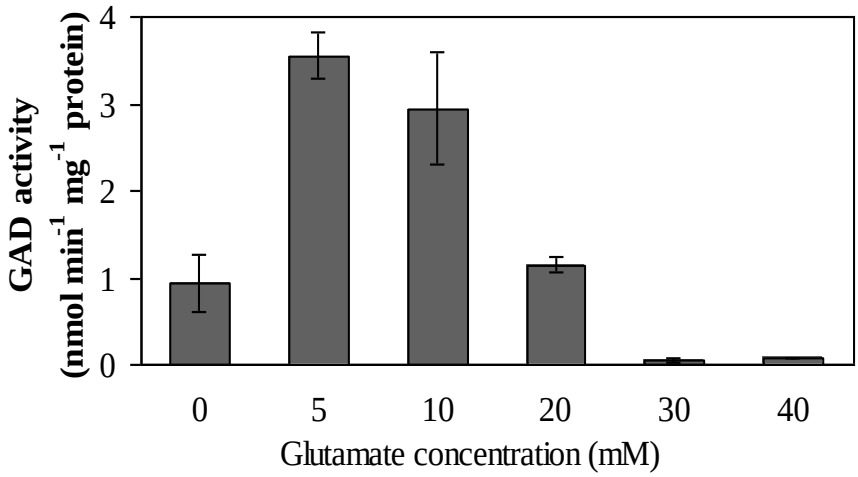


Figure 3.9 Effect of exogenous glutamate supplementation on GAD activity of *Synechocystis*. Cell suspensions in BG-11 medium were supplemented with indicated concentrations of glutamate for 24 h before determination of GAD activity. Means ± S.D. (n=3).

3.2 GABA production in *Synechocystis* under various conditions

3.2.1 Effect of osmotic stress on GABA content

GAD activity in *Synechocystis* was found to be increasing under short term osmotic stress conditions. It was then tested whether GABA levels in *Synechocystis* could also increase under osmotic stress induced by NaCl and sorbitol. The experiment was done by adapting *Synechocystis* cells under various concentrations of NaCl and sorbitol as described earlier in materials and methods section. GABA content was determined in terms of ng g^{-1} dry weight (DW) of cells. *Synechocystis* cells were observed to accumulate GABA content under osmotic stress induced by both NaCl and sorbitol at maximum concentrations of 350 mM and 300 mM respectively (Figure 3.10A, B). NaCl at 550 mM was not found to induce GABA accumulation in *Synechocystis.* Sorbitol induced osmotic stress caused a gradual increase in GABA accumulation from lowest to highest concentration used in this study, resulting in 5- fold increase of GABA content as compared to control.

3.2.2 Effect of alternative carbon sources on GABA content

LL phase grown cells of *Synechocystis* were tested for GABA accumulation in response to various carbon sources. Cells were grown in C deprived and modified BG-11 media with alternative carbon sources for 24 h and GABA content were tested under all conditions. 0.1 % (w/v) of glucose as carbon source resulted in highest GABA production inside the cells (Figure 3.11). Whereas C-deprived condition and other carbon sources including fructose, lactose and sucrose has little or no effect on GABA content of *Synechocystis* cells.

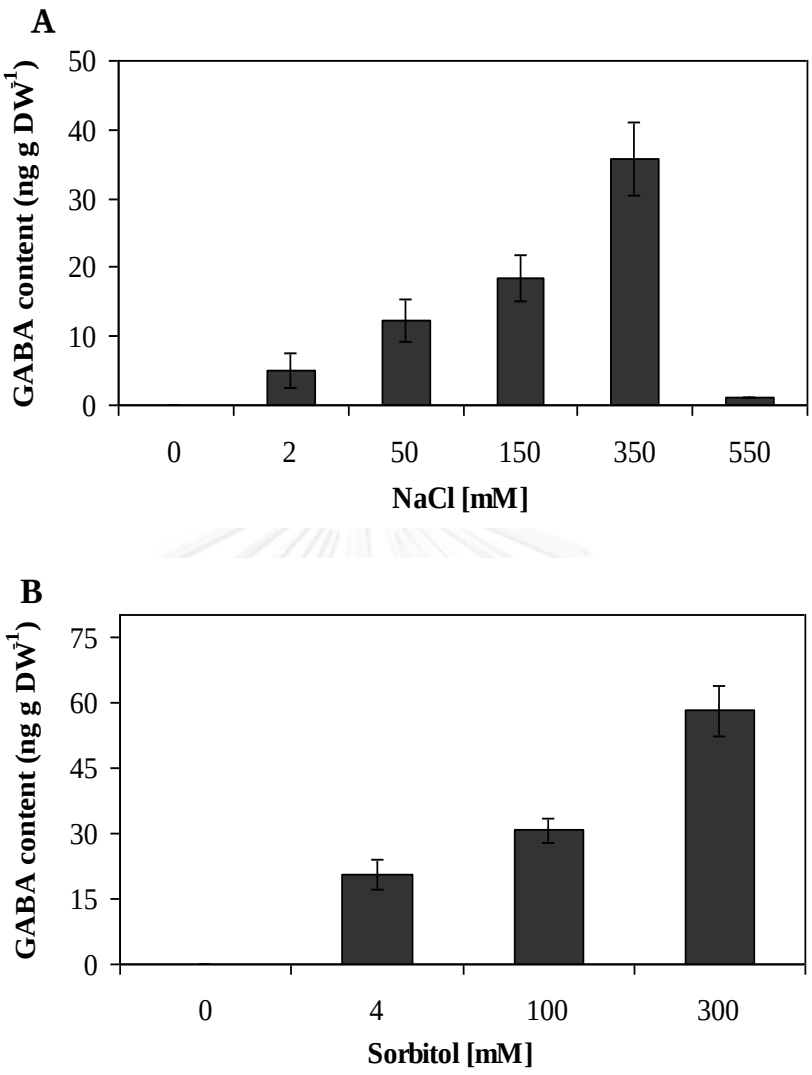


Figure 3.10 GABA content in *Synechocystis* in response to osmotic stress induced by **A)** NaCl and **B)** sorbitol at various concentrations. Means ± S.D. (n=3).

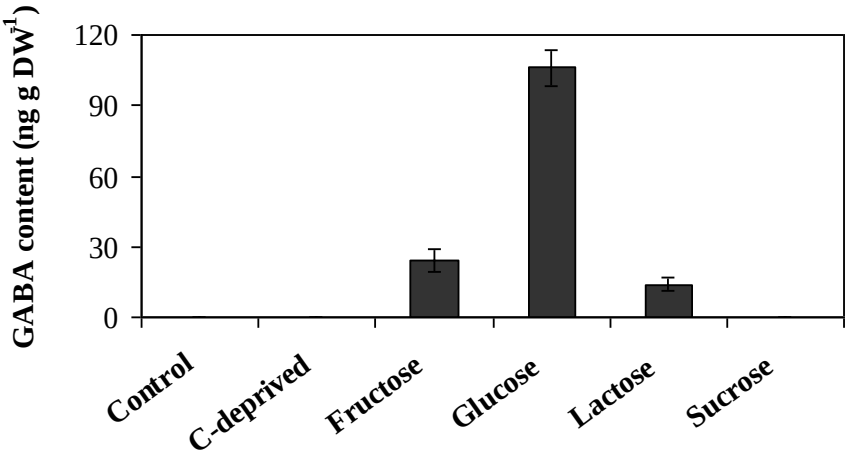


Figure 3.11 GABA content in *Synechocystis* grown for 24 h in modified BG-11 media containing no C-source (C-deprived) or five different C-sources, Na_2CO_3 (control) , fructose, glucose, lactose and sucrose at 0.1 % (w/v). Means ± S.D. (n=3).

3.2.3 Effect of alternative nitrogen sources on GABA content

Effect of alternative nitrogen sources on GABA content of *Synechocystis* cells was examined by cell adaptation in modified BG-11 media containing various N-sources or no nitrogen source (N-deprived) according to the method described earlier for GAD activity determination. Nitrate as nitrogen source resulted in 10 times more GABA accumulation as compared to N-deprived medium (Figure 3.12). Nitrite was observed to contribute almost equally in GABA production inside the cells, followed by ammonium and urea.

3.2.4 Effect of UV-B irradiation on GABA content

Effect of UV-B irradiation was studied on GABA accumulation. *Synechocystis* cells harvested at LL phase were exposed to UV-B radiation for 60 and 120 min, followed by GABA determination. Control group without UV-B treatment was used in parallel. Cells were observed to accumulate GABA at 60 min of UV-B exposure resulting in 30-fold increment in terms of GABA yield (Figure 3.13). UV-B exposure extended for 120 min resulted in the decline of GABA levels inside the cells.

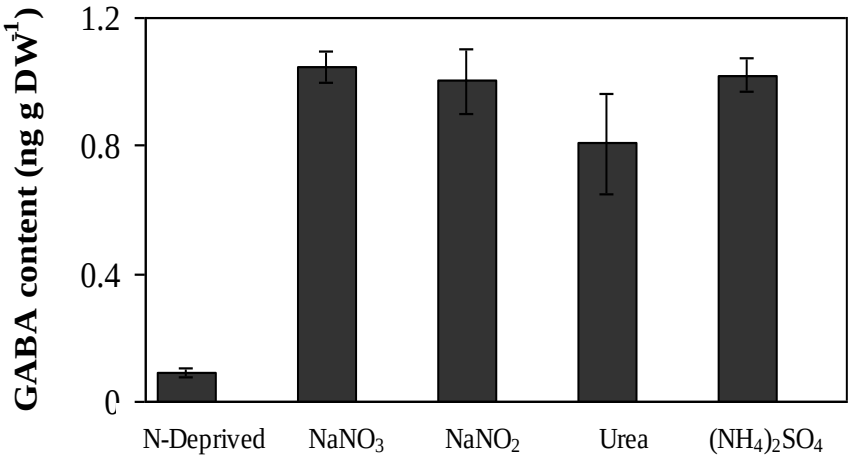


Figure 3.12 GABA content in *Synechocystis* grown for 24 h in modified BG-11 media containing no N-source (N-deprived) or various N-sources, $NaNO_3$, $NaNO_2$, urea and $(NH_4)_2SO_4$. Means ± S.D. (n=3).

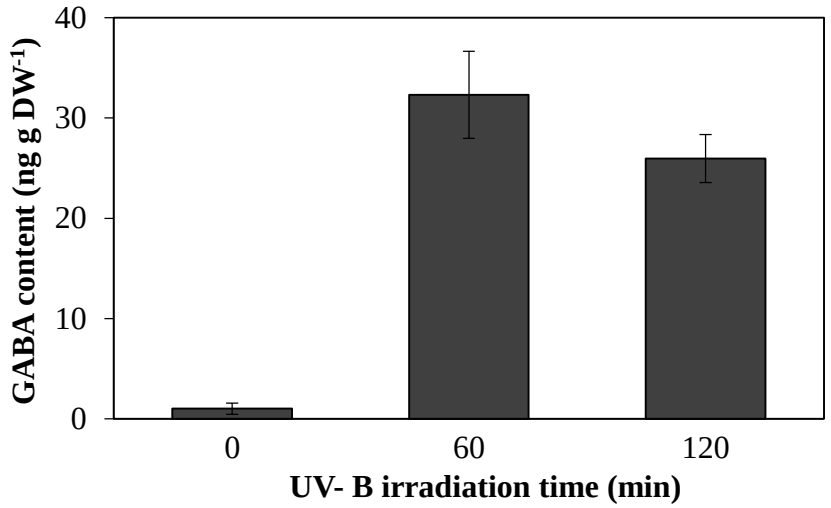


Figure 3.13 GABA content in *Synechocystis* in response to 60 and 120 min of UV-B irradiation. Means ± S.D. (n=3).

3.2.5 Effect of polyamine supplementation on GABA content

GABA content in *Synechocystis* was determined in response to polyamine supplementation. Cells were adapted in BG-11 media containing 0.5 mM of Put and Spd. Both Put and Spd addition were found to enhance GABA accumulation inside the cells but Spd resulted in about 5- fold increment in GABA content as compared to control having no polyamine in medium (Figure 3.14).

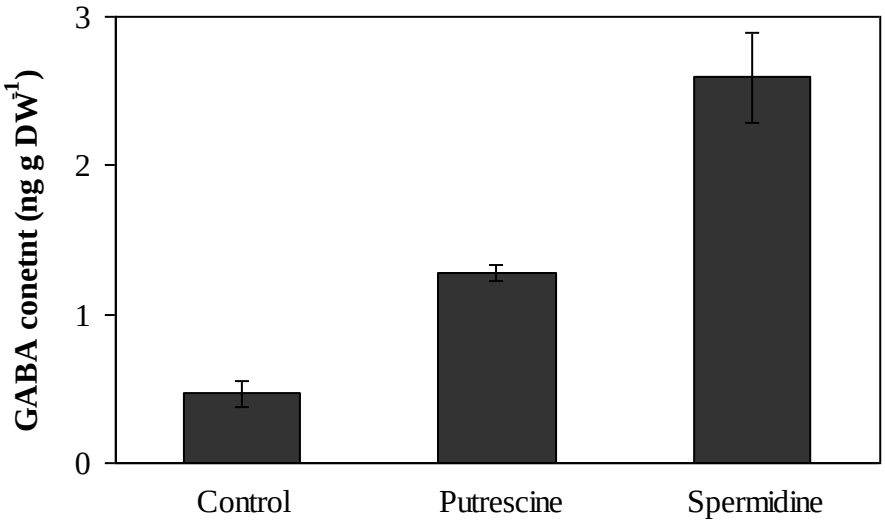


Figure 3.14 Effect of polyamine supplementation on GABA content of *Synechocystis.* Means ± S.D. (n=3).

3.2.6 Effect of exogenous glutamate supplementation on GABA content

GABA productivity in *Synechocystis* cells was tested under glutamate supplementation at various concentrations in BG-11 medium. GABA content was found to increase gradually at 5 and 10 mM of glutamate (Figure 3.15A). Further increase in glutamate concentration resulted in a decline of GABA levels inside the cell. Furthermore intracellular glutamate levels were also studied under same conditions. Cells show higher glutamate content at all the concentrations of exogenous glutamate tested as shown in the figure 3.15B.

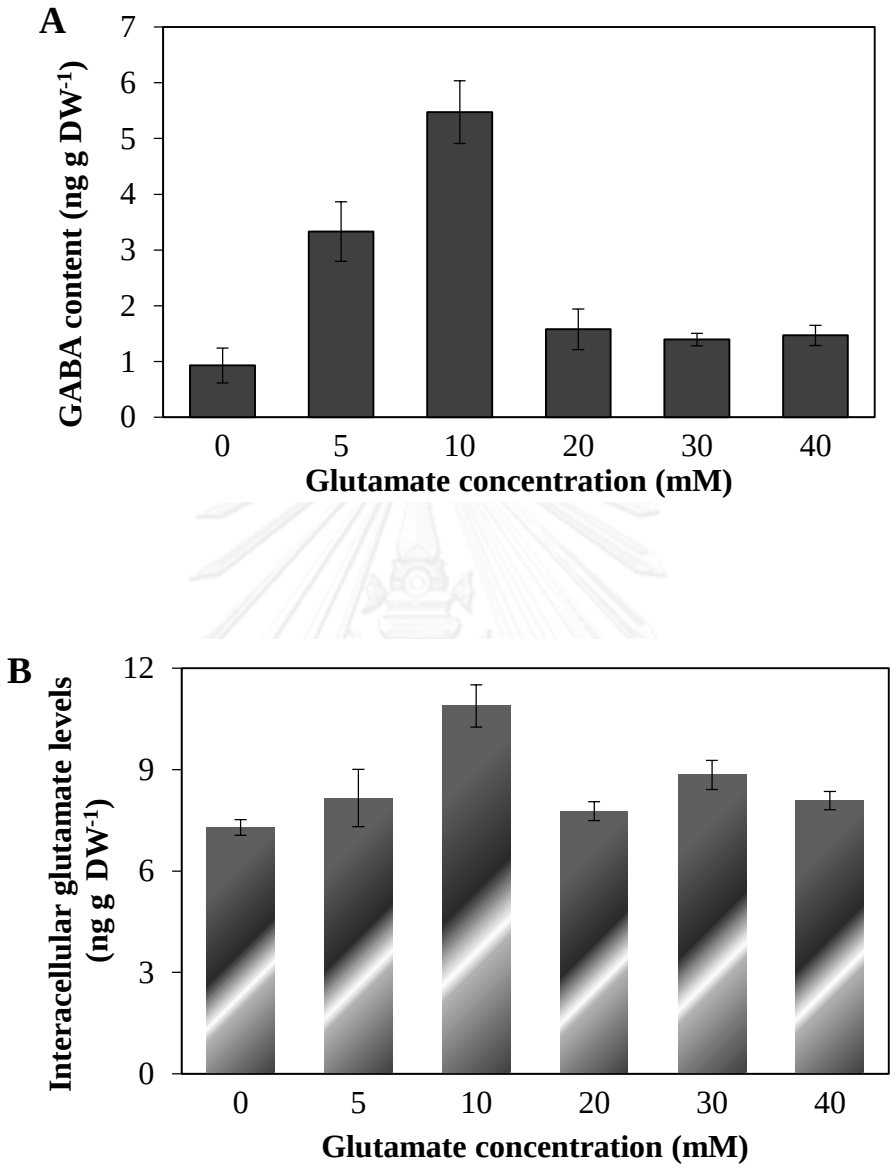


Figure 3.15 Effect of various concentrations of exogenous glutamate supplementation on **A)** GABA content, and **B)** intracellular glutamate levels of *Synechocystis.* Means ± S.D. (n=3).

3.3 Transcript analysis of gene encoding GAD enzyme under various conditions

3.3.1 Effect of osmotic stress on transcription level of *gad* gene

Transcription analyses of gene encoding GAD was done to gain more information about GAD regulation under osmotic stress conditions. Figure 3.16A showed RT-PCR products using RNA isolated from cells grown for 24 h in BG-11 medium supplemented with either NaCl or sorbitol at various concentrations. Intense bands for *gad* transcript levels were detected in cells grown under NaCl induced osmotic stress conditions gradually from 50 to 350 mM. Sorbitol induced osmotic stress also induced the transcription of *gad* as compared to the control (cells grown without NaCl and sorbitol supplementation) but induction in RNA expression was not as much as shown by NaCl condition.

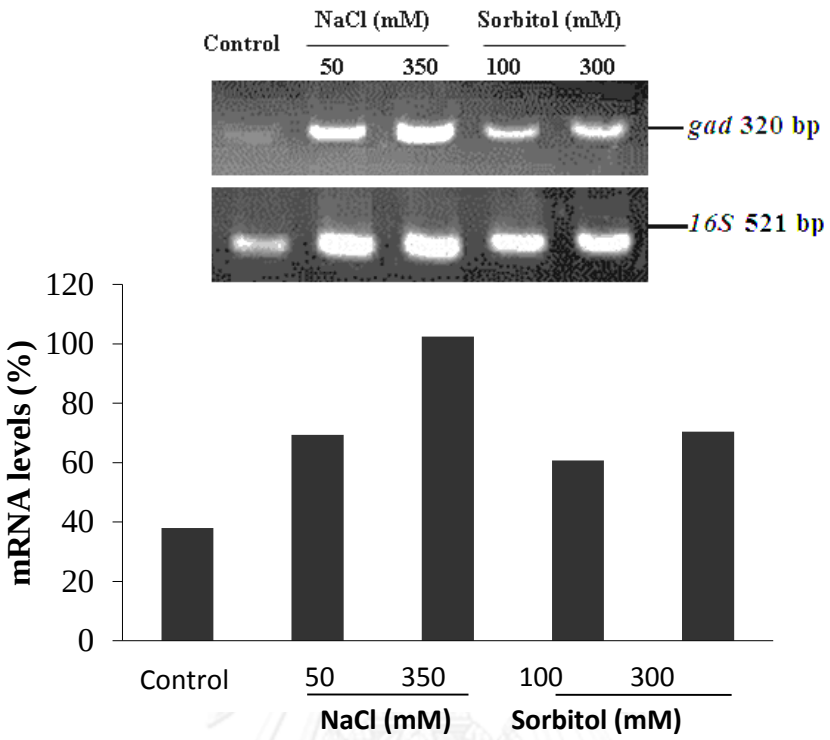
3.3.2 Effect of alternative carbon sources on *gad* transcript levels

The expression analysis of *gad* encoding GAD enzyme was done by reverse transcription-PCR (RT-PCR) in response to various C-sources. Total RNA was isolated from *Synechocystis* cells adapted for 24 h in BG-11 medium containing Na_2CO_3 as carbon source, C-deprived BG-11 medium (-C) or modified BG-11 medium containing 0.1 % (w/v) of fructose, glucose, lactose and sucrose. Faint bands for *gad* transcript levels were observed in response to various C-sources tested except for glucose (Figure 3.16B). The presence of 0.1 % (w/v) glucose in BG-11 medium could significantly enhance transcription of *gad* in contrast to other C-sources.

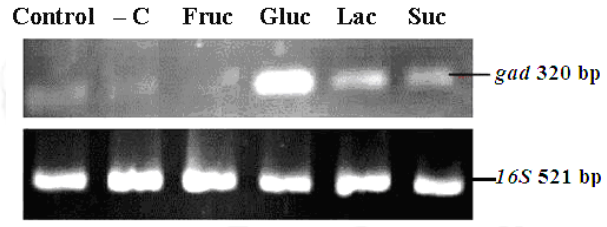
3.3.3 Effect of UV-B irradiation on *gad* transcript levels

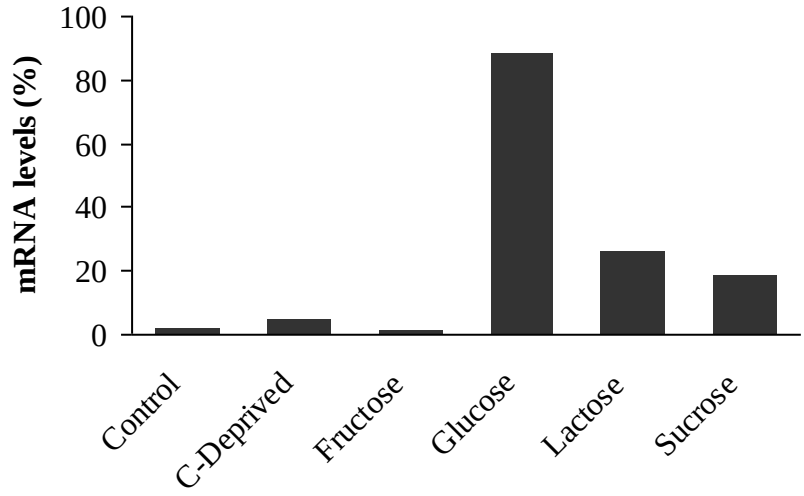
Effect of UV-B irradiation on *gad* transcript levels was investigated in *Synechocystis.* Total RNA was isolated from LL phase grown cells exposed to UV-B for 30, 60 and 120 min. UV-B irradiation was found to be drastically declining the *gad* transcript levels even at 30 min of exposure (Figure 3.16C). Whereas increase in time of UV-B exposure to cells resulted in further down-regulation of *gad* transcripts.

A



B





C

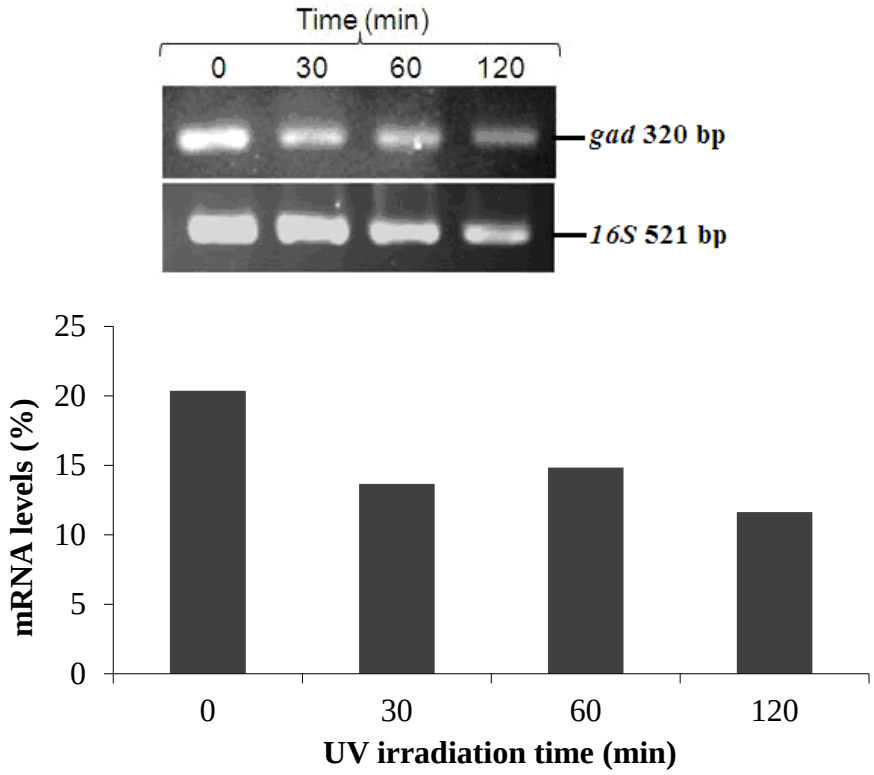


Figure 3.16 RT-PCR analyses of *gad* transcript levels in *Synechocystis* grown under **A)** NaCl and sorbitol induced osmotic stress condition, **B)** alternative C-sources, and **C)** UV-B irradiation. Effect of osmotic stress on transcript levels of gene encoding GAD was investigated by RT-PCR using total RNA isolated from cells grown in BG-11 medium supplemented with either NaCl (50 and 350 mM) or sorbitol (100 and 300 mM) for 24 h. Effect of alternative C-sources was investigated by isolating total RNA from cells adapted for 24 h in BG-11 medium containing Na_2CO_3 as C-source, or C-deprived BG-11 medium (-C), or BG-11 medium containing 0.1 % fructose (Fruc), glucose (Gluc), lactose (Lac) and sucrose (Suc). Effect of UV-B irradiation on *gad* transcript levels was studied by isolating total RNA from LL phase grown cells exposed to UV-B radiation for 30, 60 and 120 min. PCR amplifications using cDNA of *gad* gene was performed using specific primers. Transcript levels of 16S rRNA were used as internal reference gene. Experiments were repeated at least three times. Negative controls without reverse transcriptase in the RT reaction prior to the PCR were tested for all samples.

3.4 Characterization of *Synechocystis* Δ*gad* mutant

3.4.1 Construction of Δ*gad* mutant

Recombinant plasmid called as pGADSpSm possessing disrupted *gad* gene was constructed previously by Dr. Wanthanee Khetkorn and transformed into *Synechocystis* wild type strain to generate *gad* inactivation strain called as Δ*gad* mutant strain according to the method described earlier in materials and methods section (2.7.7 and 2.7.8). Design of constructed pGADSpSm plasmid and confirmation of complete segregation of Δ*gad* mutant is shown in Figure 3.17A and B respectively. The PCR result revealed that the DNA fragment isolated from *Synechocystis* Δ*gad* mutant was larger than *Synechocystis* wild-type strain.

3.4.2 Comparison of growth rate and GABA production between Δ*gad* mutant and wild type strain of *Synechocystis*

The *Synechocystis* wild type was grown in BG-11 medium as described in section 3.1.1 and Δ*gad* mutant strains was grown in BG-11 medium supplemented with streptomycin and spectinomycin antibiotics at concentration of 10 μgml^{-1} and 20 μgml^{-1}, respectively. Cell growth was monitored by checking the OD at 730 nm every two days. The results showed that mutant strain grew slightly slower than wild type (Figure 3.18A).

Cells were harvested at LL phase of growth (approximately 8th day) and GABA contents were determined in both wild type and mutant strains. Mutant showed fairly low levels of GABA inside the cells (0.02 ng g DW^{-1}) as compared to wild type (~0.5 ng g DW^{-1}) as shown in Figure 3.18B.

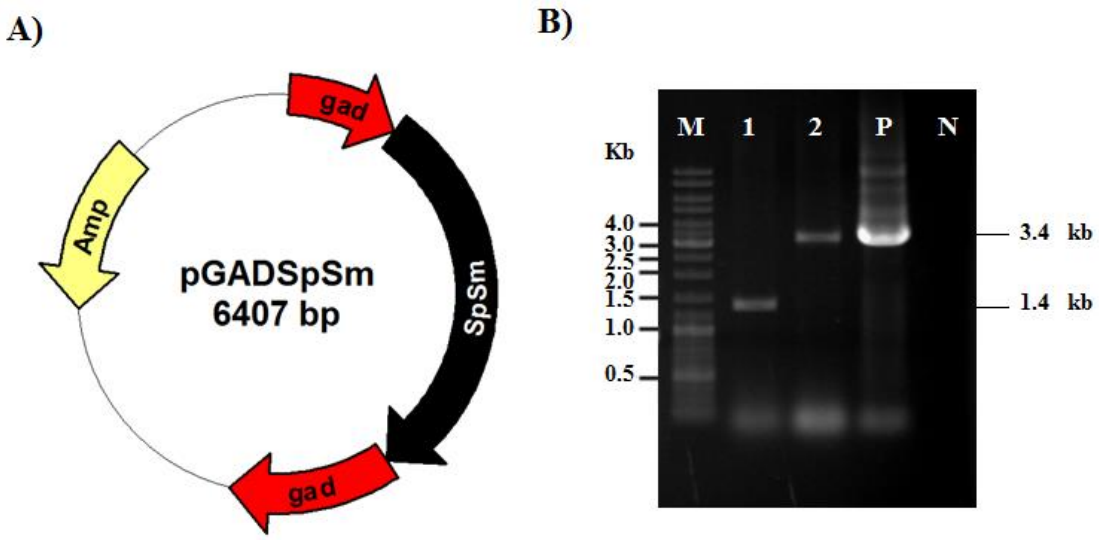


Figure 3.17 A) Design and construction of plasmid to inactivate GAD in *Synechocystis* sp. PCC 6803 created by interrupting *gad* with spectinomycin antibiotic resistant cassette. **B)** Complete segregation of mutant strain was confirmed by performing colony PCRs and analyzed by 0.8 % agarose gel electrophoresis. Primer pair specific to *gad* was used.

Lane M: GeneRuler^{TM} DNA ladder (Fermentas)

Lane 1: PCR product of *gad* using genomic DNA of wild type as template

Lane 2: PCR product of *gad* using genomic DNA of recombinat colonies as template

Lane P: Positive control using pGADSpSm plasmid as template

Lane N: Negative control using H_2O as template

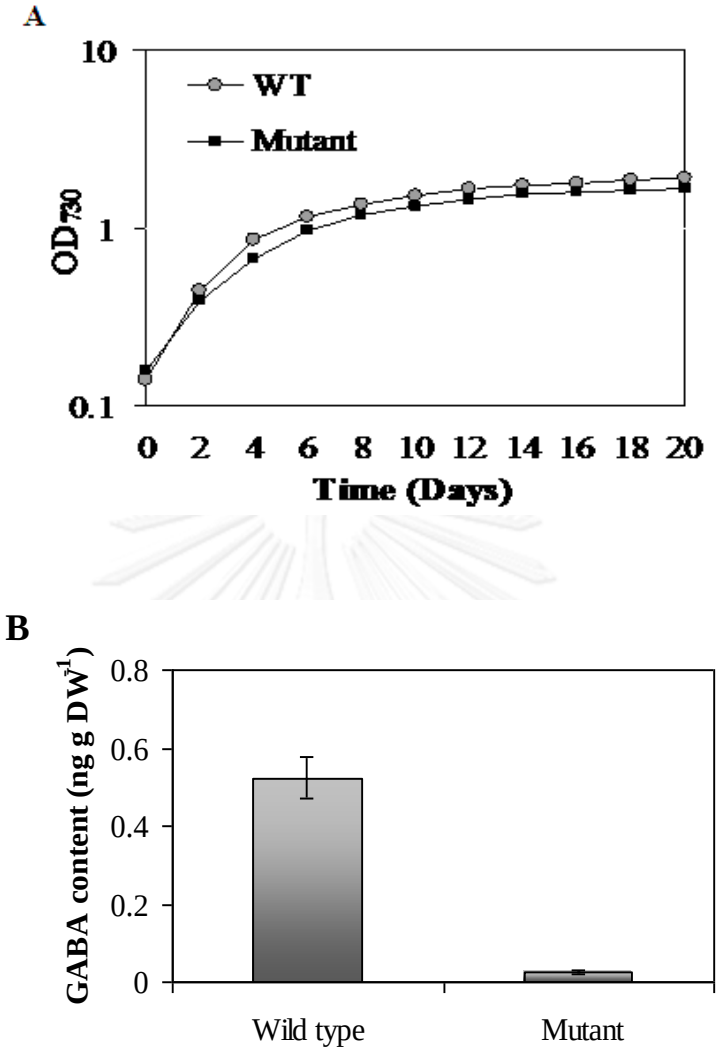


Figure 3.18 A) Comparison of growth of *Synechocystis* wild type grown in BG-11 medium and mutant strain grown in BG-11 supplemented with streptomycin (10 $\mu g ml^{-1}$) and spectinomycin (20 $\mu g ml^{-1}$) by measuring the optical density of culture at 730 nm. **B)** Comparison of GABA productivity between *Synechocystis* wild type and mutant strain grown till LL phase of cell growth. Means ± S.D. (n=3).

3.4.3 Effect of alternative nitrogen sources on GABA accumulation in mutant

GABA content in mutant strain in response to alternative N-sources was examined. LL phase cells of mutant were grown for another 24 h in BG-11 medium without nitrogen (N-deprived) and BG-11 medium containing equimolar N concentration of various N-sources, $NaNO_3$, $NaNO_2$, Urea and $(NH_4)_2SO_4$. Nitrate as nitrogen source resulted in highest GABA accumulation followed by ammonium (Figure 3.19). *Δgad* mutant with knocked out *gad* gene showed very low GABA levels when compared with wild type strain that showed highest GABA levels of 1.1 ng g DW^{-1} (Figure 3.12).

3.4.4 Effect of UV-B irradiation on GABA content of mutant

GABA content in mutant strain was investigated in response to UV-B irradiation. Cells harvested at LL phase were irradiated with UV-B for 60 and 120 min, followed by GABA determination. GABA content was observed to be increasing gradually from 60 to 120 min of UV-B irradiation (Figure 3.20). But GABA levels in mutant were fairly low as compared to wild type that showed 30 times higher GABA production inside the cells.

3.4.5 Effect of polyamine supplementation on GABA accumulation in mutant

GABA accumulation in mutant strain was determined in response to polyamine supplementation. LL phase grown cells were adapted in BG-11 medium containing 0.5 mM Put or Spd. Both Put and Spd were found to induce GABA accumulation in mutant as shown in Figure 3.21. Spd was found to be more effective in elevating GABA levels.

3.4.6 Effect of exogenous glutamate supplementation on GABA content of mutant

Effect of exogenous glutamate supplementation on GABA productivity of *Δgad* mutant was investigated by supplementing BG-11 medium with various concentrations of glutamate, 0, 5, 10, 20, 30 and 40 mM. GABA content was observed to be increasing as compared to control grown in BG-11 medium without glutamate supplementation (Figure 3.22A). Mutant showed quite less GABA accumulation in contrast to wild type where higher GABA accumulation was observed under glutamate supplementation as

shown in Figure 3.15A. Mutant accumulated very high levels of intracellular glutamate under exogenous glutamate supplementation up to 20 mM (Figure 3.22B). Glutamate supplementation at higher concentration (30 and 40 mM) was observed to slow down the growth of wild type whereas growth of mutant was completely inhibited (Figure 3.22C).

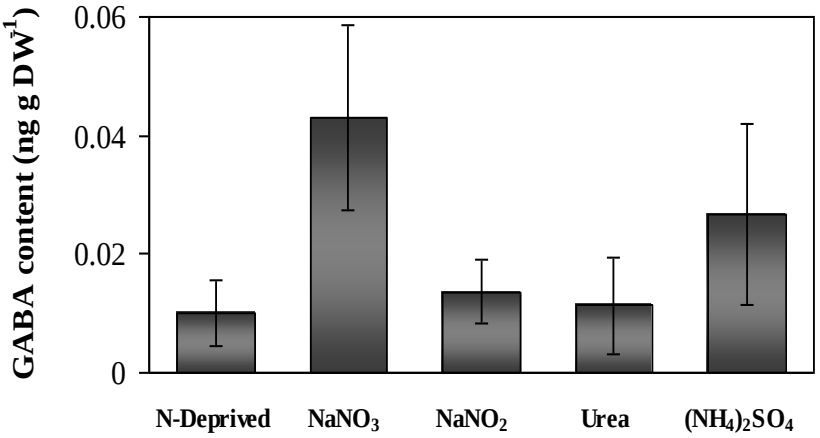


Figure 3.19 GABA content in *Synechocystis* mutant strain grown for 24 h in modified BG-11 media containing no N-source (N-deprived) or various N-sources, $NaNO_3$, $NaNO_2$, urea and $(NH_4)_2SO_4$. Means ± S.D. (n=3).

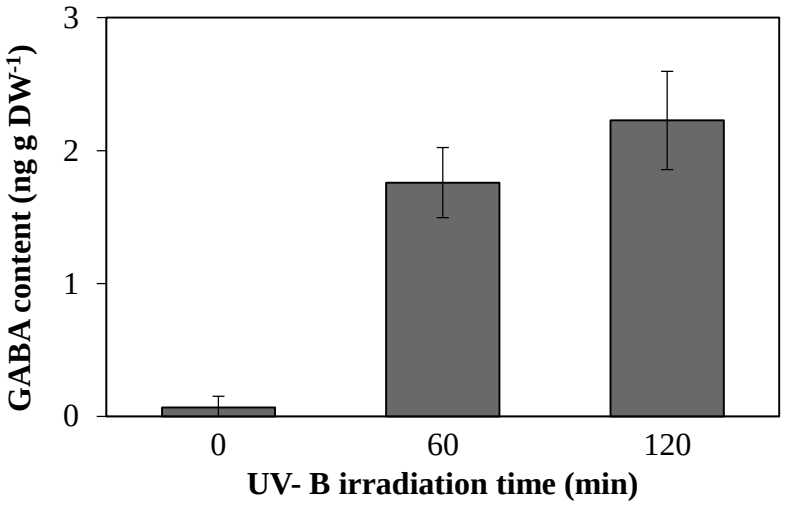


Figure 3.20 GABA content in *Synechocystis* mutant strain in response to 60 and 120 min of UV-B irradiation. Means ± S.D. (n=3).

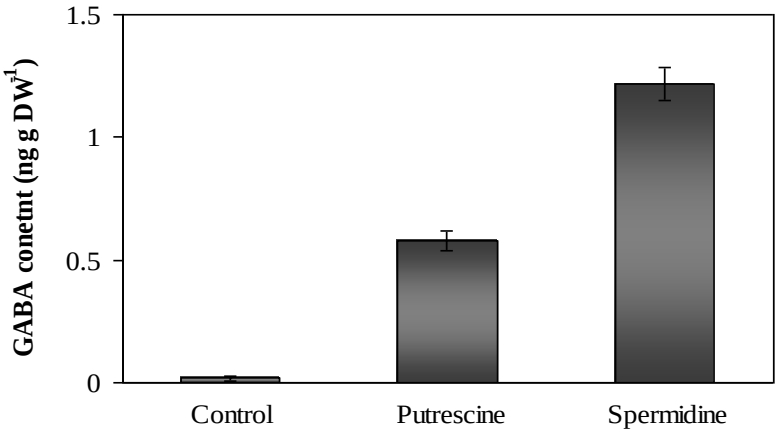


Figure 3.21 GABA content in *Synechocystis* mutant strain in response to polyamine supplementation. Means ± S.D. (n=3).

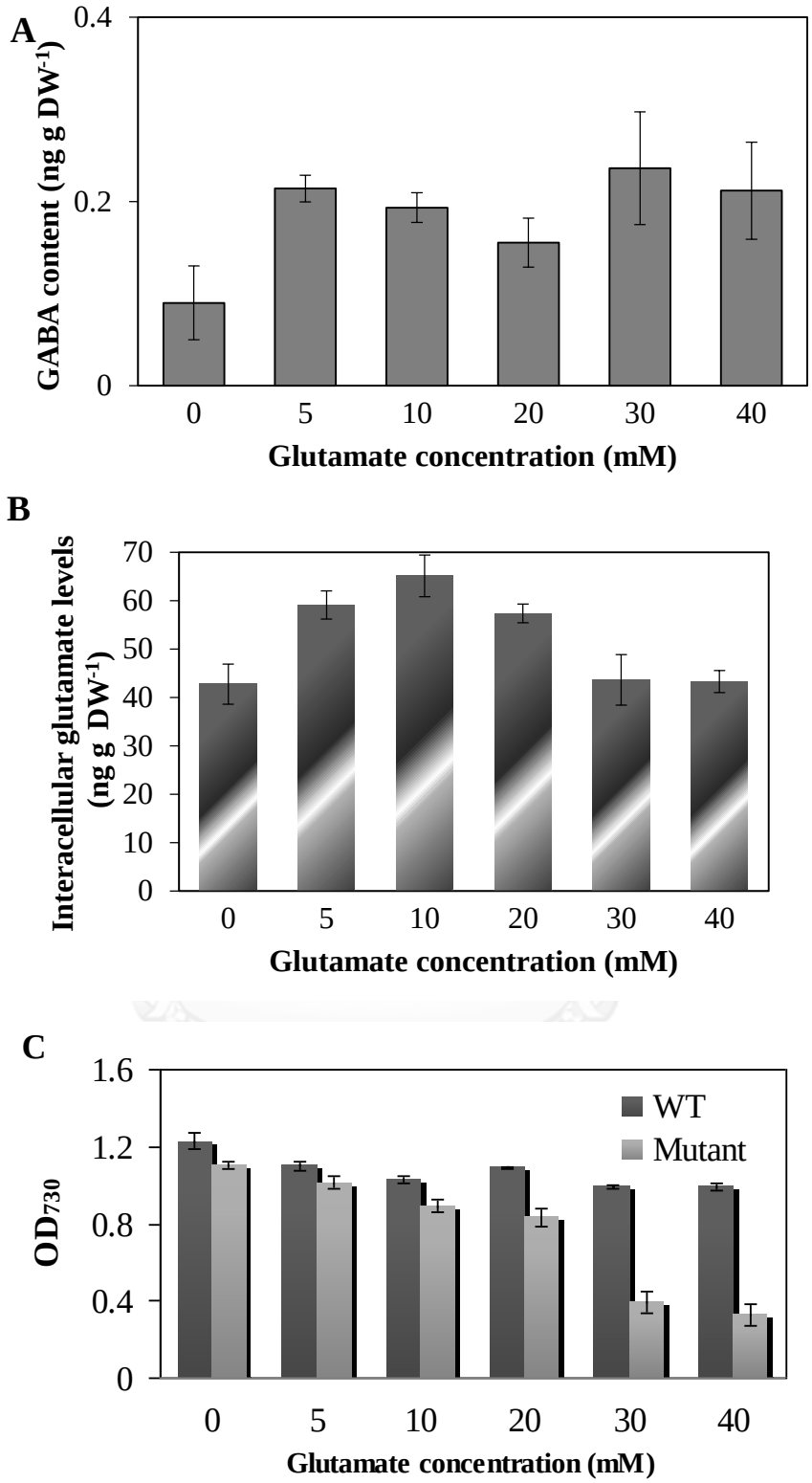


Figure 3.22 Effect of various concentrations of exogenous glutamate supplementation for 24 h on **A)** GABA content of *Synechocystis* mutant strain, **B)** Intracellular glutamate

levels of *Synechocystis* mutant strain, and **C)** Growth of *Synechocystis* mutant strain. Means ± S.D. (n=3).

3.5 Characterization of *Synechocystis* GAD protein

3.5.1 Construction of a pTAGAD recombinant plasmid containing the *gad*

The *gad* gene of *Synechocystis* was amplified using genomic DNA as a template with the aid of specific primers, designed from the complete sequence data in cyanobase (Kaneko *et al.* 1996). PCR amplification was performed as described earlier in materials and methods section 2.7.2. A prominent PCR product of *gad* gene with the approximated size of 1.4 kb was obtained as shown in Figure 3.23. PCR product was purified from gel using PCR purification kit. The purified PCR product and pTA vector were ligated together at 22 ˚C for 10 min and subsequently transformed into *E. coli* DH5α by heat shock method. Recombinant clone was selected on LB agar plate containing ampicillin and X-Gal. A single white colony containing the recombinant clone was selected on LB agar plate containing ampicillin and X-Gal followed by inoculation in LB broth containing ampicillin. After growing the culture overnight, bacterial plasmid was extracted and digested with *Nde*I and *Xho*I restriction enzymes to prove the correction of recombinant plasmid. Reactions were analyzed by 0.8 % agarose gel electrophoresis. Design of constructed pTAGAD and confirmation of recombinant plasmid harbouring *gad* gene is shown in Figure 3.24A and B respectively. Two bands of pTA vector and *gad* gene were observed at 2.7 and 1.4 kb, respectively

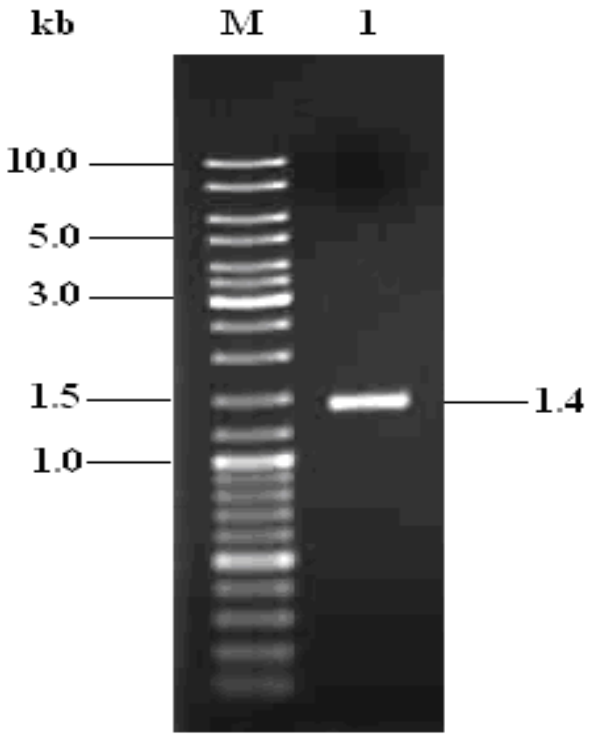


Figure 3.23 Agarose gel electrophoresis of the amplified gad gene. The PCR product was separated on a 0.8 % agarose gel and visualized by ethidium bromide staining. Lane M is a DNA marker and lane 1 is the PCR product of gad.

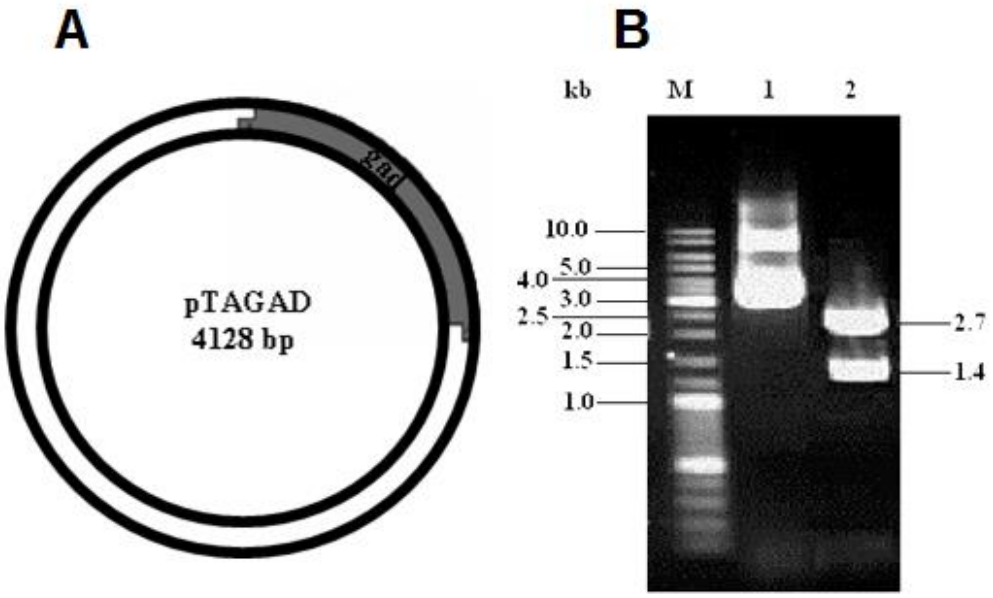


Figure 3.24 Agarose gel electrophoresis of the recombinant *gad* in pTA vector (pTAGAD). **A)** Design and construction of pTAGAD. **B)** The analysis of pTAGAD. Lane M is a DNA marker, lane 1 is pTAGAD and lane 2 is pTAGAD digested with *Nde*I and *Xho*I. The digested fragments were separated on a 0.8 % agarose gel and then visualized by ethidium bromide staining.

3.5.2 Construction of a pETGAD recombinant plasmid for the expression of *gad*

The pET 22b+ vector was double digested with *Nde*I and *Xho*I restriction enzymes. The *gad* obtained from pTAGAD and linear pET 22b+ vector were ligated together and the ligation mixture was transformed by heat shock method into competent cells of *E. coli* BL21 (DE3) for *gad* gene expression. After selecting a single white colony of transformants on LB agar plate containing ampicillin, plasmid was extracted and digested with *Nde*I and *Xho*I restriction enzymes to confirm the recombinant plasmid (pETGAD) harboring *gad* gene. Reactions were analyzed by 0.8 % agarose gel electrophoresis and pETGAD plasmid was confirmed by observing two bands of pET 22b+ vector and *gad* gene at 5.4 and 1.4 kb, respectively (Figure 3.25).

Moreover, to confirm the correction of inserted fragment sequence in transformed vectors, both pTAGAD and pETGAD were subjected to DNA sequencing by commercial service and subsequently compared to the *sll1641* gene in Cyanobase by

using the ClustalW program. The comparison results showed 100 % homology of *gad* gene to *sll1641* gene.

3.5.3 Expression and immunoblotting of recombinant *Synechocystis* GAD (rGAD)

The recombinant *E. coli* BL21(DE3) cells harboring pETGAD were grown at 37 ˚C in LB medium containing 100 µg/ml ampicillin. Protein production was induced according to the method described earlier in materials and methods section 2.8.1. Both supernatant and pellet fraction obtained from the crude extract of sonicated cells were analyzed by 12 % SDS-PAGE (Figure 3.26). The induction of an approximate 53 kDa recombinant protein occurred after 1 hour of induction by 1 mM IPTG at 37 ˚C and gradually increased over the 6 hour of induction. The result shown in Figure 3.27 illustrated that recombinant *Synechocystis* GAD (rGAD) protein was expressed in *E. coli* strain BL21 (DE3) as a soluble protein. The recombinant protein rGAD was confirmed by immunoblotting using a monoclonal antibody against His-tag at a dilution of 1:3,000 and a secondary antibody conjugated goat anti mouse immunoglobulin G at a dilution of 1:5,000. After incubating with secondary antibody, the membrane was developed. The immunoblotting analysis indicated that rGAD had an apparent molecular mass of 53 kDa corresponding to the size of the predicted rGAD (Figure 3.27).

3.5.4 Purification of rGAD protein

The recombinant GAD protein was purified by Ni-Sepharose column according to the method described earlier in materials and methods section 2.8.4. The purified protein was solubilized in 1X loading dye and then analyzed by 12 % SDS-PAGE. Coomassie blue stained gel revealed the rGAD protein with 53 kDa of expected size (Figure 3.28).

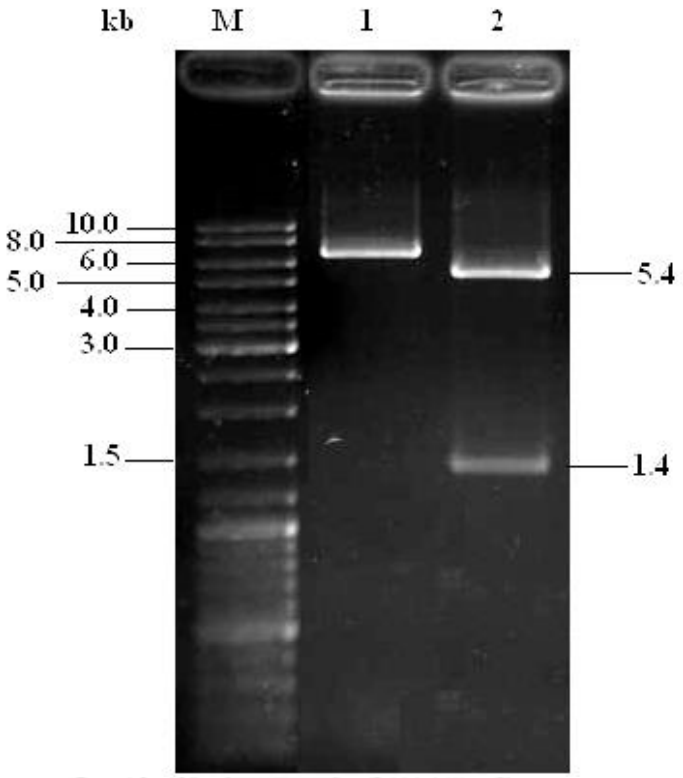


Figure 3.25 Agarose gel electrophoresis of the recombinant *gad* gene in pET-22b(+) vector for the construction of pETGAD. Lane M is a DNA marker, lane 1 is pETGAD and lane 2 is pETGAD digested with *Nde*I and *Xho*I. The digested fragments were separated on a 0.8 % agarose gel and then visualized by ethidium bromide staining.

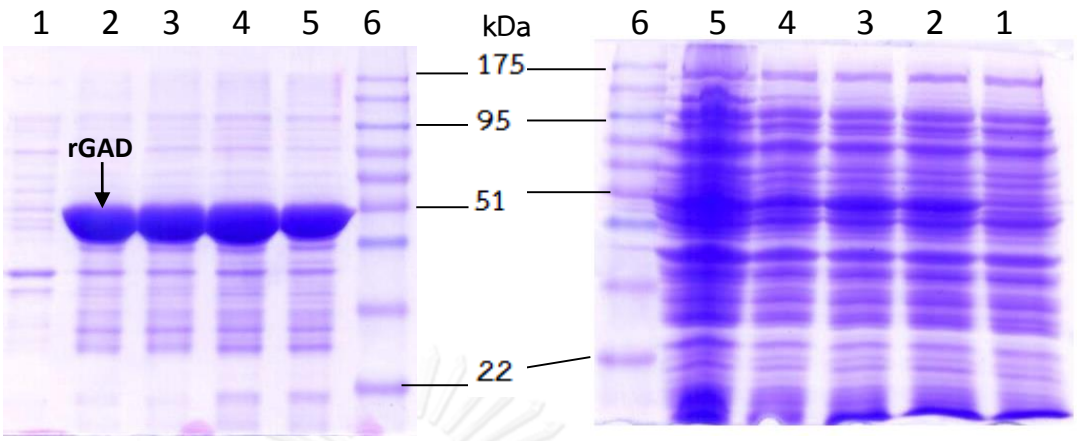


Figure 3.26 Coomassie-stained 12 % SDS polyacrylamide gel analysis of recombinant GAD protein induced by addition of 1 mM IPTG. Culture medium was collected after induction at 0, 1, 2, 3, 6 hours. Gel on left hand side contains the protein samples from supernatant fraction showing the prominent band of rGAD. Gel on right hand side is the protein samples from pellet fraction. Lane 1-5: *E. coli* cells contained plasmid pETGAD induced for 0, 1, 2, 3, 6 hours, respectively. Lane 6: Molecular weight marker in kilodaltons.

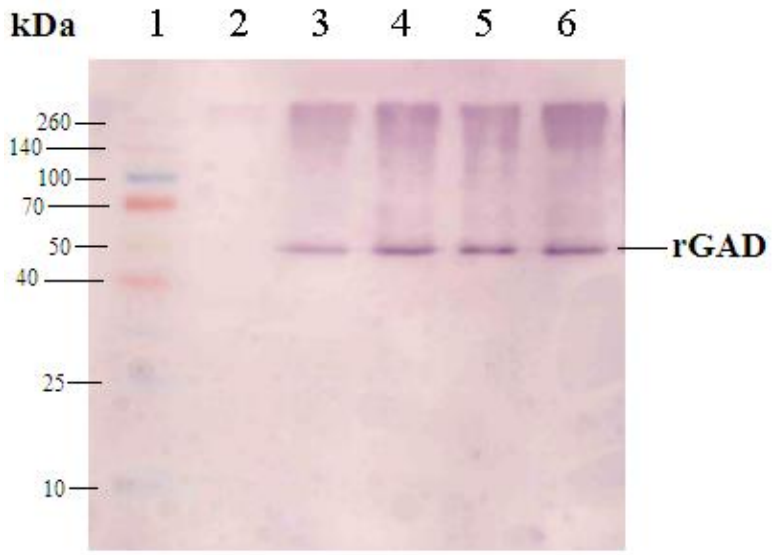


Figure 3.27 Immunoblot analyses of 20 µg of non-purified His-tagged GAD protein (rGAD) having approximately 53 kDa of molecular weight, probed with monoclonal anti-His_6 antibody. Lane 1: Molecular weight marker in kilodaltons. Lane 2-6: Supernatant fraction of *E. coli* cells containing plasmid pETGAD induced for 0, 1, 2, 3, 6 hours, respectively.

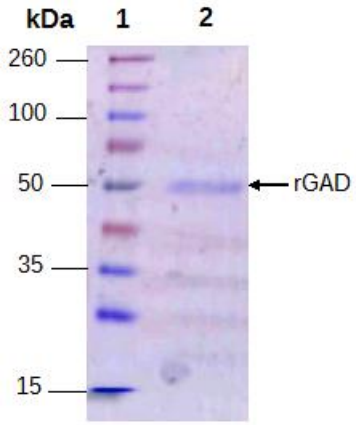


Figure 3.28 Coomassie-stained 12 % SDS polyacrylamide gel analysis of rGAD eluted fraction from Ni-Sepharose column. Lane 1: Molecular weight marker in kilodaltons. Lane 2: Purified rGAD with approximately 53 kDa of molecular weight eluted with buffer containing 500 mM imidazole.

3.5.5 Determination of Enzyme Kinetics

The kinetic behavior of rGAD was determined by measuring the initial rate of substrate conversion under the conditions of 50 mM sodium phosphate buffer (pH 5.8) at 37 °C for 30 min incubation. Various concentrations of glutamate were incubated with the recombinant GAD, and the reaction was terminated at 30 min. The Michaelis constant (Km) of rGAD for glutamate were calculated from Lineweaver-Burk plot. The calculated Km and V_{max} values of the enzyme were 19.6 mM and 21.5 nmol min^{-1} mg^{-1} respectively (Figure 3.29).

3.5.6 Effect of PLP on rGAD activity

To determine the effect of different concentrations of PLP on recombinant GAD activity, the purified rGAD was incubated with substrate solution containing 50 mM sodium phosphate buffer (pH 5.8), 30 mM glutamate, and various concentrations (0-28 μM) of PLP at 37 °C for 30 min. rGAD activity was greatly affected by different concentration of PLP. Enzyme did not show activity in the absence of PLP or at low concentration of PLP, 4 μM. GABA production was observed to be increasing with increase in PLP concentration with the highest rate of production observed when 28 μM of PLP was applied (Figure 3.30).

3.5.7 Effect of pH on rGAD activity

The effect of pH on rGAD activity was investigated in pH range of 4.5-7.5 by incubating the recombinant enzyme in reaction mixture containing 50 mM sodium phosphate buffer (pH 5.8), 30 mM glutamate, 28 μM PLP, in various pHs of 50 mM citrate-phosphate buffer (pH 4.5) and sodium phosphate buffer (pH 5.8, 6.5 and 7.5) 37 °C for 30 min. The enzyme showed optimal activity at pH 5.8 (Figure 3.31). Increase in pH values at 6.5 and 7.5 were observed to decline the rGAD activity.

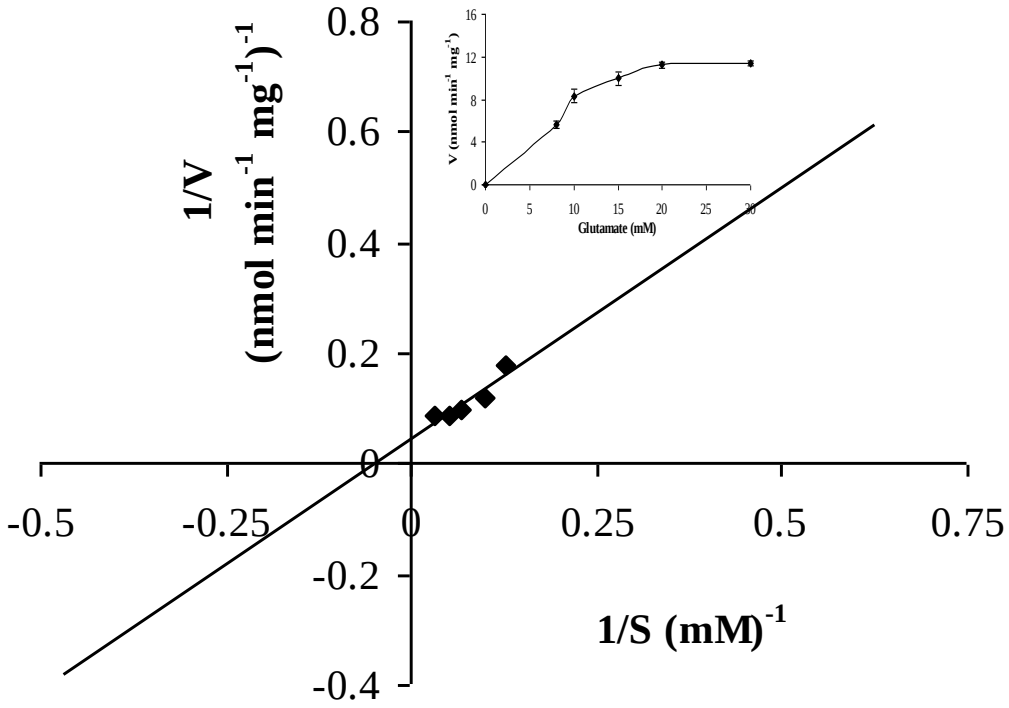


Figure 3.29 Evaluation of Michaelis constant of the recombinant GAD for glutamate in 50 mM sodium phosphate buffer (pH 5.8), by using Lineweaver-Burk plot. Means ± S.D. (n=3).

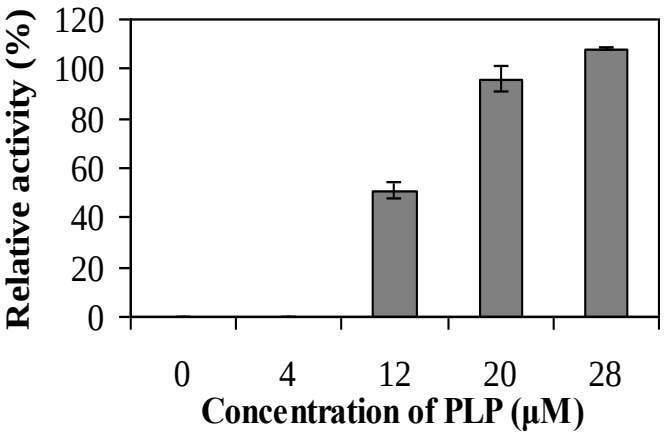


Figure 3.30 Effect of PLP concentrations on the rGAD activity in the presence of a series of PLP concentration (0-28 mM). The enzyme reaction mixture contained 30 mM glutamate, PLP (0-28 µM), 50 mM sodium phosphate buffer (pH 5.8) at 37 °C. Velocity is given in relative activity (set the rGAD relative activity value at 20 µM PLP in the reaction system as 100). Means ± S.D. (n=3).

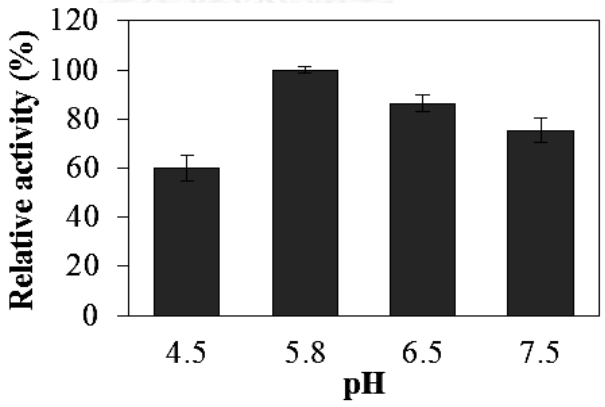


Figure 3.31 Effect of pH on rGAD activity. Optimum pH of enzymatic activity was measured in various pHs of citrate-phosphate buffer (pH 4.5) and sodium phosphate buffer (pH 5.8, 6.5 and 7.5) each at 50 mM concentration at 37 °C. Velocity is given in relative activity (set the maximal value tested as 100). Means ± S.D. (n=3).

CHAPTER IV Discussion

Metabolic pathways in plants and microorganisms are altered in response to abiotic stresses. In present study GAD enzyme is characterized and its roles are studied under various physicochemical factors and abiotic stress conditions using *Synechocystis* sp. PCC 6803 as a model organism. GABA content in wild type and GAD deficient mutant strain of *Synechocystis* was investigated and its possible function corresponding to GAD activity was investigated. Activity of GAD in *Synechocystis* was observed to be dependent on growth stage of organism. Cells exhibited highest GAD activity at late log phase of cell growth (Figure 3.1B), hence late log phase grown cells were used to study GAD in subsequent experiments.

Growth factors including pH, temperature and light were optimized for the growth of organism to achieve higher GAD activity (Figure 3.3A, B and C). GAD activity was found maximum in the cells grown at pH 7.5, which is the normal pH for BG-11 medium, followed by pH 8.5. Acidic pH of medium was not found suitable for GAD activity, so also the alkaline pH. Previously several proteins are identified in *Synechocystis* whose expression levels alter in response to the external pH fluctuations. GAD in prokaryotes being a cytosolic enzyme (Bouche and Fromm 2004) exhibits negligible changes like other cytosolic proteins unlike periplasmic proteins that show remarkable adaptations in response to variations in the external pH (Kurian *et al.* 2006). Though some experimental studies in *E. coli* reported a pronounced GAD activity by accommodating the protons under severe acidic conditions (Castanie-Cornet *et al.* 1999) but in this study low pH doesn't seem to provide a good environment for the cells to flourish and exhibit GAD activity. *In vitro* GAD activity among cells exposed to variable temperatures was found higher at 40 ˚C in comparison to normal temperature condition i.e. 30 ˚C. GAD activity was found reduced in the cells exposed to lower temperature that was also observed by Wallace (1984) while studying the effect of lower temperature on GABA accumulation in soyabean leaves (Wallace *et al.* 1984). Effect of light variation also had effect on GAD. Cells grown at normal light condition (2,000 lux) as compared to dark, lower (1,000 lux) and higher light (3,500 lux) illumination depicted higher GAD activity. GAD activity in cells grown under normal light was more than two times higher than in cells adapted to dark condition for 24 h. But still some activity was expressed by the cells grown under dark that correlates with the findings of Jordan and Givan (1979), who studied the effect of light on glutamate

metabolism in *Vicia faba* L. and reported that under dark conditions glutamate is rapidly metabolized into GABA via GAD enzyme (Jordan and Givan 1979) . It is of-course not approaching the activity of enzyme in cells grown under normal light conditions. These results suggested that *Synechocystis* cells grown under normal pH and light condition exhibit better GAD activity. While a temperature stress of 40 ̊C could be followed to achieve even higher GAD activity.

Cyanobacteria are considered as one of the earliest photosynthetic organisms who have undergone the evolution of competent mechanisms to survive against various biotic and abiotic stresses including stress exerted by harmful UV radiations (Whitton and Potts 2001). GABA shunt is considered as one of the pathways known to be involved in relieving stress against UV. UV radiations are known to be altering GABA shunt metabolites in plants (Bouché *et al.* 2003). GAD activity and GABA content were examined in *Synechocystis* wild type and mutant strain in response to UV-B exposure for 60 and 120 min duration. Increase in GAD activity with concomitant increase in GABA production inside the cells was observed in wild type strain when exposed to UV-B for 60 min (Figure 3.4). The increment in GABA content was about 30-fold in UV treated cells as compared to the untreated cells (Figure 3.13). Over production of GABA might supply more substrate for succinate semialdehyde dehydrogenase (*gabD)* that is a key enzyme of GABA degradation pathway and is rapidly involved in the restriction of free radicals by producing NADH and succinate (Bouché *et al.* 2003, Fait *et al.* 2005). Succinate is further incorporated into TCA cycle to provide enough carbon skeleton that could help the cell to defend against detrimental effects of UV-B. When UV-B exposure was extended to 120 min, GABA levels were observed to be declining, seems like UV exposure for longer duration could not help to elevate GABA content inside the cells. It could be due to the direct effect of UV-B on GAD because reduction in GAD activity was observed at 120 min of UV-B irradiation on cells. Interestingly, mutant strain showed an up-regulation of GABA production under UV-B stress (Figure 3.20), despite of the absence of a functional GAD. Here it is worth mentioning that apart from GAD activity, GABA is also known to be produced by polyamines degradation pathway (Bhatnagar *et al.* 2002) and previously it is reported that *Synechocystis* shows an increase in Put content but a decrease in Spd levels in response to UV-B irradiation (Jantaro *et al.* 2011). Increase in GABA levels in mutant might be due to Spd degradation under UV-B to protect cell against detrimental effects of UV radiations.

Nitrogen is an essential nutrient for the growth of *Synechocystis* and there are several reports revealing the importance of N-source for GAD activity and GABA production in various organisms (Wang *et al.* 2003, Binh *et al.* 2014). Catabolism of N-

source leads to glutamate formation and amino acids via glutamine synthetase and glutamate synthase (GS/GOGAT) cycle. Glutamate is further processed to serve as a substrate for GABA formation. Results from Figure 3.5 and 3.12 showed that nitrate is the preferable N-source for GAD activity and GABA accumulation in *Synechocystis* followed by nitrite and ammonium. Urea did not contribute much in GAD activity and GABA formation. On the other hand, mutant did not show any increase in GABA content in response to all nitrogen sources used (Figure 3.19). This suggested that nitrogen assimilation provides significant raw material for GABA synthesis via GAD pathway.

Previously, it is reported that optimization of C-sources had great influence on GABA production (Su *et al.* 2003, Wang *et al.* 2003). Both the GAD activity and GABA accumulation in *Synechocystis* were also observed to be depending on C-source used for the growth of organism. Among various C-sources tested, 0.1 % (w/v) glucose in BG-11 medium was found to increase the GAD activity with parallel increase in GABA content as shown in Figure 3.6A and 3.11 respectively. Fructose, lactose, sucrose and even Na_2CO_3, the C-source in basic BG-11 medium was not observed to positively affect the GAD activity and GABA content inside the cells. In order to optimize the concentration of glucose to achieve higher GAD activity, various w/v concentrations of glucose were used. Highest GAD activity was observed at 0.2 % glucose (Figure 3.6B). *Synechocystis* is able to utilize various kinds of carbohydrates as C-source (Mikkat *et al.* 1997) and the results showed that GABA production could be improved in *Synechocystis* by varying the type and concentration of C-source. Glucose is also reported as best C-source for GABA production in Lactic acid bacteria (Li and Cao 2010).

GABA is reported to be produced in large quantities in organisms under abiotic stress conditions (Kubicek *et al.* 1979, Castanie-Cornet *et al.* 1999, Kinnersley and Turano 2000). Yet its mode of action against abiotic stresses is highly obscure in plants and microorganisms. GAD activity and GABA levels in *Synechocystis* were investigated under osmotic stress induced by NaCl or sorbitol. GAD activity was observed to be increasing under short term osmotic stress caused by 50 mM NaCl (Figure 3.7A) whereas 100 mM sorbitol induced GAD activity to 5.5- fold (Figure 3.7B). Results were consistent with previous experimental study reporting increase in GABA levels in salt-sensitive cultivar of tomato plant in response to osmotic stress induced by 140 mM NaCl (Bolarín *et al.* 1995). Contrary to the activity of GAD, GABA content were found to be increasing gradually from lower NaCl and sorbitol concentrations to highest, except for 550 mM NaCl, that showed a drastic decline in GABA levels inside the cells (Figure 3.10A). GABA levels were not observed to be effected negatively even at highest concentration of sorbitol, 300 mM (Figure 3.10B). Sorbitol induced osmotic stress is reported to be

inducing GABA levels in different plants such as tomato and wheat (Bartyzel *et al.* 2003, Kolarovič *et al.* 2006). As mentioned earlier about the possibility of GABA formation via polyamines degradation, the enhanced GABA content in *Synechocystis* under osmotic stress despite the low GAD activity might be attributed to polyamine metabolism. This assumption was supported by a previous report in grapevine that showed enhanced activity of polyamine degradation enzymes under osmotic stress conditions (Hatmi *et al.* 2014). GABA might play the role of signalling molecule to regulate glucosylglycerol synthesis, a molecule that is known to accumulate largely in *Synechocystis* under osmotic stress (Mikkat *et al.* 1997). Nevertheless, more investigation regarding polyamine degradation linked to GABA pool under osmotic stress in *Synechocystis* would be required to understand the precise mechanism.

Effect of polyamine supplementation on GAD activity was also investigated. Apart from various physiological roles of polyamines in plants and bacteria, involvement of polyamines in transcriptional regulation of GAD system in *E. coli* under acid stress is recently revealed (Chattopadhyay and Tabor 2013). In *Synechocystis* 0.5 mM Put and Spd were found to upgrade the GAD activity resulting in more than 2- fold increment of GAD as compared to control (Figure 3.8). The results were quite consistent with the GABA levels in both *Synechocystis* wild type and mutant strains. Put and Spd supplementation was found to enhance GABA content inside the cells, especially Spd supplementation was observed to have a pronounced effect on stimulation of GABA accumulation in wild type as well as mutant strain (Figure 3.14 and 3.21). It is evident from bioinformatic analysis that *Synechocystis* possesses putative genes encoding enzymes for Spd degradation to γ -aminobutanal and GABA. But genes for enzymes of Put degradation to Spd or γ -aminobutanal are not reported yet. *Synechocystis* is also reported to gather higher Spd content in the cells that are harmful for normal growth of cells (Raksajit *et al.* 2009). Therefore, elevated levels of GABA content in both wild type and mutant strains under Spd supplementation might be attributed to Spd degradation into GABA via γ -aminobutanal. This could be a normal function of cell to convert a toxic (Spd) compound into non-toxic (GABA), because high levels of GABA are never reported harmful for the cell.

Glutamate is always considered as a key compound to optimize for higher GABA production in microorganisms (Yang *et al.* 2008). Glutamate is also known to activate GAD enzyme (Scott-Taggart *et al.* 1999). In *Synechocystis* GAD activity was observed to be affected by glutamate concentration in growth medium. Glutamate supplementation up to 10 mM was found to up-regulate the activity of GAD and also

GABA content (Figure 3.9 and 3.15A). Higher concentrations of glutamate at 20 mM and above resulted in the inhibition of GAD activity. Mutant showed far low levels of GABA and higher accumulation of intracellular glutamate as compared to wild type (Figure 3.22A and B). Wild type cells were observed to grow slowly at higher concentrations of glutamate whereas mutant growth at higher glutamate concentration was severely inhibited (Figure 3.22C). It strongly validates that GABA plays very important role in connecting C/N metabolism in *Synechocystis,* and a functional GAD is inevitable for maintaining intracellular glutamate levels.

GAD regulation at transcriptional level and regulation of GABA in *Synechocystis* was further investigated. Current study revealed that *gad* transcript levels are regulated under osmotic stress imposed by the cell's environment and C- source utilized by the organism for growth (Figure 3.16A and B). Significant correlation between the expression pattern of *gad* gene and GAD activity was observed in *Synechocystis* owing to the stress induced by low concentration of osmolytes. On the other hand, decline in GAD activity but up-regulation of *gad* gene at higher concentrations of NaCl and sorbitol, might be due to their inhibitory effect on enzyme activity but not on the amount of enzyme produced. Expression pattern of *gad* under variable C-sources utilized by organism was well correlated to the GAD activity under similar conditions, showing the highest expression when 0.1 % (w/v) glucose was used as a C-source. Hence, glucose could be used as a C-source for *Synechocystis* growth for up-regulation of *gad* and ultimately to obtain high GABA yield. UV-B irradiation resulted in decline in *gad* transcript level (Figure 3.16C) that was contrary to the GAD activity. This indicated that *Synechocystis gad* is possibly post-translationally regulated to overcome the detrimental effects of UV-B.

GAD is known to be regulated in microorganisms under abiotic stress conditions by balancing the intracellular ionic homeostasis, glutamate levels or by altering GABA production. Due to variable properties of glutamate decarboxylases, optimal conditions for GABA production also vary in different microorganisms, hence purification and characterization of GAD in *Synechocystis* was required further to elucidate roles of GAD and optimization of conditions for highest GABA production. From the complete sequence of *Synechocystis* genome reported by Kaneko *et al*. (Kaneko *et al.* 1996), sll1641 has been annotated as the gene encoding GAD. From the blast search, it is indicated that *Synechocystis* GAD has little sequence similarities with GAD of *E. coli* (48.8 % identity), *Neurospora crassa* (45.6 % identity) and human (30.8 % identity). Multiple alignments of these protein sequences showed conserved amino acids possessing a lysine residue that is responsible for interaction with cofactor PLP

(Fig. 4.1). Also the motif [H(I/V)DAA(S/W)GG], that is known to be conserved in PLP-dependent decarboxylase was discovered.

In order to express the *Synechocystis* GAD, *gad* gene (sll1641) was amplified by using gene specific primer pairs. 100 % homology of amplified sequence to *gad* gene published in cyanobase (http://genome.kazusa.or.jp/cyanobase) was shown by blastn sequence-alignment result. The *gad* gene was cloned and expressed in *E. coli* BL21(DE3) as a recombinant His_6-tagged protein (rGAD). Over-expressed His-tagged GAD showed an apparent molecular mass of 53 kDa on SDS-PAGE gel. The *Synechocystis* GAD was expressed as a monomer and soluble protein. The purfied rGAD from cells expressing pETGAD was tested for the activity. The rGAD activity was 11.345 nmol GABA min^{-1} mg^{-1} protein, which was 20 times higher than that of the *Synechocystis* GAD (0.5 nmol GABA min^{-1} mg^{-1} protein). The rGAD activity was tested at various concentrations of substrate ranging from 0 to 30 mM glutamate. A Km value at 19.6 mM glutamate was obtained from double reciprocal plot of glutamate concentration v/s velocity (Figure 3.29) which is quite higher than Km of *E. coli* GAD (Strausbauch and Fischer 1970). Dependency of rGAD was also analyzed by varying PLP concentrations in reaction mixture. The rGAD exhibited increase in activity with increase in PLP concentration, giving the highest activity at 28 μM of PLP (Figure 3.30). No activity was observed in the absence of PLP showing that the rGAD is highly dependent on PLP for glutamate conversion into GABA. The optimal pH for the activity of rGAD was 5.8 (Figure 3.31) that is similar to plant GAD (Schales and Schales 1946, Weinberger and Clendenning 1952, Satyanarayan and Nair 1985). However, it was contrary to the bacterial (*E. coli*) GAD that shows an optimum pH in acidic range (Strausbauch and Fischer 1970). Whereas, *Synechocystis* rGAD showed a decline in activity at pH 4.5, that depicts dependency of GAD properties on type of organisms having different optimum environmental conditions for growth.

Figure 4.1 Multiple sequence alignment of glutamate decarboxylase (GAD) from various sources. The amino acid sequences of *Synechocystis* GAD (SynGAD, sll1641), *E. coli* GAD (EcGADB, ECD 01451), *Neurospora crassa* GAD (NcGAD, NCU00678), and human GAD (HsGAD, NP_000809) were aligned with the CLUSTAL W program using default parameters. The conserved lysine residue and the motif [H(I/V)DAA(S/W)GG] is indicated by an arrow and the upper line, respectively.

Asterisk (*) = residues at that position are exactly the same.

Colon (:) = residues at that position are very similar.

Dot (.) = residues are more or less similar.

Future perspectives: The results obtained from this thesis designated the role of GAD for GABA formation under abiotic stresses. However, studies regarding GAD regulation and more investigation of properties of purified enzyme would be required to obtain a highly GABA producing strain of *E. coli* using cyanobacterial GAD. Moreover further work should be continued to define a precise mode of action of GABA to overcome stress conditions.

CHAPTER V Conclusion

Optimization of growth conditions for GAD activity and high GABA production in *Synechocystis* were investigated in this work. Different environmental factors like pH, temperature, light, nitrogen source, carbon source, glutamate and polyamine supplementation were found to alter GAD activity and GABA content. Nitrate and 0.1 % (w/v) glucose were found to be the best nitrogen and carbon source respectively for GAD activity and GABA production. *Synechocystis* GAD was also observed to alter its activity in response to osmotic and UV-B stress with alterations in GABA levels. Differential levels of *gad* expression in response to UV-B irradiation, osmotic stress and variable carbon sources depicted the regulation of *gad* under environmental factors.

Mutant strain with interrupted GAD was also used in this study to investigate the possible function of GAD in *Synechocystis* specifically under abiotic stress conditions. Study of effect of glutamate and polyamine supplementation on wild type and mutant strain revealed the importance of GAD in maintaining intracellular glutamate levels and also the function of GABA in connecting C and N metabolism in *Synechocystis.* Expression of the recombinant GAD was successfully done followed by the characterization that provided useful information about the properties of *Synechocystis* GAD. Altogether, this study not only helped in finding the optimum levels of factors affecting the GAD activity, but also proved to be useful in elucidating the possible role of GAD and GABA accumulation for relieving certain kinds of physiological stresses.

REFERENCES

Anderson, J. A., V. H. Cheldelin and T. E. King (1961). "Glutamic decarboxylase of ergot, Claviceps purpurea." J Bacteriol **82**: 354-358.

Bartyzel, I., K. Pelczar and A. Paszkowski (2003). "Functioning of the γ -aminobutyrate pathway in wheat seedlings affected by osmotic stress." Biol Plantarum **47**(2): 221-225.

Battaglioli, G., H. Liu and D. L. Martin (2003). "Kinetic differences between the isoforms of glutamate decarboxylase: implications for the regulation of GABA synthesis." J Neurochem **86**(4): 879-887.

Baum, G., Y. Chen, T. Arazi, H. Takatsuji and H. Fromm (1993). "A plant glutamate decarboxylase containing a calmodulin binding domain. Cloning, sequence, and functional analysis." J Biol Chem **268**(26): 19610-19617.

Bhatnagar, P., R. Minocha and S. C. Minocha (2002). "Genetic manipulation of the metabolism of polyamines in poplar cells. The regulation of putrescine catabolism." Plant Physiol **128**(4): 1455-1469.

Binh, T., W.-T. Ju, W.-J. Jung and R.-D. Park (2014). "Optimization of γ -amino butyric acid production in a newly isolated *Lactobacillus brevis*." Biotechnol Lett **36**(1): 93-98.

Blankenhorn, D., J. Phillips and J. L. Slonczewski (1999). "Acid- and base-induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis." J Bacteriol **181**(7): 2209-2216.

Bolarín, M. C., A. Santa-Cruz, E. Cayuela and F. Pérez-Alfocea (1995). "Short-term solute changes in leaves and roots of cultivated and wild tomato seedlings under salinity." J Plant Physiol **147**(3–4): 463-468.

Bouché, N., A. Fait, D. Bouchez, S. G. Møller and H. Fromm (2003). "Mitochondrial succinic-semialdehyde dehydrogenase of the γ -aminobutyrate shunt is required to restrict levels of reactive oxygen intermediates in plants." P Natl Acad Sci **100**(11): 6843-6848.

Bouche, N. and H. Fromm (2004). "GABA in plants: just a metabolite?" Trends Plant Sci **9**(3): 110-115.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-254.

Britton, H. and R. Robinson (1931). "CXCVIII.-Universal buffer solutions and the dissociation constant of veronal." J Chem Soc: 1456-1462.

Castanie-Cornet, M. P., T. A. Penfound, D. Smith, J. F. Elliott and J. W. Foster (1999). "Control of acid resistance in *Escherichia coli*." J Bacteriol **181**(11): 3525-3535.

Chattopadhyay, M. K. and H. Tabor (2013). "Polyamines are critical for the induction of the glutamate decarboxylase-dependent acid resistance system in *Escherichia coli*." J Biol Chem **288**(47): 33559-33570.

Coleman, S. T., T. K. Fang, S. A. Rovinsky, F. J. Turano and W. S. Moye-Rowley (2001). "Expression of a glutamate decarboxylase homologue is required for normal oxidative stress tolerance in *Saccharomyces cerevisiae*." J Biol Chem **276**(1): 244-250.

Cozzani, I., A. Misuri and C. Santoni (1970). "Purification and general properties of glutamate decarboxylase from *Clostridium perfringens*." Biochem J **118**(1): 135-141.

Denner, L. A., S. C. Wei, H. S. Lin, C. T. Lin and J. Y. Wu (1987). "Brain L-glutamate decarboxylase: purification and subunit structure." Proc Natl Acad Sci U S A **84**(3): 668-672.

Fait, A., A. Yellin and H. Fromm (2005). "GABA shunt deficiencies and accumulation of reactive oxygen intermediates: insight from *Arabidopsis* mutants." FEBS Lett **579**(2): 415-420.

Flores, H. and P. Filner (1985). "Polyamine catabolism in higher plants: Characterization of pyrroline dehydrogenase." Plant Growth Regul **3**(3-4): 277-291.

Florey, E. (1953). "Über einen nervösen Hemmungsfaktor in Gehirn und Rückenmark." Naturwissenschaften **40**(10): 295-296.

Foerster, H. F. (1972). "Spore pool glutamic acid as a metabolite in germination." J Bacteriol **111**(2): 437-442.

Hao, R. and J. C. Schmit (1991). "Purification and characterization of glutamate decarboxylase from *Neurospora crassa* conidia." J Biol Chem **266**(8): 5135-5139.

Hao, R. and J. C. Schmit (1993). "Cloning of the gene for glutamate decarboxylase and its expression during conidiation in *Neurospora crassa*." Biochem J **293 (Pt 3)**: 735-738.

Hatmi, S., P. Trotel-Aziz, S. Villaume, M. Couderchet, C. Clement and A. Aziz (2014). "Osmotic stress-induced polyamine oxidation mediates defence responses and reduces stress-enhanced grapevine susceptibility to *Botrytis cinerea*." J Exp Bot **65**(1): 75-88.

Inatomi, K. and J. C. Slaughter (1975). "Glutamate decarboxylase from barley embryos and roots. General properties and the occurrence of three enzymic forms." Biochem J **147**(3): 479-484.

Jannoey, A., H. Niamsup, S. Lumyong, Suzuki. H, T. Katayama and H. Chairote (2010). "Comparison of gamma-aminobutyric acid production in Thai rice grains." World J Microbiol Biotechnol **26**(2): 257–263.

Jantaro, S., A. Pothipongsa, S. Khanthasuwan and A. Incharoensakdi (2011). "Short-term UV-B and UV-C radiations preferentially decrease spermidine contents and arginine decarboxylase transcript levels of *Synechocystis* sp. PCC 6803." Curr Microbiol **62**(2): 420-426.

Jordan, B. R. and C. V. Givan (1979). "Effects of light and inhibitors on glutamate metabolism in leaf discs of *Vicia faba* L: Sources of ATP for glutamine synthesis and photoregulation of tricarboxylic acid cycle metabolism." Plant Physiol. **64**: 1043-1047.

Jydegaard-Axelsen, A. M., P. E. Hoiby, K. Holmstrom, N. Russell and S. Knochel (2004). "CO2- and anaerobiosis-induced changes in physiology and gene expression of different *Listeria monocytogenes* strains." Appl Environ Microbiol **70**(7): 4111-4117.

Kaneko, T., S. Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirosawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K. Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda and S. Tabata (1996). "Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions." DNA Res **3**(3): 109-136.

Kanwal, S., R. Rastogi and A. Incharoensakdi (2014). "Glutamate decarboxylase activity and gamma-aminobutyric acid content in *Synechocystis* sp. PCC 6803 under osmotic stress and different carbon sources." J Appl Phycol 1-7.

Karatzas, K. A., L. Suur and C. P. O'Byrne (2012). "Characterization of the intracellular glutamate decarboxylase system: analysis of its function, transcription, and role in the acid resistance of various strains of *Listeria monocytogenes*." Appl Environ Microbiol **78**(10): 3571-3579.

Kaupmann, K., K. Huggel, J. Heid, P. J. Flor, S. Bischoff, S. J. Mickel, G. McMaster, C. Angst, H. Bittiger, W. Froestl and B. Bettler (1997). "Expression cloning of GABA(B) receptors uncovers similarity to metabotropic glutamate receptors." Nature **386**(6622): 239-246.

Kinnersley, A. M. and F. J. Turano (2000). "Gamma minobutyric acid (GABA) and plant responses to stress." Crit Rev Plant Sci **19**(6): 479-509.

Kolarovič, L., M. Luxová and P. Valentovič (2006). "Effect of osmotic stress in early stages of ontogenesis on root respiration, growth, sugar content, and cell injury in maize seedlings differing in drought sensitivity." J Integr Plant Biol **48**(7): 814-822.

Kondo, T., C. A. Strayer, R. D. Kulkarni, W. Taylor, M. Ishiura, S. S. Golden and C. H. Johnson (1993). "Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria." Proc Natl Acad Sci U S A **90**(12): 5672-5676.

Krnjević, K. (1974). "Chemical Nature of Synaptic Transmission in Vertebrates. " Physiol Rev **54** (2): 418-540.

Kubicek, C. P., W. Hampel and M. Röhr (1979). "Manganese deficiency leads to elevated amino acid pools in citric acid accumulating *Aspergillus niger*." Arch Microbiol **123**(1): 73-79.

Kurian, D., T. Jansen and P. Maenpaa (2006). "Proteomic analysis of heterotrophy in *Synechocystis* sp. PCC 6803." Proteomics **6**(5): 1483-1494.

Labarre, J., P. Thuriaux and F. Chauvat (1987). "Genetic analysis of amino acid transport in the facultatively heterotrophic cyanobacterium *Synechocystis* sp. strain 6803." J Bacteriol **169**(10): 4668-4673.

Lakin-Thomas, P. L. (2006). "New models for circadian systems in microorganisms." FEMS Microbiol Lett **259**(1): 1-6.

Li, H. and Y. Cao (2010). "Lactic acid bacterial cell factories for gamma-aminobutyric acid." Amino Acids **39**(5): 1107-1116.

Lindberg, P., S. Park and A. Melis (2010). "Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism." Metab Eng **12**(1): 70-79.

Matsuyama, A., K. Yoshimura, C. Shimizu, Y. Murano, H. Takeuchi and M. Ishimoto (2009). "Characterization of glutamate decarboxylase mediating gamma-amino butyric acid increase in the early germination stage of soybean (*Glycine max* [L.] Merr)." J Biosci Bioeng **107**(5): 538-543.

Meeks, J. C. and J. Elhai (2002). "Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states." Microbiol Mol Biol Rev **66**(1): 94-121; table of contents.

Mikkat, S., U. Effmert and M. Hagemann (1997). "Uptake and use of the osmoprotective compounds trehalose, glucosylglycerol, and sucrose by the cyanobacterium *Synechocystis* sp. PCC6803." Arch Microbiol **167**(2/3): 112-118.

Nomura, M., I. Nakajima, Y. Fujita, M. Kobayashi, H. Kimoto, I. Suzuki and H. Aso (1999). "*Lactococcus lactis* contains only one glutamate decarboxylase gene." Microbiology **145** (Pt 6): 1375-1380.

Oh, S. H., W. G. Choi, I. T. Lee and S. J. Yun (2005). "Cloning and characterization of a rice cDNA encoding glutamate decarboxylase." J Biochem Mol Biol **38**(5): 595-601.

Raksajit, W., P. Yodsang, P. Maenpaa and A. Incharoensakdi (2009). "Characterization of spermidine transport system in a cyanobacterium, *Synechocystis* sp. PCC 6803." J Microbiol Biotechnol **19**(5): 447-454.

Rastogi, R. P. and R. P. Sinha (2009). "Biotechnological and industrial significance of cyanobacterial secondary metabolites." Biotechnol Adv **27**(4): 521-539.

Renault, H., V. Roussel, A. El Amrani, M. Arzel, D. Renault, A. Bouchereau and C. Deleu (2010). "The *Arabidopsis* pop2-1 mutant reveals the involvement of GABA transaminase in salt stress tolerance." BMC Plant Biol **10**: 20.

Riccardi, G., E. de Rossi and A. Milano (1989). "Amino acid biosynthesis and its regulation in cyanobacteria." Plant Sci **64**(2): 135-151.

Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman and R. Y. Stanier (1979). "Generic assignments, strain histories and properties of pure cultures of cyanobacteria." J Gen Microbiol **111**(1): 1-61.

Rodriguez-Ezpeleta, N., H. Brinkmann, S. C. Burey, B. Roure, G. Burger, W. Loffelhardt, H. J. Bohnert, H. Philippe and B. F. Lang (2005). "Monophyly of primary photosynthetic eukaryotes: green plants, red algae, and glaucophytes." Curr Biol **15**(14): 1325-1330.

Satyanarayan, V. and P. M. Nair (1985). "Purification and characterization of glutamate decarboxylase from *Solanum tuberosum*." Eur J Biochem **150**(1): 53-60.

Schales, O. and S. S. Schales (1946). "Glutamic acid decarboxylase of higher plants; enzymatic determination of 1 (+)-glutamic acid." Arch Biochem **11**(3): 445-450.

Schriek, S., C. Ruckert, D. Staiger, E. K. Pistorius and K. P. Michel (2007). "Bioinformatic evaluation of L-arginine catabolic pathways in 24 cyanobacteria and transcriptional analysis of genes encoding enzymes of L-arginine catabolism in the cyanobacterium *Synechocystis* sp. PCC 6803." BMC Genomics **8**: 437.

Scott-Taggart, C. P., O. R. Van Cauwenberghe, M. D. McLean and B. J. Shelp (1999). "Regulation of γ -aminobutyric acid synthesis in situ by glutamate availability." Physiol Plantarum **106**(4): 363-369.

Shelp, B. J., A. W. Bown and M. D. McLean (1999). "Metabolism and functions of gamma-aminobutyric acid." Trends Plant Sci **4**(11): 446-452.

Shukuya, R. and G. W. Schwert (1960). "Glutamic acid decarboxylase. I. Isolation procedures and properties of the enzyme." J Biol Chem **235**: 1649-1652.

Smith, J. E. and E. R. Waygood (1961). "Resolution of the apoenzyme and coenzyme of L-glutamic acid decarboxylase from sunflower cotyledons." Can J Biochem Physiol **39**(6): 1055-1059.

Strausbauch, P. H. and E. H. Fischer (1970). "Chemical and physical properties of *Escherichia coli* glutamate decarboxylase." Biochemistry **9**(2): 226-233.

Su, Y. C., J. J. Wang, T. T. Lin and T. M. Pan (2003). "Production of the secondary metabolites gamma-aminobutyric acid and monacolin K by *Monascus*." J Ind Microbiol Biotechnol **30**(1): 41-46.

Ueno, Y., K. Hayakawa, S. Takahashi and K. Oda (1997). "Purification and characterization of glutamate decarboxylase from *Lactobacillus brevis* IFO 12005." Biosci Biotechnol Biochem **61**(7): 1168-1171.

Wallace, W., J. Secor and L. E. Schrader (1984). "Rapid accumulation of γ -aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation." Plant Physiol. **75**: 170-175.

Wang, J. J., C. L. Lee and T. M. Pan (2003). "Improvement of monacolin K, gamma-aminobutyric acid and citrinin production ratio as a function of environmental conditions of *Monascus purpureus* NTU 601." J Ind Microbiol Biotechnol **30**(11): 669-676.

Weinberger, P. and K. A. Clendenning (1952). "Glutamic carboxylase of the mature wheat leaf." Can J Bot **30**(6): 755-763.

Whitton, B. and M. Potts (2002). Introduction to the Cyanobacteria. The Ecology of Cyanobacteria. B. Whitton and M. Potts, Springer Netherlands**:** 1-11.

Whitton, B. A. and M. Potts (2001). "The ecology of cyanobacteria: their diversity in time and space (reviewed by T. Bailey Watts)." Diversity and Distributions **7**(4): 203-204.

Wu, J. Y., T. Matsuda and E. Roberts (1973). "Purification and characterization of glutamate decarboxylase from mouse brain." J Biol Chem **248**(9): 3029-3034.

Yang, S. Y., F. X. Lu, Z. X. Lu, X. M. Bie, Y. Jiao, L. J. Sun and B. Yu (2008). "Production of gamma-aminobutyric acid by *Streptococcus salivarius* subsp. thermophilus Y2 under submerged fermentation." Amino Acids **34**(3): 473-478.

Zhang, H., H. Y. Yao and F. Chen (2006). "Accumulation of gamma-aminobutyric acid in rice germ using protease." Biosci Biotechnol Biochem **70**(5): 1160-1165.

APPENDICES

APPENDIX A

BG-11 medium

BG-11 medium (1 liter)

	Solid medium	Liquid medium
Deionized H_2O	Adjust volume to 1 liter	972 ml
Becto-agar	15 g	-
TES (1M)	10 ml	-
30 % $Na_2S_2O_3.5H_2O$	10 ml	-
$NaNO_3$ (1.77M)	10 ml	10 ml
KH_2PO_4 (0.29 M)	1 ml	1 ml
$MgSO_4.7H_2O$ (0.3 M)	1 ml	1 ml
$CaCl_2.2H_2O$ (0.244 M)	1 ml	1 ml
Na_2CO_3 (0.19 M)	1 ml	1 ml
Citric acid (mM)	1 ml	1 ml
Na_2EDTA (2.7 mM)	1 ml	1 ml
0.6 g/100 ml Feric ammonium citrate	1 ml	1 ml
**Trace element A5 solution	1 ml	1 ml
1 M HEPES-NaOH, pH 7.5	10 ml	10 ml

****Trace element A5 solution preparation (1 liter)**

H_3BO_3 (27 mM)	2.86 g
$MnCl_2.4H_2O$ (9.1 mM)	1.81 g
$Na_2MoO_4.2H_2O$ (1.6 mM)	0.39 g
$CuSO_4.5H_2O$ (0.3 mM)	0.080 g
$Co(NO_3)_2.6H_2O$ (0.17 mM)	0.049 g
$ZnSO_4.7H_2O$ (0.76 mM)	0.221 g

APPENDIX B

LB medium

LB medium (1 liter)

	Solid medium	Liquid medium
Bacto tryptone	10 g	10 g
NaCl	5 g	5 g
Yeast extract	5 g	5 g
Agar	15 g	-

All compositions were dissolved together with 800 ml of distilled water; and then the mixture was adjusted to pH of 7.0 with 10 M NaOH. The total volume of solution was then adjusted to 1 liter with deionized water. The medium was sterilized by autoclaving at 15 lb/in_2 for 15 min.

APPENDIX C

Bradford protein determination

Bradford stock solution	
95 % ethanol	100 ml
88 % phosphoric acid	200 ml
Serva Blue G	350 mg
Stable indefinitely at room temperature	

Bradford working buffer	
distilled water	425 ml
95 % ethanol	15 ml
88 % phosphoric acid	30 ml
Bradford stock solution	30 ml
Filter through Whatman No. 1 paper, Store at room temperature. Usable for several week, but may need to be refiltered.	

Assay

1. Prepare protein solution 20 µl into tube.
2. Add 1 ml Bradford working buffer and vortex.
3. Read OD_{595} within 10 min - 1 hour.

APPENDIX D

Polyacrylamide gel electrophoresis

Stock solutions

30 % (w/v) Acrylamide, 0.8 % (w/v) bis-acrylamide, 100 ml

Acrylamide 29.2 g

N,N′-methylene-bis-acrylamide 0.8 g

Distilled water was added to make a total volume of 100 ml and stirred until completely dissolved.

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 6.06 g

Adjusted pH to 6.8 with concentrated HCl slowly. Added distilled water to the total volume of 100 ml.

1 M Tris-HCl buffer pH 6.8, 100 ml

Tris (hydroxymethyl)-aminomethane 2.1 g

Adjusted pH to 6.8 with concentrated HCl slowly. Added distilled water to the total volume of 100 ml.

2 M Tris-HCl buffer pH 8.8, 100 ml

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with concentrated HCl slowly. Added distilled water to the total volume of 100 ml.

20 % w/v SDS, 100 ml (stored at room temperature)

SDS 20 g

Distilled water was added to make a total volume of 100 ml.

10 % Ammoniumpersulfate (APS), 1 ml

APS 0.1 g

Distilled water was added to make a total volume of 1 ml.

0.5 % Bromophenol blue

Bromophenol blue 0.5 g

Distilled water was added to make a total volume of 100 ml.

20 % v/v Glycerol

Glycerol	20 ml

Distilled water was added to make a total volume of 100 ml.

Working solutions

Solution B (for SDS-PAGE), 100 ml

2 M Tris-HCl, pH 8.8	75 ml
10 % SDS	4 ml

Distilled water was added to make a total volume of 100 ml.

Solution C (for SDS-PAGE), 100 ml

1 M Tris-HCl, pH 6.8	50 ml
10 % SDS	4 ml

Distilled water was added to make a total volume of 100 ml.

SDS-PAGE

12 % Separating gel (for 2 gels)

30 % Acrylamide Solution	4.17 ml
Distilled water	3.33 ml
Solution B	2.5 ml
10 % APS	50 µl
TEMED	5 µl

5 % Stacking gel (for 2 gels)

30 % Acrylamide Solution	1.67 ml
Distilled water	5.8 ml
Solution C	2.5 ml
10 % APS	50 µl
TEMED	5 µl

Laminii solution 50 ml

0.5 M Tris-HCl pH 6.8	13.8 ml
50 % glycerol	20.0 ml

20 % SDS	10.8 ml

Distilled water was added to make a total volume of 50 ml.

Solubilizing buffer

Laminii solution	900 µl
2-mercaptoethanol	100µl
0.5 % Bromophenol blue	5 µl

* The ratio of sample and sample buffer is 1:1. Mixture was incubated at 65 ℃ for 10 min. Then, centrifuged at 10,000 g for 1 min, samples were loaded into the gel.

Electrophoresis buffer, 1 liter (25 mM Tris, 192 mM glycine, 0.1 % SDS)

Tris (hydroxymethyl)-aminomethane	3.0 g
Glycine	14.4 g
SDS	1.0 g

Distilled water was added to make a total volume of 1 liter.

Commassie gel stain, 1 L

Coomassie blue R-250	1.0 g
Methanol	900 ml
Glacial acetic acid	100 ml

Distilled water was added to make a total volume of 1 liter.

Commassie gel destain, 1 L

Methanol	100 ml
Glacial acetic acid	100 ml

Distilled water was added to make a total volume of 1 liter.

APPENDIX E

Western blotting reagents

Blotting buffer

Tris	0.582 g
Glycine	0.293 g
Methanol	20 ml

Distilled water was added to make a total volume of 100 ml.

10X PBS (Phosphate buffer saline), pH 7.2

NaCl	80 g
KCl	2 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g

Distilled water was added to make a total volume of 1 liter. Use 1X.

PBS-T

1X PBS

0.05 % Tween-20

Blocking buffer

5 % Anlene ® milk in 1X PBS buffer

Antibody buffer

5 % Anlene ® milk in PBS-T buffer

Alkaline phosphatase buffer (100 ml)

0.1 M Tris HCl (pH 9.5)

0.1 M NaCl

5 mM $MgCl_2$

NBT

50 mg NBT in 1 ml DMF (Dimethyl formamide)

BCIP

50 mg BCIP in 1 ml DMF (Dimethyl formamide)

Developing buffer

NBT	66 µl
Alkaline phosphatase buffer	10 ml
BCIP	33 µl

APPENDIX F

Protein purification buffers

Washing buffer

20 mM sodium phosphate buffer pH 5.8, 3 mM Na-EDTA

Binding buffer

20 mM sodium phosphate buffer pH 7.4, 500 mM NaCl, 20 mM imidazole

Elution buffer

20 mM sodium phosphate buffer pH 7.4, 500 mM NaCl, 500 mM imidazole

Dialysis buffer

20 mM sodium phosphate buffer pH 7.4

APPENDIX G

Buffers for agarose gel electrophoresis

TAE buffer, composition per 1 liter

50X: Tris-base	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA, pH 8.0	100 ml

Mix the compositions together and adjust volume up to 1 L, mix again in glass bottle and then store at room temperature. 1X and 0.5X TAE buffer is used for preparing agarose gel and running gel electrophoresis respectievly.

0.8 % agarose gel (100 ml)

Agarose gel	0.8 g
1X TAE buffer	100 ml

Warm the gel until mix completely in buffer.

20X SB buffer, composition per 1 liter

SB buffer is also used in agarose gel electrophoresis (preferably for RNA samples). It has a lower conductivity, produces sharper bands and can be run at higher speed than gels made in TAE buffer. 20X SB buffer containing Borax ($Na_2B_4O_2$) at concentration of 100 mM and adjusted pH at 7.8 with boric acid. 1X SB buffer is used for running gel electrophoresis.

APPENDIX H

Buffer for preparation of competent cells

TFB1 (pH 5.8)

Potassium acetate	1.49 g
$CaCl_2$	0.74 g
$MnCl_2$	4.95 g
Glycerol	75 ml

Adjust pH to 5.8 with 1M acetic acid (glacial acetic acid ~17.4N). Store at 4 °C.

TFB2

100 mM MOPS pH 7.8	10 ml
$CaCl_2$	1.1 g
Glycerol	15 ml

Store at 4 °C. Buffers should be ice-cold for the procedure.

APPENDIX I

Acid washed glass beads for RNA extraction

Method

1. 50 g of 0.5 mm glass beads (Sigma G-9268) were added into a 100 ml of Erlenmeyer flask. The volume of glass beads should be 1/5 of the volume of the flask.

2. 6 M HCl was poured carefully to cover the glass beads.

3. The flask was swirled to wash the glass beads with acid.

4. After washing the glass beads with 6 M HCl, acid was poured carefully into another bottle.

5. Distilled water was added to wash the glass beads to the volume 5 times the volume of the beads in flask. Glass beads were stirred up by swirling the bottle for atleast 10 sec followed by pouring off the distilled water wash in another bottle.

6. Step 5 was repeated atleast 10 times to reduce the acid concentration below 10 mM.

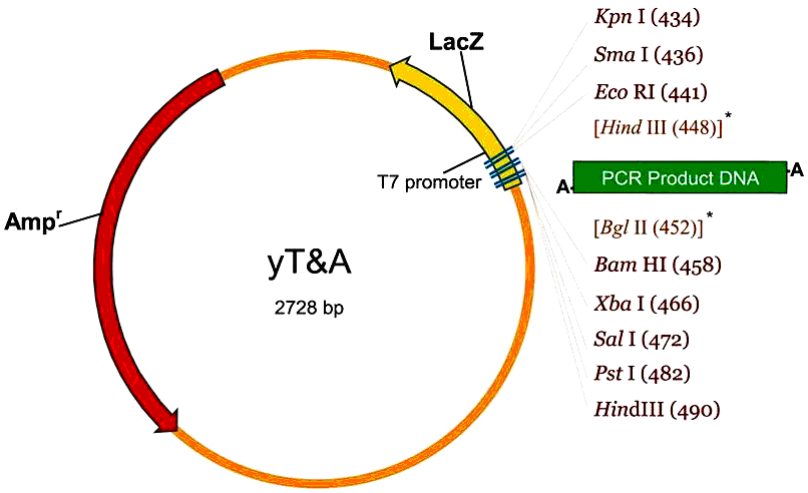
7. Glass beads were moved in to a beaker and covered with foil to autoclave.

8. Beads were oven dried at 50 °C overnight.

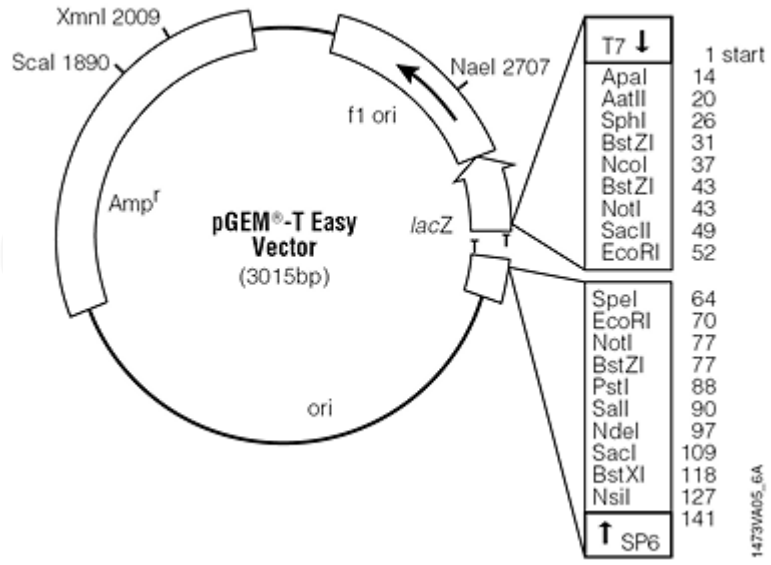
APPENDIX J

Cloning/expression vectors

J1)



J2)



J3)

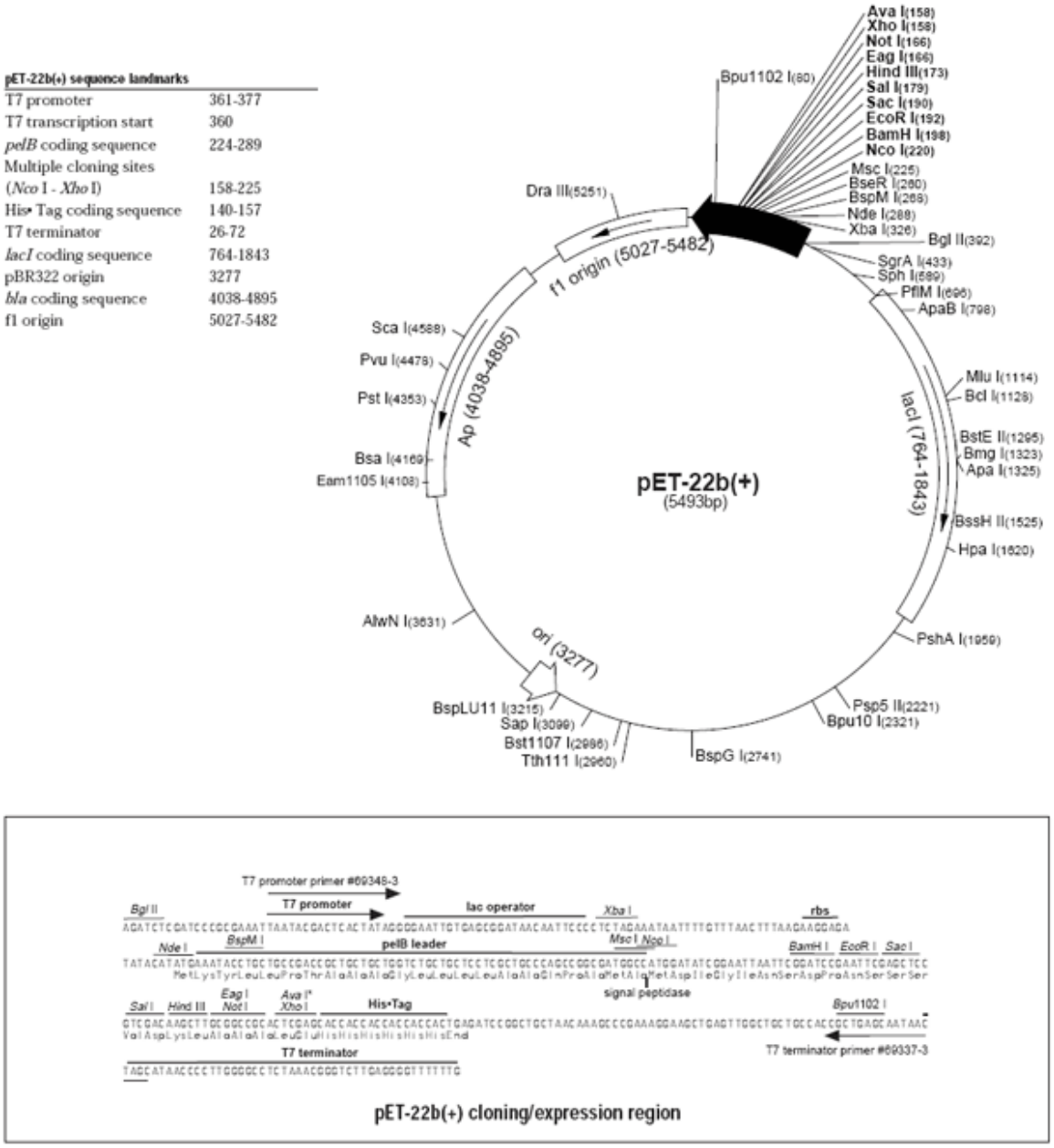


Fig J. 1) pTA vector, **2)** pGEM ®- T Easy vector, **3)** pRL 5801 and **4)** pET-22b+ vector circle map and cloning/expression region.

APPENDIX K

GABA and glutamate determination

HPLC conditions:

Column type: Zorbax Eclipse AAA analytical column

Detector type: UV

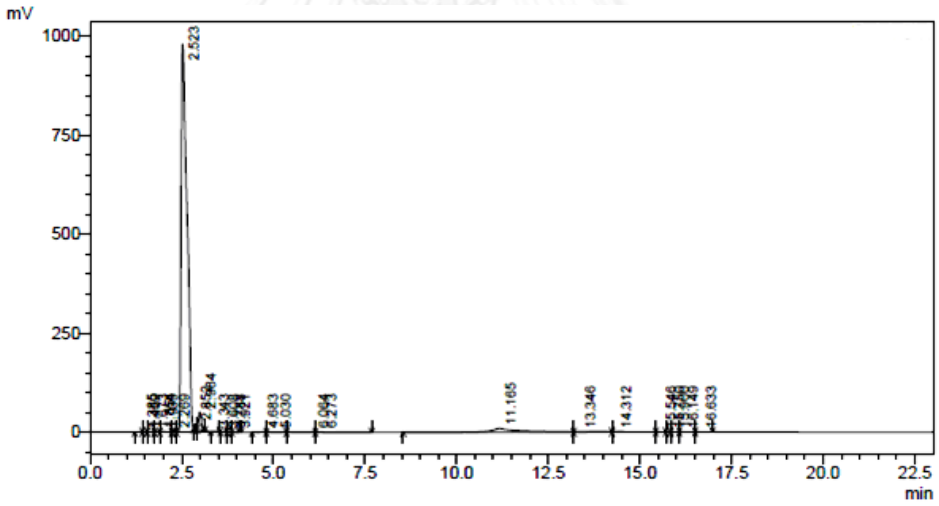
Column oven temperature: 40 °C

Mobile phase A: Acetonitrile

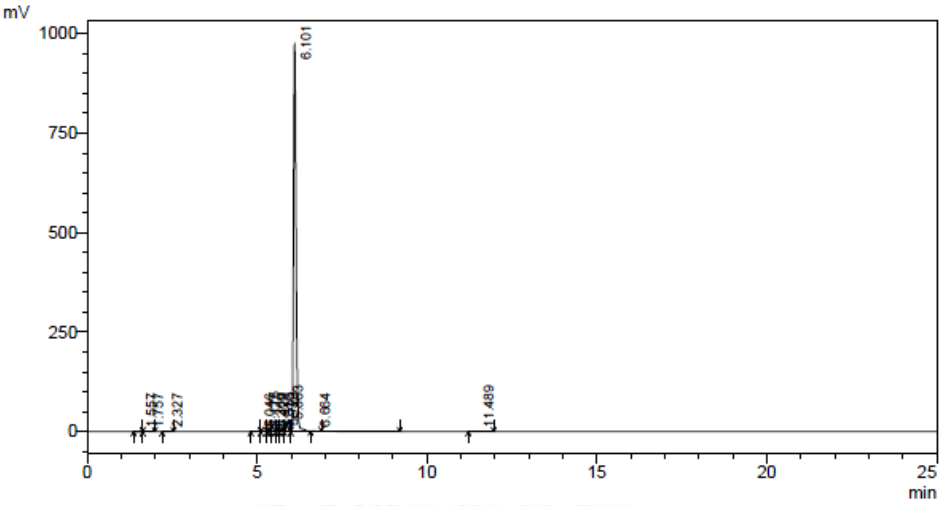
Mobile phase B: 0.1 % (v/v) acetic acid in ultra-pure water

Flow rate: 1 ml/min

K1)



K2)



K3)

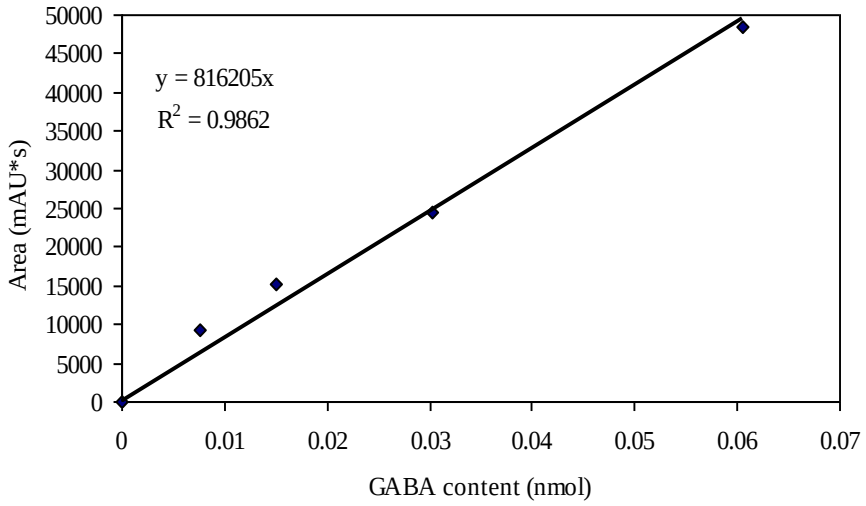


Fig K. 1) Standard glutamate (retention time = 2.5 min), **2)** Chromatogram of standard GABA (retention time = 6.1 min), and **3)** Standard curve of GABA.

APPENDIX L

Personal information

Field of research interest:

My dissertation focused on glutamate decarboxylase mediated GABA production and abiotic stress impacts on this mechanism. The research provides fruitful information about the possible role of this pathway in response to abiotic stresses and also ideal conditions that can prove helpful to improve GABA production. I am familiar with biochemical laboratory techniques such as isolation, purification and growth of microbial strains, DNA, RNA and protein extraction, PCR, RT-PCR, DNA cloning, recombinant protein expression, mutant strain construction, protein purification, enzymology, metabolic pathways, HPLC, bioinformatics, Microsoft Office, power point, excel as well as Photoshop Adobe.

Publications

1. **Kanwal, S.,** Khetkorn, W., and Incharoensakdi, A. (2014) GABA accumulation in response to different nitrogenous compounds in unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Curr. Microbiol. (Accepted).
2. **Kanwal, S.,** Rastogi, R,P., and Incharoensakdi, A. (2014) Glutamate decarboxylase activity and gamma-aminobutyric acid content in *Synechocystis* sp. PCC 6803 under osmotic stress and different carbon sources. J. Appl. Phycol. Doi: 10.1007/s10811-014-0259-9.
3. Haq, I.U., Ullah, N., Bibi, G., **Kanwal, S.**, Ahmad, M.S., and Mirza, B. (2012) Antioxidant and cytotoxic activities and phytochemical analysis of *Euphorbia wallichii* root extract and its fractions. I.J.P.R. 11: 241-249.
4. **Kanwal, S.,** Ullah, N., Haq, I.U., Afzal, I., and Mirza, B. (2011) Antioxidant, antitumor activities and phytochemical investigation of *Hedera nepalensis* k.koch, an important medicinal plant from Pakistan. Pak. J. Bot. 43: 85-89.

Academic Experiences

1. 18th Biological Sciences Graduate Congress, University of Malaya, Malaysia, 6 - 8 January 2014. (Oral presentation) “UV-B induced acclimation of GAD system in unicellular cyanobacterium *Synechocystis* sp. PCC 6803”.

2. Course completion on American Chemical Society Leadership Development Courses Engaging Colleagues in Dialogue and Fostering Innovation, 29 August 2013. American Chemical Society Office of International Activities. BKK, Thailand.
3. 17th Biological Sciences Graduate Congress, Chulalongkorn University, Thailand, 8 - 10 December 2012. (Oral presentation) "Activity and transcriptional regulation of glutamate decarboxylase in *Synechocystis* sp. PCC 6803 under ionic and osmotic stress".
4. Staff of the 13th FAOBMB International Congress of Biochemistry and Molecular Biology, 25 - 29 November 2012. The Biochemistry and Molecular Biology Section Science Society of Thailand Under the Patronage of His Majesty the King, Chulalongkorn University and Mahidol University, Thailand.
5. Course completion on Biosecurity/Food Safety Enhancment Program, Quaid e Azam University, Pakistan, February/March 2009.
6. 6th International Bhurban Conference on Applied Sciences and Technology, Quaid e Azam University, Pakistan, 19 – 22 January 2009. (Oral presentation) "Biological & pharmacological evaluation of *Hedera nepalensis* plant extract and its fractions".
7. 9th Biennial PSBMB Conference on Advances in Biochemistry and Molecular Biology, PMAS Arid Agriculture University, Pakistan, 17 – 20 December 2008.

Honors and Awards

1. The Bronze medal winner for Oral Presentation (Cell and Molecular Biology) "UV-B induced acclimation of GAD system in unicellular cyanobacterium *Synechocystis* sp. PCC 6803" In: The 18th Biological Sciences Graduate Congress, 6 - 8 January 2014, University of Malaya, Malaysia.
2. Gold medal winner in M.Sc. (2004-2005), Biological sciences from Quaid e Azam University, Pakistan.

References:

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VITA

Miss Simab Kanwal was born on July 22, 1985 in Azad Jammu & Kashmir, Pakistan. She has graduated with a Master of Science (M.Sc.) degree in Biological Sciences and Master of Philosophy degree (M.Phil.) in Biochemistry from Faculty of Biological Sciences, Quaid e Azam University in 2007 and 2009, respectively. She has further studied for the Doctor of Philosophy (Ph.D.) degree in Biochemistry, Faculty of Science, Chulalongkorn University, Thailand since 2010.