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Full Length Research Paper

Enhancement of glutamine synthetase activity in *Paenibacillus polymyxa* by optimization of different growth conditions

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Biological nitrogen fixation is highly regulated at the transcriptional level by sophisticated regulatory networks that respond to multiple environmental signals. Glutamine synthetase (GS) occupies a central position in cellular metabolism and offer to the cell, a potential point for regulation of biosynthetic function. The aim of this work is to enhance the activity of GS in *Paenibacillus polymyxa* through altering the constituents of the growth medium, thereby increasing the nitrogen fixation capability. Two bacterial strains were identified as *P. polymyxa* by 16S rRNA gene (Accession No AB727983). High GS activity was recorded in the two strains, in presence of the divalent cations Mg^{+2} and Mn^{+2}. Western blot analysis confirmed the presence of the GS at approximately ~60 kDa. GS activity was found to be affected by growth medium, carbon source, nitrogen source and sodium chloride. LB supplemented with 7% glycerol, 0.4% asparagine and 0.15% sodium chloride gave the highest GS activity.

Key words: Glutamine, *Paenipacillus polymyxa*, 16S rRNA, nitrogen fixation, western blot, Mg^{+2} and Mn^{+2}.

INTRODUCTION

The aerobic, rod-shaped, endospore-forming genus *Bacillus* is a systematically diverse taxon (Claus and Berkeley, 1986). The 16S rRNA gene sequence analysis had identified at least 10 phylogenetic groups in the genus *Bacillus* (Shida et al., 1997). The rRNA group 1 (*Bacillus cereus*) includes the type species *B. subtilis*, and the rRNA group 3 was proposed as the new genus *Paenibacillus* by Ash et al. (1991), which tend to accumulate in soils with high organic contents. Many species of bacteria could obtain their nitrogen from a single widely available source for example, ammonia. In addition, many of these organisms could use organic compounds, such as amino acids, as their sole source of nitrogen. In each case, substitution of ammonia by one of these compounds depends on the ability of the organism to produce the permeases and enzymes specifically required for the conversion of this compound to glutamate and ammonia, which in turn, cells are supplied with ammonia, and their unnecessary synthesis would exert a severe strain on cellular metabolism. It was therefore no surprise that the cells possess a highly efficient system for the regulation of nitrogen assimilation. A remarkable trimeric protein called P_{II} was an essential component of this regulatory system in bacteria (Ninfa and Atkinson, 2000).

P_{II} protein was neither a response regulator nor a sensor; rather, it serves as the central processing unit to coordinate information concerning the intracellular

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Abbreviations: GS, Glutamine synthetase; **MSM,** minimal salt medium; **NB,** nutrient broth medium; **NF,** nitrogen fixation medium; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **PTS,** phosphotransferase transport system; **GS,** glutamine synthetase; **GOGAT,** glutamine: 2-oxoglutarate amidotransferase; **GDH,** glutamate dehydrogenase.

concentration of glutamine and 2-ketoglutarate for the appropriate regulation of the activity of glutamine synthetase (GS). Thomas et al. (1970a) noted that the glutamine synthetase of *B. subtilis* has an absolute requirement for divalent cation Mg^{+2} or Mn^{+2}, which serve as an activator, although each affects various catalytic parameters differently. Due to the tight coupling of metabolism and signaling, three major metabolic functions can be attributed to glutamine; (1) As primary product of ammonium assimilation, (2) as nitrogen donor in various biosynthetic reactions, and (3) as one of the 20 standard amino acids needed for protein synthesis (Forchhamer, 2008). Given these important functions of glutamine, the regulation of glutamine synthase activity is essential for bacterial growth.

MATERIALS AND METHODS

Microorganisms

Two isolates of *Paenibacillus polymyxa* were isolated from green house soil of Agriculture Genetic Engineering Research Institute (AGERI), Cairo, Egypt, and were named as M and G isolates.

Protein analysis

Growth curves for both *P. polymyxa* isolates (M and G) were carried out to determine the maximum growth time at which cells will be harvested for protein analysis. Protein banding patterns and total protein content were carried out according to Laemmli (1970) and Lowry et al. (1951), respectively.

Detection and activity of glutamine synthetase

Western blot technique

To determine the molecular weight of GS, protein banding patterns was carried out according to Laemmli (1970). Western blot technique was carried out according to manufacturer's instructions of western blot kit (BIOBASIC INC, Toronto) using first antibody specific to GS (Forchhammer Lab, Tubingen University).

Assay of glutamine synthetase activity

Glutamine synthetase was extracted by a modified method of Dean et al. (1977). Minimal salt medium, supplemented with 0.2% glutamine was inoculated with 2 ml of *P. polymyxa* isolates (optical density 0.8 at 660 nm). After 8 h, 150 ml was centrifuged at 7000 × *g* for 20 min, then the pellet washed twice by 30 ml of 50 mM imidazole hydrochloride. The pellets were resuspended in 5 ml of the same buffer and lysed by sonicator. Glutamine synthetase activity was assayed in crude extract by a modified method of Elliot (1995). From crude extract, 0.2 ml was added to 1.8 ml of the reaction mixture (50 mM imidazole, 10 mM ATP, 100 mM glutamate, 40 mM hydroxylamine and 10 mM manganese chloride or 40 mM magnesium sulphate). The reaction mixture was mixed, then incubated at 37°C for 15 min. The reaction was stopped by adding 1 ml of ferric chloride reagent (1 M). The optical density was measured at 540 nm after removing the precipitate by centrifugation for 5 min at 2000 × *g*.

Calculations

Activity of GS (Units/ml) = (absorbance at 540 nm for sample - absorbance at 540 nm for blank) 3 × 15 / 6.22 × 0.2.

Where 3: total volume of assay in ml; 15: conversion factor to 15 min (unit definition); 6.22: mm extinction coefficient of β-NADH at 540 nm; 0.2: volume of crude extract in ml. Unit definition: One unit will convert 1.0 μmole of L-glutamate to L-glutamine in 15 min, at pH 7.1 and 37°C.

Specific activity of GS (Units/mg) = activity of GS (Units/ml) / total protein of extracted cells (mg /ml)

Amplification of 16S rRNA gene in both strains of *Paenibacillus polymyxa*

This was carried out using specific primers (Ding et al., 2005); forward primer: 5'- AGAGTTTGATCCTGGTCAGAACGCT-3' and reverse primer: 5'- TACGGCTACCTTGTTACGACTTCACCCC-3'. Bands obtained from PCR products were eluted and purified by (Qiagen elution kit). The purifed fragments were sequenced in Promega company laboratory. Nucleotide sequences of the products were edited using Bioedit version 5.0.6 (Hall, 1999).

Effect of different factors on GS activity

To determine the best medium, carbon, nitrogen sources and sodium chloride concentrations for maximum GS activity, *P. polymyxa* isolates M and G were grown in different growth media including; Luria bertani medium (LB), minimal salt medium (MSM), nutrient broth medium (NB) and nitrogen fixation medium (N.F). For evaluation of different carbon sources, LB media was used, to which 5 g/L of different carbon sources; including monosaccharide (glucose), disaccharide (fructose and lactose), polysaccharide (manitol, sucrose and starch) and alcohols sugars (sorbitol and glycerol in concentration 5 ml/L) were added. In another experiment, LB medium was used, supplied with different concentrations of glycerol. For evaluation of different nitrogen sources, LB media (containing best carbon source) was supplied with 0.4 g/L of different nitrogen sources, which included urea, peptone, asparagine, glycine, histidine and cystine. In addition, different concentrations of NaCl (0.5 to 10 g/L) were supplied to LB media (containing the best carbon and nitrogen source).

Statistical analysis

Results are expressed as the mean ± S.D. The statistical analyses were carried out using (F-test), followed by Dunnett's multiple comparison tests, with statistical significance set at $p < 0.05$. All analyses were performed with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA) version (2007).

RESULTS

Due to the heavy use of chemicals, natural environment has been seriously deteriorated. Furthermore, the increasing prizes for chemical fertilizers are threatening smallholder agriculture. Therefore, the use of living bio-fertilizers (nitrogen fixing bacteria) provides an attractive replacement for mineral fertilizers.

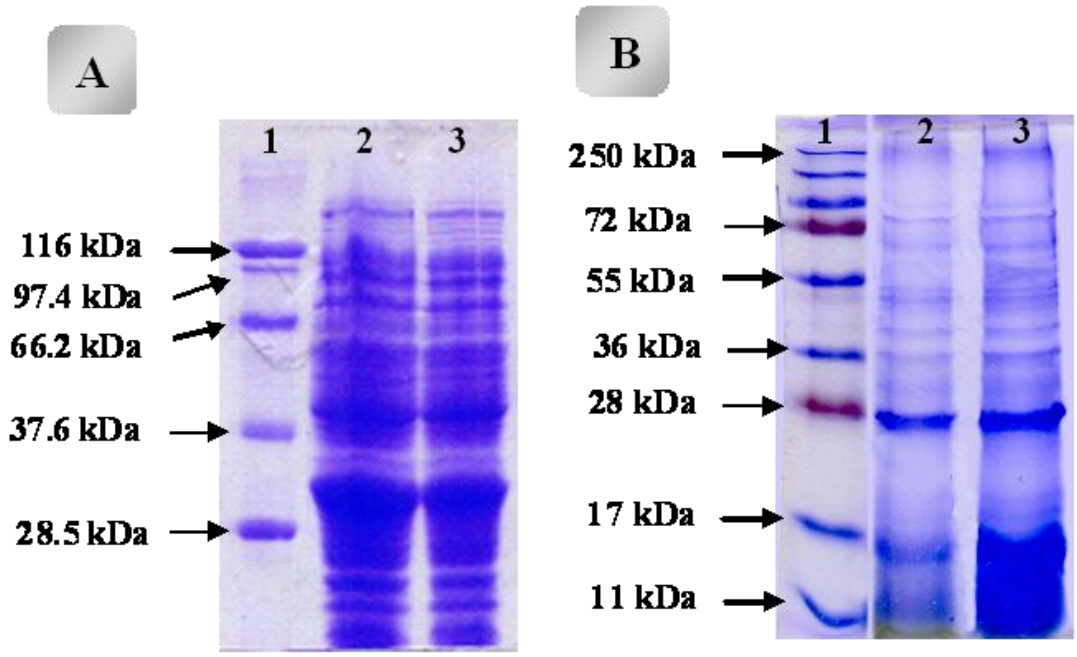

Figure 1. SDS- PAGE analysis of total cellular protein of M and G strains. (A) Grown in LB medium, and (B) grown in MSM medium. Lane 1: protein marker; lane 2: M strain; lane 3: G strain.

Table 1. Determination of activity and specific activity of glutamine synthetase from *Paenibacillus polymyxa* M and G by Mn^{+2} and Mg^{+2} cation ion (at de-adenylylation and adenylylation stage).

Media containing	*Paenibacillus Polymyxa* isolate	Total protein (mg/ml)	Activity (u/ml)		Specific activity (u/mg)	
			Mg^{+2}	Mn^{+2}	Mg^{+2}	Mn^{+2}
Glucose 0.3% (w/v)	M	0.29	113	58	392	251
	G	0.37	261	162	702	215
Glucose 0.3% (w/v) + Glutamine 0.3% (w/v)	M	0.31	69	72	229	291
	G	0.40	204	79	514	1293

Characterization of different strains of *Paenibacillus polymyxa* producing glutamine synthetase

Different protein patterns of the total cellular protein for *P. polymyxa* isolates using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis were observed. Strains of *P. polymyxa* (M and G) were grown in both LB and MSM media for 18 h at 30°C, 150 rpm and pH 7. The pattern of total cellular protein of both strains by SDS-PAGE is illustrated in Figure 1a and b. Although there was no difference of the protein pattern for both isolates in the same medium, there was a clear difference in the protein pattern when growing on different media.

Enzymatic assay

To assay specific activity, the total protein was estimated as shown in Table 1. Specific activities were 392 and 702 u/mg, with Mg ion for M and G isolates, respectively, while with Mn ion, the specific activities were 251 and 215 u/mg for M and G isolates, respectively. However, with carbon and nitrogen sources, the activity and specific activity were higher with manganese than magnesium ion. Specific activities were 229 and 514 u/mg with Mg ion for M and G isolates, respectively, while with Mn ion the specific activities were 291 and 1293 u/mg for M and G isolates, respectively.

Western blot technique

To detect the molecular weight of glutamine synthetase from the harvest cells of *P. polymyxa* isolates (M and G) grown in MSM medium, western blot technique, with specific antibody raised in rabbit against glutamine synthetase was used. Data illustrated in Figure 2 showed

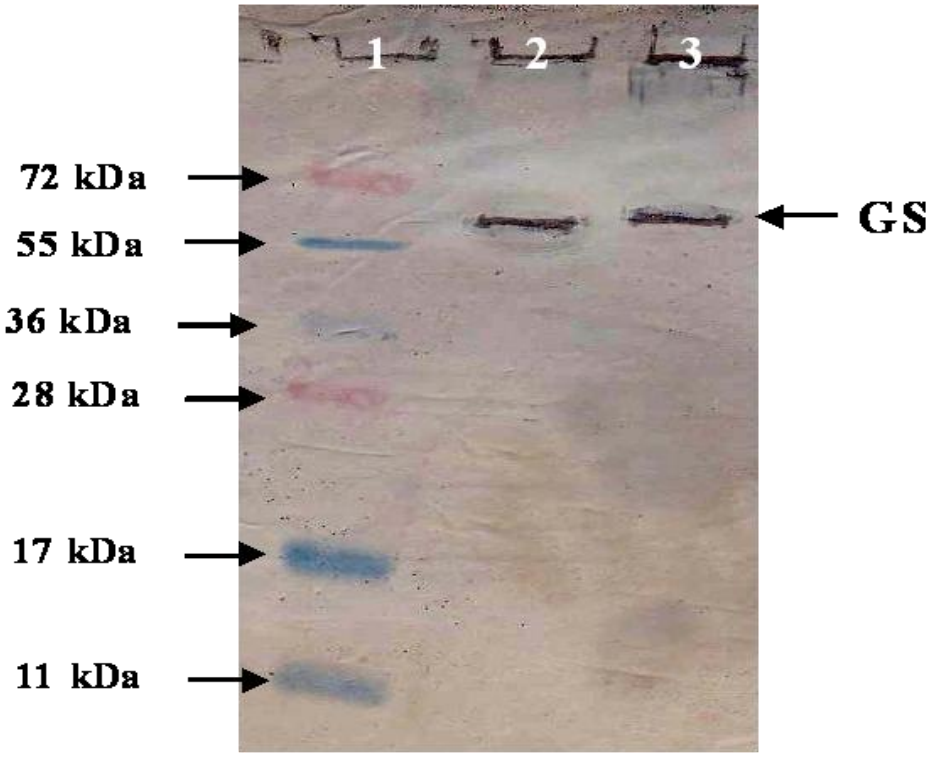

Figure 2. Western blot technique for detection the glutamine synthetase enzyme. Lane 1; Protein marker; lane 2 and 3: GS from M and G isolates, respectively.

a sharp band detected at ~60 kDa.

Amplification of 16S rRNA gene

M and G isolates were identified by amplification of 16S rRNA gene using specific primers, as described in the method section. The obtained band was at expected size at 1500 bp. The obtained 1500 bp PCR product was eluted from agarose gel and sequenced. Identity of nucleotide sequences, and similarities to data base entries of the obtained products were edited using BioEdit version 5.0.6 (Hall, 1999), and analyzed using Blastn (Altschul et al., 1997) at (http://www.ncbi. nlm.nih.gov/BLAST/ (Sequence was submitted in GenBank with Accession No AB727983). Neighbor-joining). Neighbor-joining (NJ) phylogenetic tree was constructed using PHYLIP (3.69) (Felsenstein, 1993). *B. subtilis* was used as an out-group. The sequence results demonstrated that both M and G were clustered with *P. polymyxa* PPL-3 by 100% bootstrap value Figure 3.

Growth medium for GS activity

An experiment was carried out to evaluate the GS in the two strains under study (*P. polymyxa* M and G) using different media: LB, NF, MSM and NB media. The strains were grown in optimum incubation conditions as described in methods. Data illustrated in Figure 4 showed that the values of GS activity were higher in LB medium, followed by NB and NF, while the lower activity was detected on MSM medium. LB medium showed the highest GS activity. Data shown in Figure 4 indicated that regarding Mn^{+2} ion, M strain recorded (172 u/ml) and gave higher than G strain (157 u/ml). On the other hand, G strain showed higher GS activity (151 u/ml) than M strain (145 u/ml) with Mg^{+2} ions. The lowest GS activity was in MSM medium with Mn^{+2} ions; M strain had (95 u/ml) higher than G strain (88 u/ml). Also, with Mg^{+2} ions, M strain had GS activity (81 u/ml) higher than G strain (70 u/ml). LB medium was the best medium for growth of both strains, which showed optical densities, 0.54 and 0.53, for M and G strains, respectively. From the data in Figure 4, it could be concluded that the relation between GS activity and growth was exponential. There was no significant difference between NF and MSM media but there was a significant difference between M and G strains in these two media. Also, a significant difference was found between LB and NB media, however, there was no significant difference between M and G strains in the two medium.

Effect of different carbon sources on GS activity

From the results given in Figure 5, using different carbon sources, the data indicates that the values of GS activity

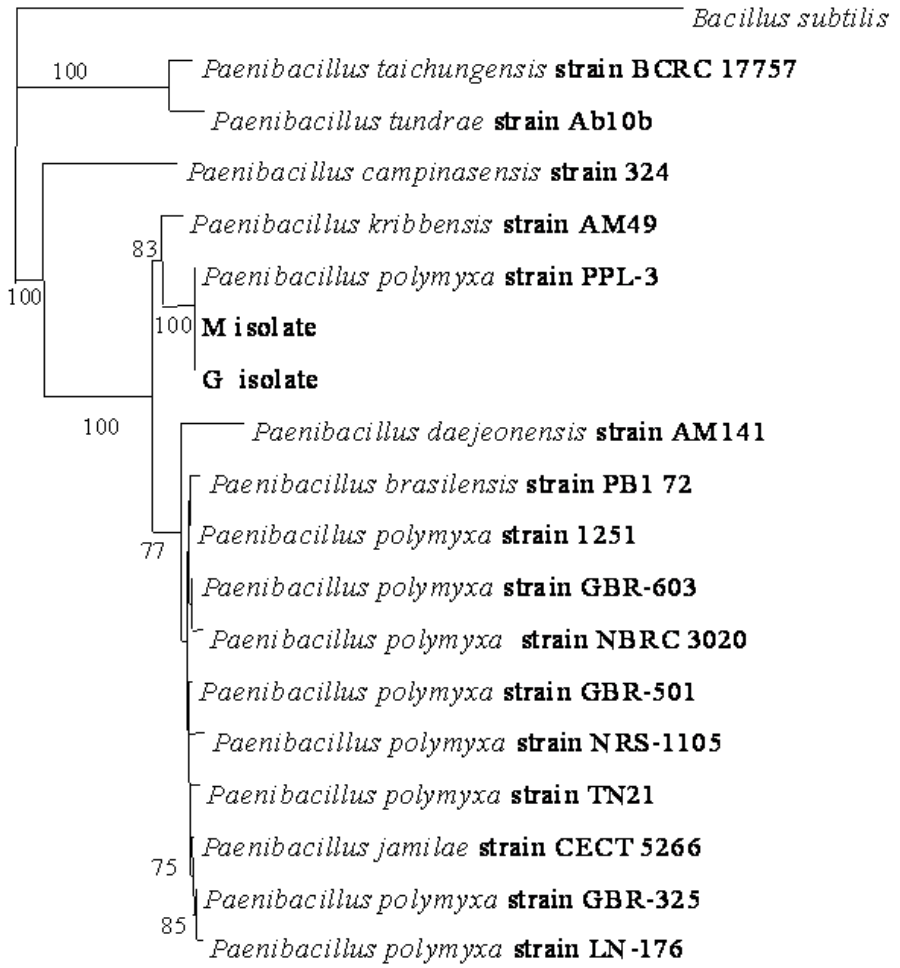

Figure 3. Distance neighbor-joining phylogenetic tree of isolates M and G. Consensus bootstrap with values of over 50% are shown on the nodes.

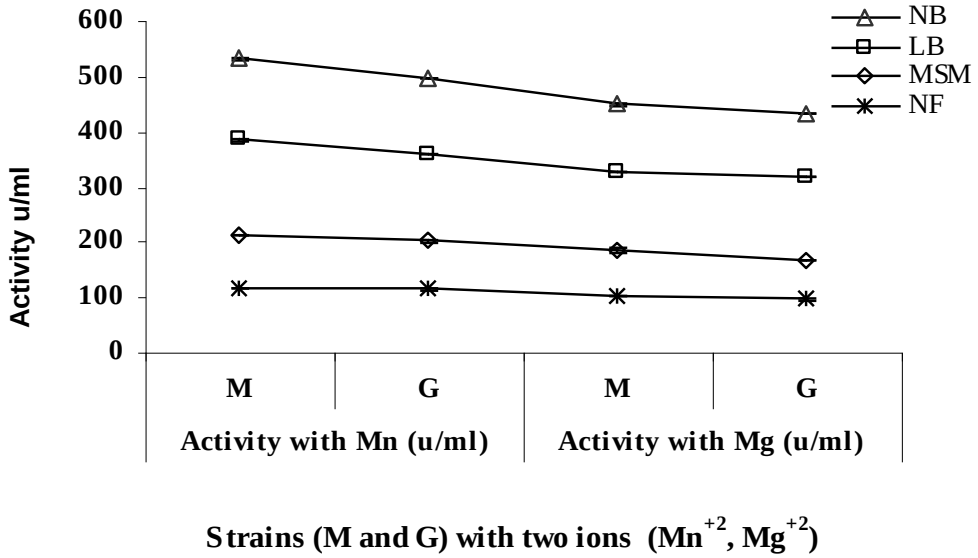

Figure 4. Effect of different media on GS activity from *Paenibacillus polymyxa* (M and G strains) with Mn^{+2}, Mg^{+2} ions.

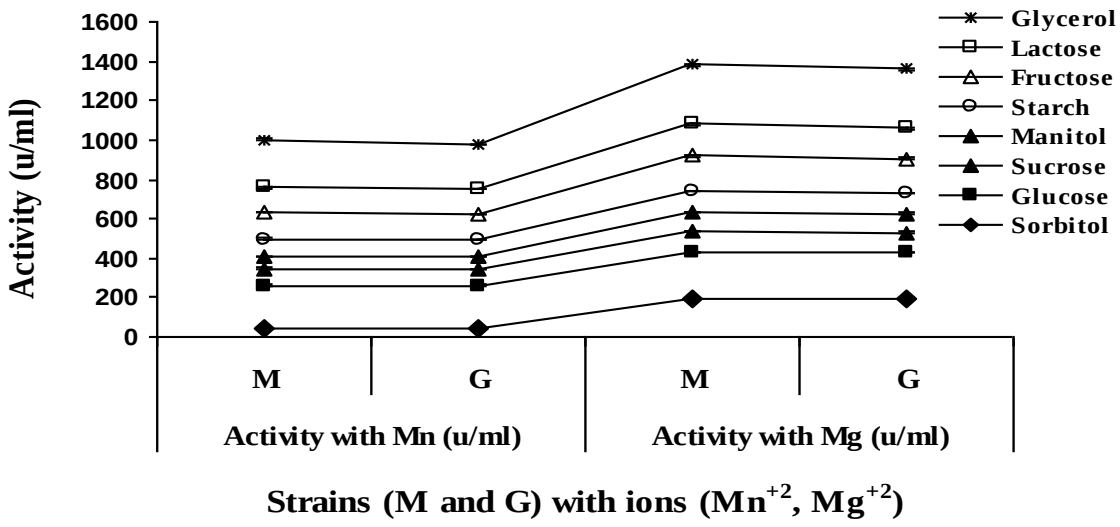

Figure 5. Effect of different carbon sources on GS activity of *Paenibacillus polymyxa* (M and G strains) with two ions (Mn^{+2} and Mg^{+2}).

were in descending order: glycerol > glucose > lactose > fructose > starch > sucrose > manitol > sorbitol. The highest GS activity was recorded with glycerol, and activity with Mg^{+2} ions was higher than with Mn^{+2} ions. In M strain with Mg^{+2}, it was (298 u/ml), G strain was (299 u/ml), while with Mn^{+2} M strain was (245 u/ml) and G strain was (227 u/ml). The lowest activity was with sorbitol in presence of Mg^{+2} and Mn^{+2} ions. With M strain, Mg^{+2} ion, the GS activity was (199 u/ml), G strain (198 u/ml), while in Mn^{+2} ion, M strain was (43 u/ml) and G strain (43 u/ml). On the other hand, there was no correlation between growth and activity of GS, however sorbitol had low GS activity while it had high growth, and M strain also had higher growth than G strain which recorded, 0.18 and 0.16, respectively.

Effect of different concentrations of glycerol on GS activity

The data in Figure 6 demonstrates that different concentrations of glycerol (3, 5, 7, 9, 11, 13 and 15%) influence GS activity. Gradual increases in GS activity were observed with concentrations of 3, 5 and 7%; however, increasing the concentration beyond 7% decreased the activity. The highest GS activity was detected with 7% glycerol supplemented with Mg^{+2} ion in M and G strains (380 and 376 u/ml, respectively). While with Mn^{+2} ions in M strain, the GS activity was 268 u/ml and in the G strain, it was 26 u/ml. The lowest activity was at 15% of glycerol; however it had the highest growth.

Effect of different nitrogen sources on GS activity

The data given in Figure 7 indicates that the values of GS activity were in descending order: asparagine > urea >peptone > histidine > glycine > cystine. Aspragine recorded the highest GS activity with Mg^{+2} and Mn^{+2} ions; Mn^{+2} being higher than Mg^{+2}. Activities were: in M strain with Mn^{+2} (397 u/ml), G strain (396 u/ml), while with Mg^{+2}, M strain (362 u/ml), G strain (378 u/ml). The lowest activity was with cystine in Mg^{+2} and Mn^{+2} ions. With M strain, Mg^{+2} ion, the GS activity was (273 u/ml), G strain (270 u/ml), while in Mn^{+2} ion, M strain, it was (286 u/ml), G strain (284 u/ml). On the other hand, there was no correlation between growth and activity of GS, cystine had low GS activity and urea had high growth but also still, M strain had higher growth than G strain.

Effect of different concentrations of asparagine on GS activity

From the data in Figure 8, using aspragine with different concentrations (0.0, 0.2, 0.4, 0.6, 0.8 and 1% wt/v), it was illustrated that the values of GS activity gradually increased using 0.2 and 0.4% wt/v, and decreased thereafter till 1% wt/v. The highest GS activity was with 0.4% (wt/v) of aspragine, Mg^{+2} ion with M strain recorded (306 u/ml), G strain (304 u/ml). While, with Mn^{+2} ion and M strain, GS activity was (329 u/ml), in G strain (329 u/ml). The lowest activity was at 1% (wt/v) in Mg^{+2} and Mn^{+2} ions. With M strain, Mg^{+2} ion, the GS activity was (245 u/ml), G strain (243 u/ml) while with Mn^{+2} ion, M and G strains were (261 u/ml). Also, the highest growth was at 1% (wt/v).

Effect of different concentrations of sodium chloride on GS activity

Results given in Figure 9, using different concentrations

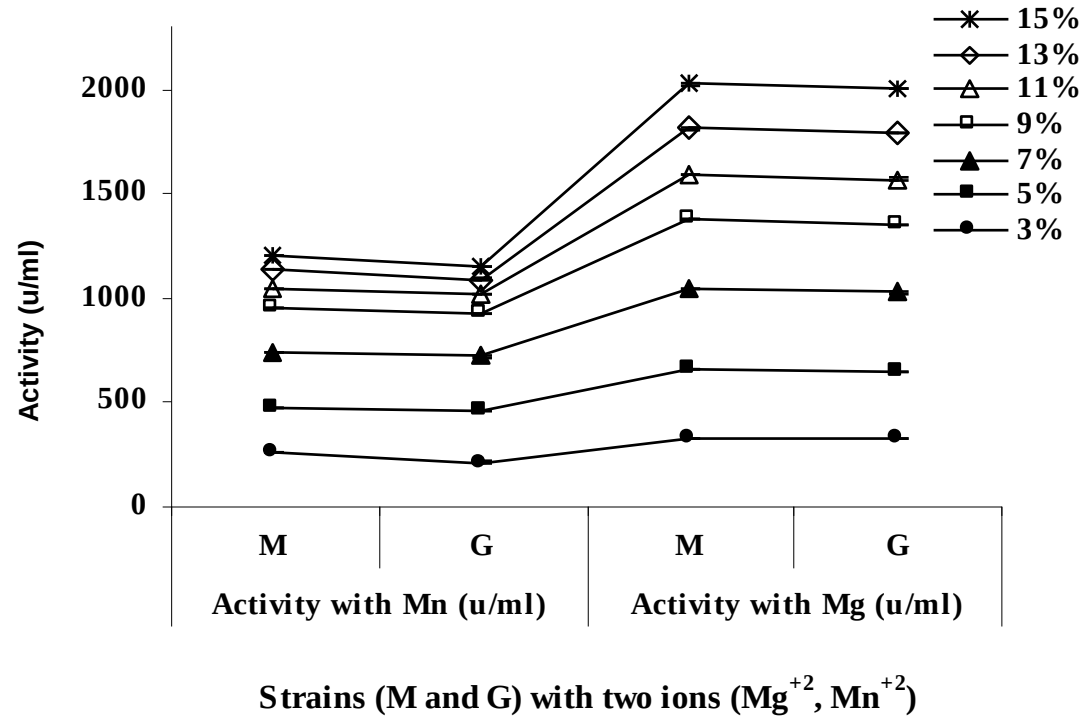

Figure 6. Effect of different concentrations of glycerol on GS activity of *Paenibacillus polymyxa* (M and G strain) with two ions (Mn^{+2} and Mg^{+2}).

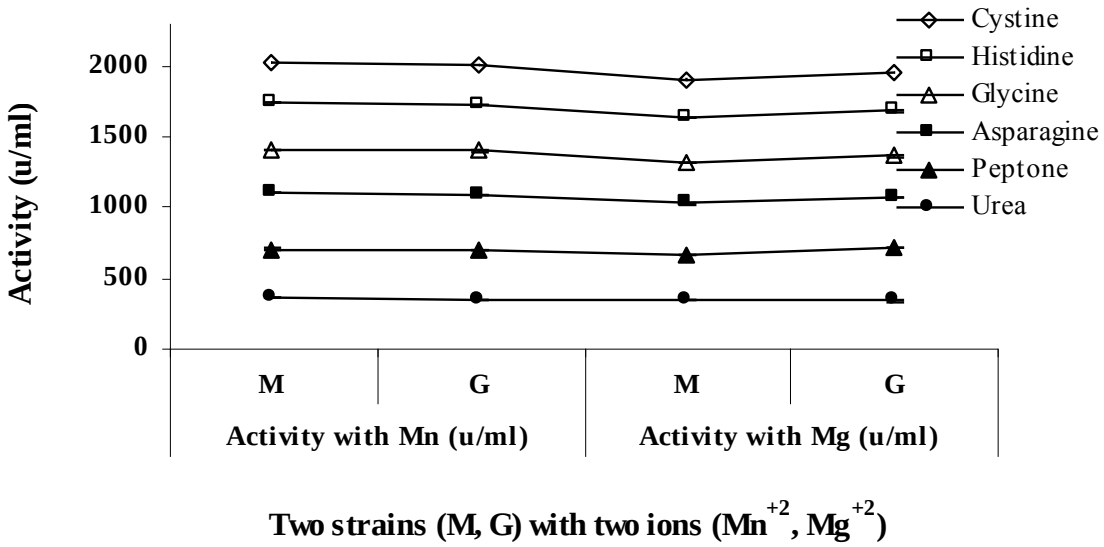

Figure 7. Effect of different nitrogen sources on GS activity of *Paenibacillus polymyxa* (M and G) with ions (Mn^{+2} and Mg^{+2}).

of NaCl (0.0, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 and 10 g/L), illustrate that the values of GS activity gradually increased using 0.0 and 1.5 g/l, and decreased thereafter till 10 g/L. The ratio of salt had great effect on growth and metabolism in bacterial culture. Thus by using different concentrations of sodium chloride, the difference appear in GS activity and growth ratio, however there was no correlation between both. The highest GS activity was with 1.5 g/L, with Mg^{+2} and Mn^{+2} ions. GS activity was higher in Mg^{+2} than Mn^{+2}. In M strain, with Mg^{+2} it was (272 u/ml), G strain (270 u/ml), while with Mn^{+2}, M strain it was (271 u/ml), G strain with

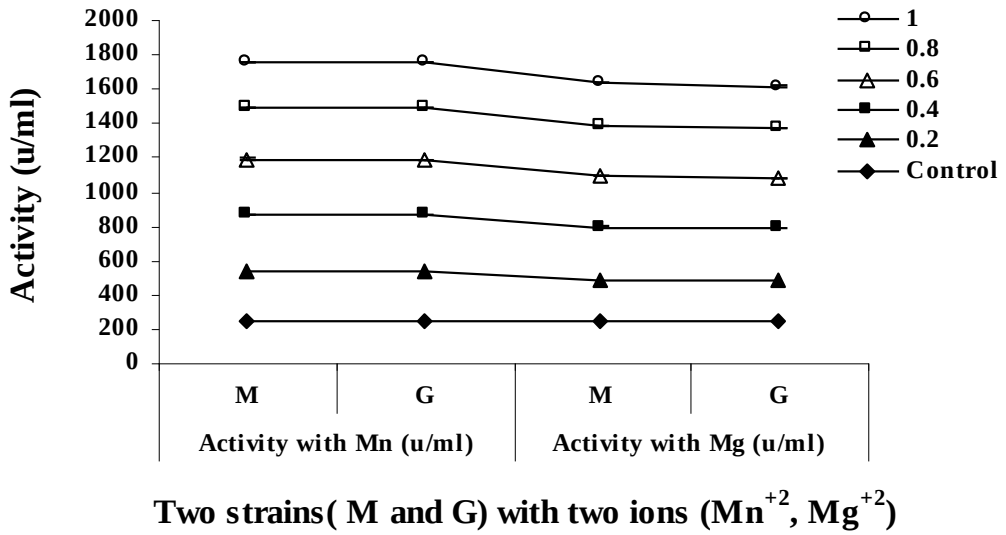

Figure 8. Effect of different concentrations of aspragine on GS activity of *Paenibacillus polymyxa* (M and G) with two ions (Mn^{+2} and Mg^{+2}).

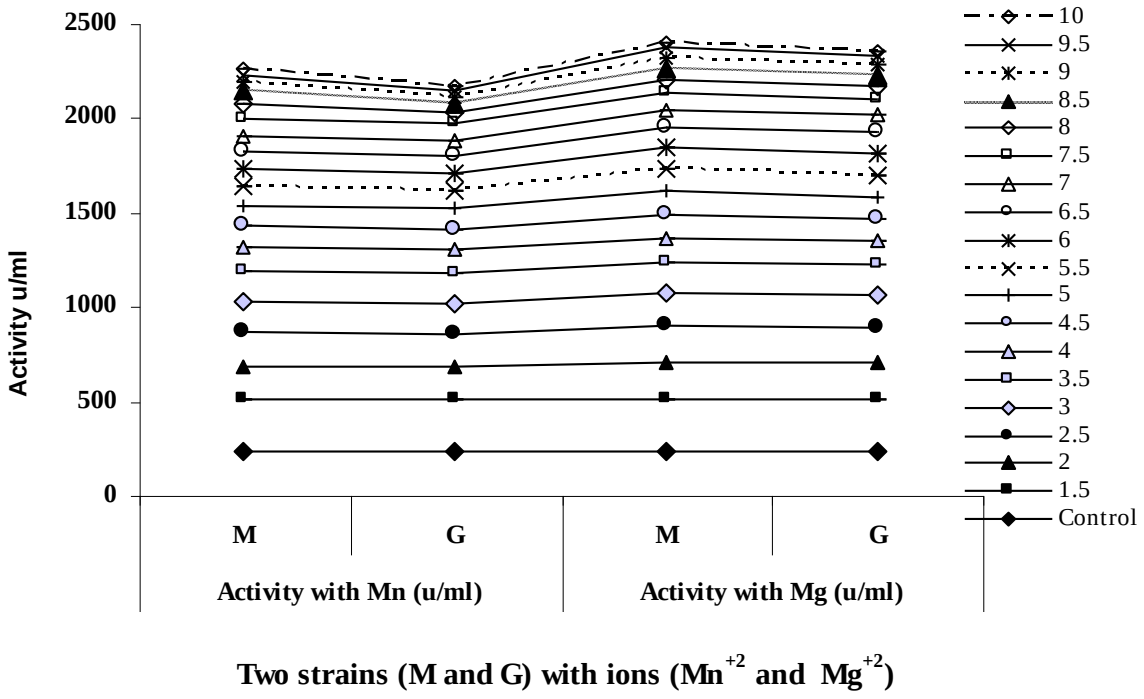

Figure 9. Effect of different concentrations of sodium chloride on GS activity of *Paenibacillus polymyxa* (M and G) with two ions (Mn^{+2} and Mg^{+2}).

Mg^{+2} (270 u/ml). The lowest activity was with 10 g/l in Mg^{+2} and Mn^{+2} ions. With M strain, Mg^{+2} ion, the GS activity was G strain (25 u/ml) while in Mn^{+2} ion, M strain was (20 u/ml), G strain (19 u/ml). At 3 g/L, the strains had high growth but still M strain had higher growth than G strain.

DISCUSSION

SDS-PAGE of total cell proteins is a powerful tool for the taxonomical discrimination of a great number of strains (Eaglesham et al., 1987; Pot et al., 1992). Glutamine synthetase assay had an absolute requirement for

divalent cation Mg^{+2} or Mn^{+2} that act as an activator, however, each affected various catalytic parameters differently. The concentration of either divalent cation required to produce half-maximal activity was a function of the ATP concentration. Data presented showed that activity and specific activity of glutamine synthetase in *P. polymyxa* isolates were generally higher with magnesium than manganese ion. Both Mn^{+2} and Mg^{+2} activate the glutamine synthetase enzyme in the biosynthesis of L-glutamine, unlike the *E. coli* system, the Mg^{+2} dependent activity was intrinsically less stable than the Mn^{+2} dependent activity (Thomas et al., 1970a). There was a remarkable similarity in the physical structures of the glutamine synthetases from *B. subtilis* and *E. coli*. The capacity of *B. subtilis* glutamine synthetase to be activated by Mn^{+2} and Mg^{+2} was apparently an intrinsic property of the enzyme; it was independent of growth conditions and was not determined by adenylylation of the enzyme as it was for the enzyme from *E. coli*. Glutamine synthetase from *B. subtilis* was inhibited by glutamine, and this inhibition varies with the divalent cation used to activate the enzyme; with Mn^{+2} activation, inhibition by glutamine was greatly potentiated by AMP. From the standpoint of cellular regulation, it seems that in both *E. coli* and *B. subtilis*, the intra-cellular level of glutamine was the most important single factor in determining glutamine synthetase activity. However, in *B. subtilis,* glutamine was a direct inhibitor of glutamine synthetase activity, whereas in *E. coli*, glutamine exerted its effect indirectly through activation and inhibition of the adenylylating and deadenylylating enzymes, respectively (Thomas et al., 1970b). The obtained data from western blot demonstrated that the molecular weight of glutamine synthetase of *P. polymyxa* isolates was ~60 kDa. This was in agreement with data obtained by Thomas et al. (1970b) and Stadtman (2001). Forchammer (2008) and Thomas et al. (1970b) mentioned that GS in many microorganisms occurs in three different isoforms (GSI, GSII and GSIII), with different molecular weights, but the GS isoform of *B. subtilis* and *E. coli* was ~60 kDa in size as determined by western immunoblot. This related to P_{II} protein system. Most free-living bacteria are capable of this prototrophic life style.

The results obtained in this study showed that the fragment of 16S rRNA (1500 bp) that was obtained using universal primers P1 and P6 with the two strains M and G, were in accordance with Ding et al. (2005). Marmur (1961) reported that genetic approaches were combined with the phenotypic studies to assist in the determination of the taxonomic position of the 16 novel isolates of *Paenibacillus* and total DNA was extracted from all studied *Paenibacillus* strains. Shida et al. (1997) proposed that the strains had their identification as members of the genus *Paenibacillus*, confirmed by PCR amplification of a 16S rRNA fragment, with the specific forward primer PAEN515F and the universal reverse primer 1377R. All isolates produced the expected fragment of 860 bp, characteristic for members of the genus *Paenibacillus* (Irene et al., 2002). By using the ClustalW program, phylogenetic distances were calculated according to the neighbour-joining method (Saitou and Nei, 1987), with the one-parameter model of Jukes and Cantor (1969). Irene et al. (2002) demonstrated the comparison of the 16S rDNA sequence of strain PB172T, with those of some *Paenibacillus* spp. available in the databases. They showed that strain PB172T clustered in amonophyletic group, together with *P. polymyxa*, *P. peoriae* and *P. jamilae* in 100% of the trees obtained. The phylogenetic similarity indicated by the 16S rDNA data was in agreement with the levels of 16S rDNA sequence similarity obtained with the novel strain and *P. polymyxa*.

As a result, LB medium was used in the following experiments. The previous data were in agreement with study carried by Thomas et al. (1970a) who used LB as optimal medium to detect GS activity from *P. polymyxa* ATCC 842, *P. massiliensis* 2301065T, *B. cereus* DSM31 and *B. megaterium* DSM 32. However, Dean et al. (1977) reported that GS activity was higher when *B. subtilis* strains were grown in minimal salt media supplemented with 0.2% glutamine as nitrogen source. Ding et al. (2005) then mentioned that the nitrogen-free medium (NF) was optimum medium. There was a significant difference between the different carbon sources with the two strains under study, and there was a significant difference between the two strains when using the same carbon source. Carbon substrates are transported into the cell either in an unmodified form or through a phosphotransferase transport system (PTS) system (major carbohydrate transport system in bacteria). Two major players interconnecting carbon and nitrogen regulation were the P_{II} proteins and the phosphotransferase system. Moreover, several DNA-binding transcription regulators sense signals were derived from both carbon and nitrogen metabolism. The regulatory networks enable the bacteria to make the appropriate metabolic responses to change nutrient availabilities in the environment features. Most compounds derive nitrogen by secondary transfers from only two central intermediates, the amino acids glutamate and glutamine. Moreover, a decrease in growth rate was the most direct indication of nutrient limitation (Forchhammer, 2008).

There was significant difference between the different concentrations of glycerol with the two strains, and there was significant difference between the two strains when using the same concentration of glycerol. Biju et al. (2008) reported that *Listeria monocytogenes* was able to efficiently utilize glycerol as a carbon source. In a defined minimal medium, the growth rate (during balanced growth) in the presence of glycerol was similar to that in the presence of glucose or cellobiose. Different glycerol concentrations between 0.5 and 5 mM were used to determine the optimal concentration of glycerol required to distinguish the growth rates of strain GD236 *Lactococcus lactis* (Alexander and Mariette, 2000).

Glycerol entered the cytoplasm by passive diffusion across the lipid bilayer (Sweet et al., 1990) or through facilitated diffusion mediated by GlpF (Heller et al., 1980). Glycerol was of low toxicity towards microorganisms. In a 16 h test with *Pseudomonas putida*, no inhibition of bacterial growth was found at concentrations between 100 and 10,000 mg/L, the information was considered sufficient to conclude that glycerol was of low toxicity to (www.henkel.com). Glycerol concentration of 7% was selected for further investigations.

Bacillus spp. can use ammonium and a number of other nitrogen-containing compounds as sole source of nitrogen (Fisher, 1993). Ammonium was assimilated through glutamine synthetase (GS), glutamine: 2-oxoglutarate amido-transferase (GOGAT), and glutamate dehydrogenase (GDH) (Schreier et al., 1993).

There was significant difference between the different nitrogen sources with the two strains, and there was significant difference between the two strains when using the same nitrogen source. Aspragine was selected for further experimentation. Regulation by the nitrogen source of the medium might be the major control system for urease and asparaginase production in *B. subtilis*, since synthesis of these enzymes was not increased by addition of urea or asparagine to the growth medium. In fact, the synthesis of these enzymes was repressed, not increased, by addition of urea or asparagine to the growth medium. In fact, the synthesis of these enzymes was repressed several fold when their enzymatic substrates were used as the nitrogen source of the growth medium (Mariette and Susan, 1991). This finding suggests that sufficient $NH4^+$ is produced by the catabolism of urea and asparagine to repress the synthesis of their degradative enzymes. The relative levels of GS and GOGAT were dependent on available sources of nitrogen. The levels of GS synthesis are dependent on the need for glutamine synthesis; they were lowest in the presence of glutamine (Colin, 1989).

A significant difference was found between the different concentrations of aspragine with the two strains, and there was significant difference between the two strains when using the same concentration of aspragine. Aspragine concentration of 0.4% wt/v was used in the further investigations. Asparagine functions in the transport and storage of nitrogen, in addition to being an essential compound of many proteins required for normal plant development (Sieciechowicz et al., 1988). Asparagine, one of the 21 important amino acids that make up proteins, was known to be synthesized from aspartate in an ATP-dependent amidation reaction; it had no toxic effect on bacterial growth (Milman and Cooney, 1979). Two mechanistically distinct asparagine synthetases were known (Susan and Sheldon, 1992). The one encoded by *asnA* utilized ammonia as amide donor, whereas the *asnB*-derived protein works with glutamine. The same was observed with different concentrations of sodium chloride. Since glutamine synthetase activity was chloride dependent, we sought to determine the influence of the anion on glutamine synthetase activity. Here, cells were grown in the presence of 2.5 M NaCl. Different chloride salts stimulated glutamine synthetase activity, and the highest activity was observed in the presence of choline chloride. Since it has been shown previously that nitrate was not inhibitory (Stephan et al., 2006) and since the effect was observed with different anions, the experiments demonstrate a Cl^- dependence of glutamine synthetase activity (Stephan et al., 2006). The glutamine synthetase was a key enzyme in the production of the compatible solutes glutamate and glutamine, but also in the production of ectoine and proline. An inactive glutamine synthetase would reduce or diminish the cells ability to synthesize compatible solutes, and therefore growth of the moderate halophile would be impaired in the absence of chloride. This interpretation was in line with the observation that the cell yield is chloride dependent; indicating that an essential, chloride-induced component(s), chloride, exerted an even more pronounced effect on the enzymatic activity of preformed enzyme.

In the absence of chloride in the assay buffer, glutamine synthetase activity was decreased by as much as 90%. These data demonstrate for the first time, a regulatory role of a component of common salt, chloride, in the biosynthesis of compatible solutes. To exclude regulation of glutamine synthetase by the salt concentration in the assay buffer, cells were grown at different salinities, and glutamine synthetase activity was measured for each culture at three different KCl concentrations in the assay buffer. Independent of the salinity used to grow the cells, glutamine synthetase activity was maximal in the presence of 1.5 M KCl in the assay buffer (Stephan et al., 2006).

Conclusion

The importance of this study was to understand the metabolism of GS in *P. polymyxa* to enhance the enzyme activity. Two nitrogen fixing bacterial isolates were isolated from the soil and were identified as *P. polymyxa* by 16S rRNA gene. Both of them were able to produce GS supplied with two important cations (magnesium and manganese). This study showed that the molecular weight of GS was ~60 kDa using western blot technique. Growth media greatly affected the activity of GS and GS specific activity in the two isolates. The constituents of the growth medium including carbon source, nitrogen source and sodium chloride concentrations were important in the activity of GS. GS activity form both isolates grown with different carbon sources were higher with magnesium than manganese. Also, when a bacterial cell was grown on different nitrogen sources, the GS activity was higher with manganese than magnesium this is due to the ability of reverse reaction of GS enzyme to adapt with stress conditions of growth.

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