

Optimized M9 Minimal Salts Medium for Enhanced Growth Rate and Glycogen Accumulation of *Escherichia coli* DH5 α

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Glycogen plays important roles in bacteria. Its structure and storage capability have received more attention recently because of the potential correlations with environmental durability and pathogenicity. However, the low level of intracellular glycogen makes extraction and structure characterization difficult, inhibiting functional studies. Bacteria grown in regular media such as lysogeny broth and tryptic soy broth do not accumulate large amounts of glycogen. Comparative analyses of bacterial media reported in literature for glycogen-related studies revealed that there was no consistency in the recipes reported. *Escherichia coli* DH5 α is a convenient model organism for gene manipulation studies with respect to glycogen. Additionally, M9 minimal salts medium is widely used to improve glycogen accumulation, although its composition varies. In this study, we optimized the M9 medium by adjusting the concentrations of nitrogen source, tryptone, carbon source, and glucose, in order to achieve a balance between the growth rate and glycogen accumulation. Our result showed that 1 \times M9 minimal salts medium containing 0.4% tryptone and 0.8% glucose was a well-balanced nutrient source for enhancing the growth and glycogen storage in bacteria. This result will help future investigations related to bacterial physiology in terms of glycogen function.

Keywords: Glycogen accumulation, M9 minimal salts medium, tryptone, glucose

Introduction

Glycogen is a widespread carbon and energy storage molecule in prokaryotes, and consists of glucosyl resi-

dues only [1, 2]. Glucosyl units in linear chains are first linked together by α -1,4-glucosyl linkages. Linear chains are then connected together by α -1,6-glucosyl linkages to form branches [3]. Recently, glycogen was confirmed as an important compound for bacterial environmental durability and pathogenicity due to its highly branched structure and unexpected connections with a variety of metabolism pathways, such as trehalose, maltose and capsular glucan, *etc.* [1, 2, 4–7]. Thus, accumulation of sufficient glycogen in liquid culture is a pre-requisite for investigating glycogen physiological functions and char-

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acterizing its structures through different techniques, such as transmission electron microscopy (TEM), size exclusive chromatography (SEC), and fluorophore-assisted carbohydrate electrophoresis (FACE), *etc.* [8]. In this short communication, we report an optimized recipe of M9 minimal salts medium for promoting *E. coli* DH5 α growth and glycogen accumulation.

Bacterial growth requires abundant and well-balanced nutrients, while glycogen accumulation normally occurs under growth-limited conditions with excessive carbon source and deficiency of other nutrients like

nitrogen and phosphorus at stationary phase [4, 9, 10]. There are normally five energy storage compounds in bacteria, which are: polyphosphate (polyP), wax ester (WE), triacylglycerol (TAG), polyhydroxybutyrate (PHB) and glycogen [2, 11]. Not all bacteria store glycogen as an energy compound, while some bacteria may accumulate multiple energy compounds [2]. For example, host-associated bacteria such as *Buchnera aphidicola*, *Wolbachia pipientis*, and *Coxiella burneti* do not have glycogen metabolism related enzymes, hence no glycogen storage [2]. Human pathogen *Mycobacterium*

Table 1. Culture media used for studying glycogen metabolism and/or accumulation in of *Escherichia coli*.

<i>E. coli</i> strains	Medium	Composition	Growth rate*	Glycogen accumulation	Methodology	Year	Ref.
K12	M9	M9 complemented with 2.7 g/l glucose	N/A	N/A	HPLC GOPOD Assay	2017	[17]
BW25113	M9	48 mM Na ₂ HPO ₄ , 22 mM KH ₂ PO ₄ , 9 mM NaCl, 19 mM NH ₄ Cl, 0.1 mM CaCl ₂ , 2 mM MgSO ₄ , 0.4% glucose or glycerol, 100 μ g/ml amino acids	5	N/A	N/A	2016	[18]
MG1665	LB	10 g/l Bacto™ tryptone, 5 g/l yeast extract, 10 g/l NaCl	N/A	N/A	Electron micrography	2016	[19]
DH5 α	M9	1.5% agarose, 0.4% glucose, 0.2% thiamine, 2 mM MgSO ₄ , and 0.1 mM CaCl ₂	12	80 ng/ μ g	Iodine vapor staining GOPOD Assay	2015	[15]
K12	M9	M9 containing 95 mM Na ₂ HPO ₄ , 44 mM KH ₂ PO ₄ , 17 mM NaCl, 37 mM NH ₄ Cl, 0.1 mM CaCl ₂ , 2 mM MgSO ₄ and 50 mM glucose	7	N/A	Iodine vapor staining	2014	[20]
K12	MOPS	MOPS containing 0.4% glucose	9	0.15 mg/ml	Theoretical calculation	2012	[21]
K12	Kornberg	1.1% K ₂ HPO ₄ , 0.85% KH ₂ PO ₄ , 0.6% yeast extract, 50 mM glucose, 1 mM MgCl ₂	N/A	N/A	Iodine vapor staining	2010	[22]
K12	Kornberg	1.1% K ₂ HPO ₄ , 0.85% KH ₂ PO ₄ , 0.6% yeast extract, 250 μ M Mg ²⁺ , 50 mM glucose	10	180 nmol/mg	Amyloglucosidase, hexokinase, and glucose-6-P dehydrogenase-based test kit	2009	[23]
EDL933	LB	LB agar plate containing 2% glucose	N/A	N/A	Iodine vapor staining	2008	[7]
K12	Kornberg	1.1% K ₂ HPO ₄ , 0.85% KH ₂ PO ₄ , 0.6% yeast extract, 50 mM glucose	N/A	Percentage to WT	Iodine vapor staining	2007	[24]
BW2511 BW1 BW2 DH5 α BL21AI RR1 MS201 IK5	M9	0.4% glucose, 0.4% Casamino acids, 0.1 mM CaCl ₂ , 0.2% MgSO ₄ ·7H ₂ O, 0.6% Na ₂ HPO ₄ , 0.3% KH ₂ PO ₄ , 0.5% NaCl, 0.1% NH ₄ Cl	12	Varied	Iodine vapor staining GOPOD Assay	2005	[10]
BL21(DE3)	M9	N/A	N/A	N/A	N/A	2004	[25]
B	LB	LB medium containing 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 3 g/l KH ₂ PO ₄ , 1g/l K ₂ HPO ₄ , and 0.2% glucose	N/A	N/A	Alpha-amylase and glucose measurement	1994	[26]
K12	Kornberg	Kornberg medium containing 0.5% glucose	N/A	N/A	Iodine vapor staining	1993	[27]
K12	Enriched media	N/A	N/A	N/A	N/A	1981	[28]

*Growth rates mean time points where exponential phase ends.

tuberculosis, on the other hand, stores both wax ester and glycogen as reserves [2]. As for when glycogen is accumulated, several bacterial species such as *Streptococcus mitis* and *Sulfolobus solfataricus* store glycogen at the unusual exponential phase [12]. Although most bacteria grow well in broths like LB (Lysogeny broth, Sigma) and TSB (Tryptic soy broth, BD), *etc.*, they require specific types of media for sufficient glycogen accumulation [13, 14]. So far, manipulation of glycogen structure at genome level has been widely studied in *E. coli* [15]. Meanwhile, *E. coli* DH5 α (Thermo Fisher Scientific) has been confirmed to be efficiently competent for plasmid transformation with high insertion stability [16]. Thus, we use *E. coli* DH5 α as a model organism for glycogen studies.

Glycogen study requires specialized medium for sufficient glycogen accumulation. We searched previously reported media used in glycogen study and found out that no consistency exists (Table 1). A variety of media, including LB, Kornberg, MOPS, and M9 are used and the supplements have varied between studies. The initial purpose of our study was to investigate how N-terminus of glycogen branching enzyme (GBE) influences glycogen structure. Thus, we constructed a set of *E. coli* DH5 α mutants with progressive truncation of GBE N-terminus, which were *E. coli* DH5 α *glgBA*90, *E. coli* DH5 α *glgBA*180, *E. coli* DH5 α *glgBA*270, and *E. coli* DH5 α *glgBA*369. A complete GBE gene knockout strain was also constructed and termed *E. coli* DH5 α Δ *glgB*. Using LB broth to culture these strains resulted in no glycogen being detected, which made our following study infeasible. Thus, it was necessary to enhance bacterial glycogen storage abilities. 1 \times M9 minimal salts medium supplemented with 0.8% glucose was then used, which was also a failure due to the slow growth rate of *E. coli* DH5 α in the medium. Thus, we then tried to optimize the tryptone to glucose ratio in order to balance growth rate and glycogen accumulation.

In summary, this study provides a clear picture about how nitrogen and carbon sources in M9 minimal salts medium influence *E. coli* DH5 α growth and glycogen accumulation. The optimized 1 \times M9 minimal salts medium (supplemented with 0.4% tryptone and 0.8% glucose) has proved to be an effective source for facilitating the structural and functional characterization of glycogen in *E. coli* DH5 α .

Materials and Methods

Bacterial strains, plasmids, cultures, and growth conditions

Bacterial strains used in this study include *E. coli* BL21 (DE3), *E. coli* DH5 α , *E. coli* RR1 and *E. coli* IK5. λ -Red homologous recombination system was used for constructing mutants in *E. coli* DH5 α , which consists of three plasmids, pKD4, pKD46, and pCP20. Lysogeny broth (Bacto™ tryptone, Bacto™ yeast extract, and NaCl) and M9 minimal salts (Sigma-Aldrich, USA) were used for culturing bacterial strains. Trace elements consisting of EDTA (1%), ZnSO₄ (0.029%), MnCl₂ (0.198%), CoCl₂ (0.254%), CuCl₂ (0.0134%), and CaCl₂ (0.147%) were added to all M9 minimal salts media. All bacteria were cultured at 37°C at 220 rpm shaking rate for growth rate measurement and glycogen accumulation, except where otherwise specified.

Mutant constructions

Five mutants of *E. coli* DH5 α expressing progressively deleted GBEs in the chromosomal position of *glgB* were constructed, which were *E. coli* DH5 α *glgBA*90, *E. coli* DH5 α *glgBA*180, *E. coli* DH5 α *glgBA*270, *E. coli* DH5 α *glgBA*369, and *E. coli* DH5 α Δ *glgB*. λ -Red recombination system (Plasmids pKD4, pKD46, and pCP20) by Datsenko and Wanner [35] was generously provided by Dr. Harry Sakellaris. Plasmid pKD46 was first transformed into *E. coli* DH5 α . Then, six primers H1P1, P2H2, P2H3, P2H4, P2H5, P2H6 with 36 nucleotides in 5' and 3' regions that correspond to homologous regions in *glgB* were used to amplify linear PCR products from plasmid pKD4. All the five linear PCR products had the length of 1.5 kb and contained a kanamycin resistance gene flanked by FRT sites. These linear PCR products were electroporated into competent *E. coli* DH5 α cells carrying pKD46. Recombination catalysed between the FRT sites and the *glgB* locus by the λ -Red recombinase resulted in the replacement of the wild type *glgB* chromosomal locus with the deleted variants. For details, please refer to Wang *et al.* [15].

Optimization of M9 minimal salt media

Sterilized 20% tryptone solution was used to supplement 1 \times M9 minimal salts medium (0.8% glucose). By adjusting tryptone/glucose ratio (T/G), we made up six

types of $1 \times M9$ minimal media, which were $1 \times M9$ minimal media with T/G ratio as 0 (0% T), 0.0125 (0.01% T), 0.0625 (0.05% T), 0.125 (0.1% T), 0.5 (0.4% T), and 1.0 (0.8% T). These media were tested for *E. coli* DH5 α and its five mutants in terms of growth rates. Glycogen accumulation amount in *E. coli* DH5 α were also measured when cultured at different $1 \times M9$ minimal salts media for 22 h.

Measurement of *E. coli* DH5 α growth curves

A single colony was picked from a selective LB agar plate and cultured in 5 ml LB broth at 37°C overnight with 220 rpm shaking rate. 250 μ l of the LB broth was used to inoculate 25 ml liquid media (LB broth and $1 \times M9$ minimal medium T/G = 1:2, etc.) in a 125 ml sterile flask, which was cultured at 37°C overnight with 220 rpm shaking rate. At selected time points, the culture was taken out of the flask aseptically and aliquoted into a 96-well microtiter plate. Then, an OD₆₀₀ value was measured and recorded by using a spectrophotometer. OD₆₀₀ readings were drawn by correlating with corresponding time points to draw growth curves.

Protein quantification

In order to quantify the protein amount in samples, a standard protein concentration curve was constructed using 0.25 mg/ml BSA (mix 125 μ l 2% BSA with 875 μ l dH₂O) and Coomassie Plus (Bradford, Australia). All sample solutions were vortexed vigorously to mix the solutions well with Coomassie assay. The protein absorbance of standards and samples was measured with a spectrophotometer at 595 nm wavelength at the same time. A standard curve was constructed based on OD₅₉₅ readings for standards, which was then used to calculate protein concentrations in samples.

Quantification of glycogen content

10 ml of bacterial culture was centrifuged at 5,000 \times g, room temperature for 10 min. Then the cell pellet was resuspended in 0.05 M Triethanolamine (TEA) buffer, which was centrifuged again at 5,000 \times g, room temperature for 10 min. The pellet was resuspended in 500 μ l sodium acetate buffer followed by adding 10 μ l 0.1 M Pefabloc (protease inhibitor). The suspension was sonicated for 10 sec on ice and rested on ice for 30 sec with 25% amplitude. The sonication procedure was repeated

for two more times. 100 μ l of the sonicated crude extract was aliquoted to a fresh Eppendorf tube with 10 μ l amyloglucosidase (200 unit/ml). The tube was incubated at 50°C for 30 min. A blank control was always kept along with the test tube. No amyloglucosidase was added into the blank control tube. After incubation, tubes were centrifuged at 14,000 \times g for 5 min and supernatants were transferred to fresh Eppendorf tubes. 1 ml of glucose oxidase/peroxidase (GOPOD, Australia) was added to each of the tubes, which were incubated at 50°C for 20 min. Then, the reaction mixture was transferred to a 96-well plate with 150 μ l solution/well and the absorbance at 510 nm wavelength (OD₅₁₀) was recorded.

Computational analysis

All data obtained from experiments were analysed statistically. Excel and R packages were used for pair-wise Student's *t*-test and graph illustrations. Statistical significance was defined as *p*-value less than 0.05.

Results and Discussion

Most of the studies have focused on how glycogen metabolism-related genes influence *E. coli* growth rates and glycogen accumulation, rather than the impacts of culture medium [17, 18, 22]. Varik *et al.* noticed the importance of culture composition and studied how amino acid composition could change *E. coli* growth rate and glycogen accumulation [18]. During our study of glycogen structure manipulation and its impact on bacterial viability, we noticed that sufficient glycogen accumulation through bacterial culture is an essential factor. Thus, we initiated the culture optimization work.

At the beginning, the bacterium was cultured in LB broth at 37°C with 220 rpm shaking rate, which turned out to be a failure due to the trace amount of glycogen stored in bacterial cells. This could be caused by abundant nutrients available in the broth that inhibit glycogen storage. We then tested the commercial M9 minimal salts (Sigma) for glycogen accumulation based on literature reports [5, 29]. The main ingredients of M9 minimal salts include Na₂HPO₄, KH₂PO₄, NaCl and NH₄Cl. However, *E. coli* DH5 α grows extremely slowly when following the manufacturer's instructions, even after supplementing 0.8% glycogen as a carbon source. The reason for such a low growth rate might be due to ammo-

nium chloride being the single nitrogen source in the medium [30, 31], which means that all essential amino acids have to be synthesized from scratch. Thus, we provided the organic nitrogen source tryptone (T), that includes free amino acids, to gradually adjust $1 \times M9$ minimal salts medium while glucose (G) concentration was fixed at 0.8%. By doing this, we may achieve a balance between bacterial cell density and glycogen storage since low nitrogen source will limit the growth of *E. coli*. 1 ml of trace elements was also added into the medium for each litre in order to ensure normal growth of *E. coli* DH5 α .

Growth curves of *E. coli* DH5 α were measured in triplicate for each of the six $1 \times M9$ minimal media. Results are shown as an average for each medium in Fig. 1. Improvement of growth rates was observed through increased T/G ratios. Higher T/G ratio led to a reduced lag and exponential phase while stationary phase was remarkably extended. For $1 \times M9$ minimal salts medium (T/G = 0), *E. coli* DH5 α grows slowly. For $1 \times M9$ minimal media (T/G = 1:80, 1:16, and 1:8), *E. coli* DH5 α grows faster and reaches higher OD₆₀₀ values at 22 h. When T/G ratio is 1:2, the bacterium grows fastest, although the highest OD₆₀₀ value that *E. coli* DH5 α can reach drops a little bit to around 1.0. For T/G = 1:1, after *E. coli* DH5 α enters into stationary phase, cells clustered together to form aggregates, which makes OD₆₀₀ measurement inaccurate. Thus, after 12 h, there is no OD₆₀₀

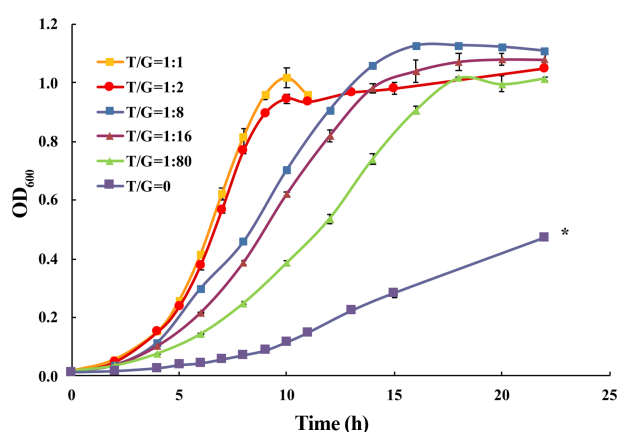


Fig. 1. Growth curves of *E. coli* DH5 α in $1 \times M9$ minimal salt medium with different T/G ratios. According to the result, the higher the T/G ratio is, the faster the bacteria grow. For each time point, three replicates were used for calculating the average. Significant difference was observed between T/G = 0 and T/G = 1:2 through two-tailed student-*t* test (*, *p*-value < 0.05).

reading in this medium (T/G = 1:1). The observed bacterial clustering might be caused by bacterial auto-aggregation because of chemotaxis induced by over-nutrition [32]. However, specific mechanisms for the clustering require further investigation and are beyond the scope of this study. No similar phenomena were observed for other media. Thus, $1 \times M9$ minimal medium (T/G = 1:1) was not considered for further experiments and $1 \times M9$ minimal medium (T/G = 1:2) is considered to be optimal for enhancing bacterial growth.

It had previously been established *in vitro* that N-terminal truncation of glycogen branching enzyme (GlgB) has an impact on glycogen chain length distribution [33, 34]. Thus, we constructed four *E. coli* DH5 α mutants (*E. coli* DH5 α *glgB* Δ 90, *glgB* Δ 180, *glgB* Δ 270, *glgB* Δ 369) with *in situ* progressive truncation of N-terminus of GlgB and a *glgB*-deficient strain *E. coli* DH5 α Δ *glgB* for glycogen structure manipulation [15]. Growth consistency for all *E. coli* DH5 α strains in M9

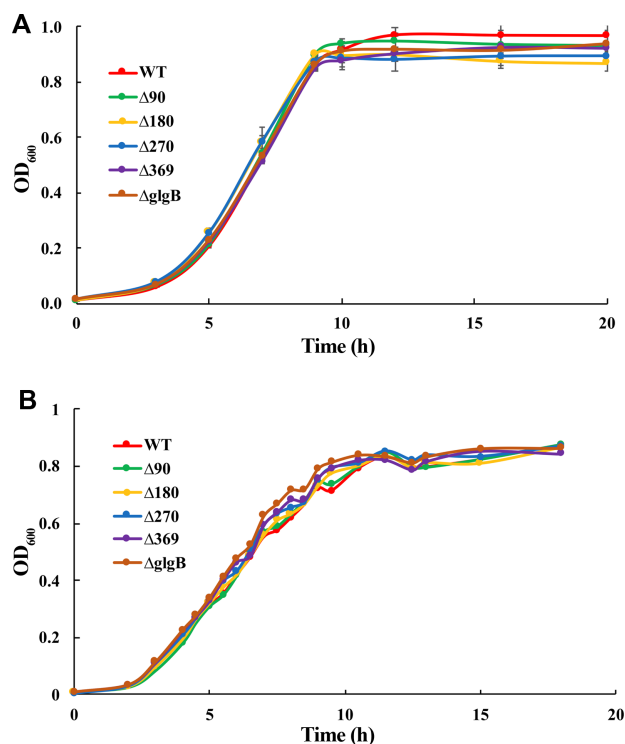


Fig. 2. Growth curves of *E. coli* DH5 α strains in (A) $1 \times M9$ minimal salt medium (T/G = 1:2) and (B) LB broth. For $1 \times M9$ minimal salts medium, three replicates were performed. Average value with standard error bar was presented. For LB growth curve, four repeats of a single culture for each strain were performed and average value was presented.

minimal salt medium (T/G = 1:2) is essential for glycogen analysis. In addition, growth rates of *E. coli* DH5 α strains in 1 \times M9 minimal salts medium (T/G = 1:2) should be comparable to these in LB broth. Thus, we compared the growth rates of all *E. coli* DH5 α in the two media. The results showed that optimized 1 \times M9 minimal salt medium (T/G = 1:2) is sufficient for all strains to grow quickly and consistently and no significant differences were observed between the two media in terms of growth rates (Fig. 2). *E. coli* RR1 and IK5 were also used for growth rate measurement and there was no significant difference observed (unpublished data). Since the current study for glycogen structure is mainly restricted by small accumulation amount, we also examined the glycogen level in *E. coli* DH5 α at 22 h in LB broth and five 1 \times M9 minimal salts media for glycogen storage optimization. Glycogen contents in bacterial strains were assayed by following the protocol specified by Wang *et al.* [15]. The ratio of glucose/protein (G/P) was used to represent the amount of glycogen accumulated inside *E. coli* DH5 α cells. LB broth showed no detectable glycogen accumulation. Fig. 3 shows that *E. coli* DH5 α in 1 \times M9 minimal salts medium (T/G = 1:2) had the highest amount of G/P ratio.

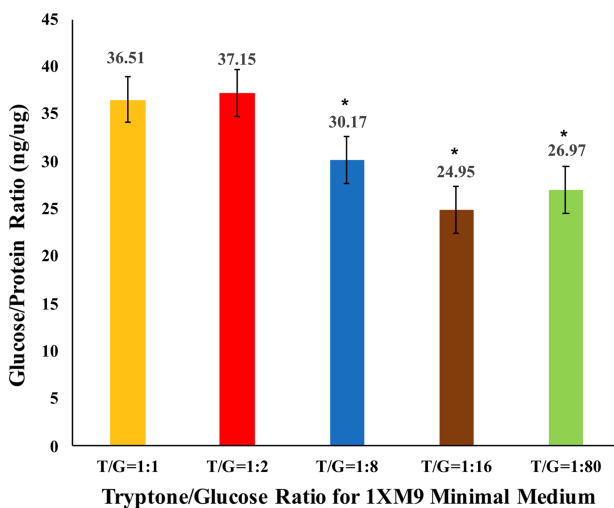


Fig. 3. Glycogen accumulation abilities of *E. coli* DH5 α cultured in five types of 1 \times M9 minimal salt media with different T/G ratio at 22 h. Three replicates were used for each medium. Glucose/Protein ratio (G/P) was used as an equivalent unit of glycogen concentration accumulated in *E. coli* DH5 α cells. Significant differences were observed based on two-tailed student-*t* test for T/G = 1:8, T/G = 1:16, and T/G = 1:80 when they were compared with T/G = 1:2, respectively (*, *p*-value < 0.05).

In sum, *E. coli* DH5 α grows quickly with comparatively short exponential phase, extended stationary phase, and high level of glycogen accumulation amount in 1 \times M9 minimal salt medium (T/G = 1:2). Thus, 1 \times M9 minimal salt medium (T/G = 1:2) is optimal to culture *E. coli* DH5 α for glycogen structure characterization and physiological function analyses.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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