Study of Ribulose 1, 5-Bisphosphate Carboxylase from S*ulfobacillus acidophilus* Strain NY-1 Isolated from Lignite Mines

Jenny Angel Stanislaus[1*] and Dhandapani Ramamoorthy[2]

[1]*Department of Biochemistry, Holy Cross College, Tiruchirappalli, Tamilnadu, India*
[2]*Department of Microbiology, Periyar University, Salem, Tamilnadu, India*

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*** Corresponding author:**
E-mail: jennyangelbiochem @hcctrichy.ac.in

ABSTRACT

One of the key compounds engaged in the carbon dioxide fixation cycle (Calvin-Benson-Bassham cycle) is Ribulose 1, 5-Bisphosphate Carboxylase/ Oxygenase (RuBisCo). These are known to act as a carbon sink thus leading to decrease of the carbon level in the atmosphere. This unique property of RuBisCo can therefore help in diminishing an Earth-wide global warming problem, a noteworthy risk in the present world. In the present study, presence of RuBisCo in *Sulfobacillus acidophilus* strain NY-1 was studied. This strain was isolated from Neyveli lignite mines and their growth parameters such as pH and temperature wereoptimized. The ideal pH and temperature for *S. acidophilus* was at pH 1.7 and at 45°C respectively. The correlation of growth of *S. acidophilus* with light, carbon dioxide and aeration was investigated by enumerating the number of cells/mL using a hemocytometer. The cell count was highest in light condition whereas no growth was observed in dark condition. At the 60th hour of incubation, a cell density of 1.60×10^6 cells/mL was observed. Similarly, in the presence of carbon dioxide the maximum cell count was 2.72×10^6 at the 40th hour of incubation with aeration. The presence of RuBisCo in *S. acidophilus* was affirmed by ion exchange chromatography technique.

1. INTRODUCTION

In the carbon cycle, the predominant part of terrestrial carbon is stored in soil, whereas only a minor part is seen in the vegetation. During the process of biomass formation and decomposition sequestration of carbon dioxide (greenhouse gas) occurs. As a result, soil organic carbon may influence and counteract global warming. Atmospheric carbon dioxide is absorbed by the enzyme RuBisCo which is one of the prominent enzymes in the Calvin-Benson-Bassham (CBB) cycle. This cycle includes three main enzymes: Ribulose Bisphosphate Carboxylase/Oxygenase, sedoheptulose-bisphosphatase and phosphoribulokinase (Sage et al., 2008). The Ribulose 1, 5-Bisphosphate Carboxylase/ Oxygenase (RuBisCo) is the most significant enzyme among the three enzymes as it acts as a carbon dioxide sink. Hence, it plays an imperative part in decreasing the carbon dioxide level which in turn will reduce global warming.

The RuBisCo plays two main roles depending on the availability of carbon dioxide/oxygen sources. One function is known as the Carboxylase activity. In this reaction, the addition of molecular carbondioxide to Ribulose 1, 5-bisphosphate (RuBP) forms two molecules of 3-phosphoglyceate (3-PGA) catalyzed by RuBisCo. This provides an opportunity for conversion of atmospheric inorganic carbon to organic carbon in our biosphere (Meenakshi and Srisudha, 2012; Ke-Quing et al., 2014). Another important function is the Oxygenase process which is considered as a competitive negative side reaction. The addition of oxygen to RuBP, forms one molecule of 3-PGA and one molecule of 2-phosphoglycolate (Ken et al., 2001; Tominaga et al., 2020). Subsequently enhancing RuBisCo's carbon-dioxide-fixing capability will thus lead to increase in carbondioxide entrapment (Fischer and Edmeades, 2010; Erb and Zarzycki, 2018).

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A wide variety of RuBisCo forms are reported in both prokaryotes and eukaryotes. RuBisCo are classified into two types-Form I and Form II-based on amino acid sequences. Form I is further classified into two groups, the Green-like group and the Red-like group. The examples for green-like include proteobacteria, cyanobacteria, green algae and plants. Red-like examples include eukaryotic non-green algae and bacteria such as *A. eutrophus* (Delwiche and Palmer, 1996; Watson and Tabita, 1997). The three-dimensional structures of RuBisCo from microorganisms such as *Sulfobacillus* (Caldwell et al., 2007); *Synechococcus* sp. strain PCC 6301 (Newman and Gutteridge, 1994); *Chromatiumvinosum* (Nakagawa et al., 1986); *Rhodospirillum rubrum* (Schneider et al., 1990); *Galdieria partita* (Shibata et al., 1996); and plants like spinach (Andersson et al., 1989) and tobacco (Chapman et al., 1988) have been investigated in detail. The complete key observations of this enzyme from the year 1947 up to 2006 was presented by Portis and Parry (2007) and will be helpful in utilization of this enzyme in a holistic way. Caldwell et al. (2007) also studied the enzymes involved in CBB cycle in *Sulfobacillus* and the genes encoding RuBisCo.

In 2007, the Intergovernmental Panel on Climate Change (a United Nations Body) published a report in which it stated that the average global atmospheric temperature is rapidly increasing. This is mainly due to greenhouse gases such as carbondioxide whose level has increased since the start of the industrial revolution. Much advancement in technology has been implemented to capture the atmospheric carbondioxide as in the case of the carbon capture and storage (CCS) system. Using this system it is possible to trap 90% of existing carbon dioxide emission underground, but environmentalists fear that entrapping could lead to earthquakes in the future (Liben et al., 2012).

Research on another aspect of trapping carbon dioxide using RuBisCo was also reported as this enzyme plays a key role in photosynthesis. This is one of the most abundant enzymes present in nature but the main limitation is that it is heat labile and inefficient. Ways have been considered in improving the efficiency of this enzyme so that it can absorb more atmospheric carbon dioxide. In this present study, an attempt was made to detect the presence of RuBisCo protein from mining bacteria, as it has been proven that such bacteria can produce heat stable proteins. Therefore, the assay of RuBisCo was assessed by ion exchange chromatography from *Sulfobacillus* sp. under optimized condition. This study may help in further manipulation of bacterial RuBisCo for effective utilization to reduce the carbon dioxide level in the atmosphere which is a significant factor in global warming.

2. METHODOLOGY

2.1 Culture conditions

Most of the acidophilic bacteria cannot be cultivated in ordinary culture media as the agar used as solidifying agent in media becomes liquefied at lower pH. Therefore, in the present work *S. acidophilus* strain NY-1 was cultivated in liquid medium (ferrous sulfate tryptone soy broth) at pH 3.0 at 37ºC for 5 days. The culture was inoculated at 5 day intervals thrice in fresh medium for adaptation in new environment. This culture was maintained as pre-cultured strain and used for further studies.

2.2 Optimization of growth parameters

In order to study the effect of various growth parameters on the growth of the organism as well to optimize the parameters the culture was grown in liquid media. Enumeration of *S. acidophilus* can be done by cell count method and spectrophotometric method, as it is difficult to cultivate in solid media.

2.2.1 Effect of temperature and pH

To ferrous sulfate tryptone soy broth, 2% (v/v) of pre-cultured strain was inoculated. It was incubated at different temperatures of 37, 45, 55 and 65ºC and shaken at 150 rpm for 5 days (Watling et al., 2008). For every 24 h, a cell count using a Neubauer chamber was performed. To study the effect of initial pH, the medium pH was adjusted with 2.0 N sulphuric acid to pH 1.7, 2.2, and 3.0 respectively and shaken at 150 rpm at 45ºC for 5 days. Every 24 h for 5 days, a cell count estimation was done. Simultaneously the turbidity was measured at 700 nm in a Systronic double beam spectrophotometer.

2.2.2 Effect of light and dark condition

The pre-cultured strain 2% (v/v) was inoculated in 100 mL ferrous sulfate tryptone soy broth and incubated under light in a shaker for 150 rpm at 45ºC for 5 days. At regular intervals the culture was subjected to cell count using a Neubauer chamber. Likewise another set of inoculated medium were grown under dark conditions and similarly the culture

was subjected to cell count by using a Neubauer chamber for 5 days (Wedel and Soll, 1998).

2.2.3 Presence and absence of carbon dioxide

The pre-cultured strain 2% (v/v) was inoculated in 100 mL ferrous sulfate tryptone soy broth supplemented with 2% sodium bicarbonate for carbon utilization as a substitute for atmospheric CO_2 and another without carbon dioxide (Maryshamya et al., 2019). The medium was incubated at 45°C for 5 days and the cultures were subjected to cell count. Aeration was provided to the culture media through a sterile tube which was connected to a motor. Air bubbles were seen to arise from the bottom of the liquid media thus providing aeration.

2.3 Determination of cell count by hemocytometer

A uniform cell suspension of 10 µL was mixed with 10 µL of 0.4 % trypan blue solution (fresh and filtered) in phosphate buffered saline. It was gently vortexed and allowed to stand for 10 min. The preparation was then observed in hemocytometer under 100X magnification. The cells that take up the stain and those that do not take were counted and expressed in terms of percentage of cell viability

$$\% \text{ Cell viability} = (\text{Total viable cells (Unstained)} / \text{Total cells (Viable + Dead)}) \times 100$$

2.4 Protein characterization

Partial purification of protein was carried out by ammonium sulfate precipitation method. The supernatant was obtained by centrifugation at 5,000 rpm for 20 min to which 80% ammonium sulfate was added and kept overnight (O'Donnelly et al., 2014). It was again centrifuged at 13,000 rpm for 20 min and the pellet was suspended in 20 mM Tris-HCL buffer (pH 7.0). The pellet was later subjected to dialysis overnight at 4°C with dialysis membrane-150 (cut off MW 5,000 to 10,000).

2.4.1 Ion exchange chromatography

The dialysate was subjected to ion exchange chromatography using DEAE cellulose column. The DEAE cellulose column was thoroughly washed with equilibrated buffer (10 mM Tris-H_2SO_4, pH-7.4). 1.0 gm of DEAE cellulose was mixed with 10 mL of 0.05 M equilibrated buffer and added to the column of 8.0 cm height. The column was packed tightly and allowed to settle well. The column was washed with equilibrated buffer once before loading the sample (Robinson et al., 1988).Care should be taken that the column remains wet throughout the experiment. One mL of the dialyzed sample was loaded and the column was washed with 10 mL of linear gradient of 0.2 to 0.5 M $(NH_4)_2SO_4$ salt (equilibrium buffer). Fractions of 3 mL of eluent were collected in a series of test tubes. The optical density of the collected eluent was measured at OD_{280} nm using Cystronics double beam spectrophotometer.

3. RESULTS AND DISCUSSION

There are many reports on RuBisCo enzymes availability in plants and it has been extensively studied. The role of this enzyme in lower form of organisms like algae, cyanobacteria and proteobacteria are also studied recently. But only a few reports are available for gram positive bacteria. Isolation of RuBisCo from Gram positive bacteria (*Sulfobacillus acidophilus*) from harsh environments will further help in manipulating this enzyme for various applications. RuBisCo is the key enzyme of the Calvin-Benson-Bassham (CBB) cycle which may be the principal route of carbon-fixing pathway for acidophiles. In the present investigation *S. acidophilus* strain NY-1 was isolated from soil in Neyveli Lignite mines (data not shown). This soil is rich in lignite fragments, pyrite, and total carbon content and secondary minerals such as gypsum. The top layers are usually of neutral pH but the subsoil remains acidic (pH 2.5-2.9). This may be due to oxidation of pyrite, soil leaching and minerals precipitation. All the above factors contribute to the isolation of *Sulfobacillus* sp. in such mining sites.

3.1 Optimization of growth parameters

Standardization of growth parameters is very essential for all microorganisms as these conditions may lead to maximum growth.

3.1.1 Effect of temperature and pH

The most predominant factors such as temperature and pH optima for the growth of *S. acidophilus* strain NY-1 were studied. The effect of temperature was studied and it was observed that the bacterium was able to tolerate temperatures up to 65°C, but maximum growth was observed at 45°C (Figure. 1). Most acidophilic organisms grow well between 28-62°C, the strain *S. acidophilus* is therefore considered as a moderately thermophilic acidophile. There are many reports on the thermophilic nature of *Sulfobacillus* sp. such as *S. thermosulfidooxidans*

which was able grow in batch culture at 45°C (Kaneda, 1991), *Sulfobacillus* sp. strain VKMB at 50°C (Tsaplina et al., 1991), *S. acidophilus* at 48°C (Norris et al., 1996), *S. thermosulfidooxidans* DSM 9293 at 45°C and *S. thermosulfidooxidans* VKM B-1269 at 50-55°C (Golovacheva and Karavaiko, 1978).

The effect of acidic pH such as 1.7, 2.2, and 3.0 was also studied and maximum growth was observed at pH 1.7 (Figure 2). The optimum pH for *S. thermosulfidooxidans* was observed between pH 1.9-2.4 as reported by Golovacheva and Karavaiko (1978). A similar report was described previously by Norris et al. (1996) about the presence of RuBisCo in *S. acidophilus* that were grown autotrophically at 45ºC on ferrous iron (50 mM) at pH 1.6. The acidic pH and high temperature tolerance seen in this strain contribute to the prevention of bacterial contamination when grown at an industrial scale.

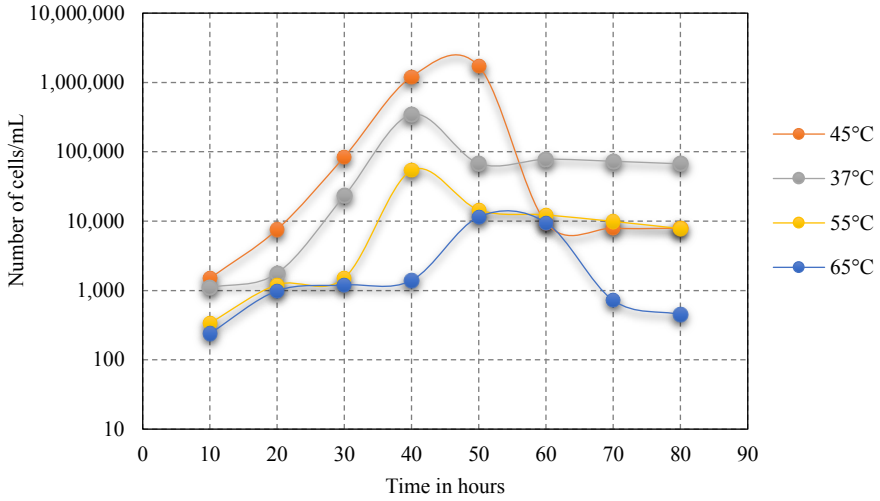

Figure 1. Growth of *Sulfobacillus acidophilus* strain NY-1in varying temperature

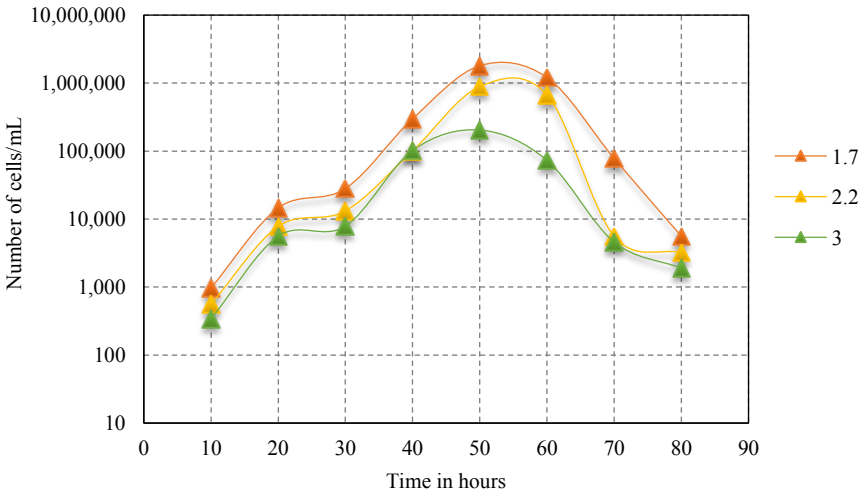

Figure 2. Growth of *Sulfobacillus acidophilus* strain NY-1 in varying pH of 1.7, 2.2, and 3.0

3.1.2 Effect of light and dark condition

The effect of light and dark condition on the growth of the strain was also studied. The maximum cell density was observed as 1.60×10^6 at the 60th hour of incubation for the strain NY-1 (Table 1). No growth was observed in dark condition which indicates that autotrophs require light for its growth. Light is also essential for RuBisCo activity as it was higher in light conditions than in dark conditions as reported by Wedel and Soll (1998). In contrast, in the absence of light *Ralstonia eutropha* was able to grow with CO_2 as its carbon source by utilizing the energy from H_2 oxidation.

3.1.3 Presence and absence of carbon dioxide

Environments where the oxygen concentration keeps changing are mainly dominated by chemolithotrophic sulphur and iron oxidizing bacteria. In acidic media enriched with metal ions, the solubility

of oxygen significantly reduces with increasing temperature. Therefore, the influence of carbon dioxide along with aeration was studied. An increase in the cell number was observed in the presence of carbon dioxide and the deposition of insoluble precipitates also increased. Comparative studies of the data showed that the cell density was higher, reaching a maximum of 2.72×10^6 cells/mL, in the presence of carbon dioxide than in its absence.

Table 1. Cell count enumeration in different conditions

Isolate	Growth rate at different conditions (number of cells/mL)	20 h	40 h	60 h	80 h	100 h	Growth rate at dark Condition
NY-1	Light condition	1.45×10^4	1.71×10^5	1.60×10^6	1.21×10^6	1.67×10^3	Nil-Absence of cells
	Presence of carbon dioxide	1.35×10^5	2.72×10^6	1.33×10^6	1.21×10^6	1.89×10^4	
	Absence of carbon dioxide	7.60×10^3	6.50×10^4	1.70×10^6	9.91×10^4	7.80×10^3	

3.2 Determination of cell count

One of main limitations in solid media cultivation of acidophiles such as *Sulfobacillus* sp. is that it cannot be cultivated in agar medium. Therefore, assessment of cell growth can be elucidated by cell count and spectrophotometric method. The FeSYE media is the most preferred media as it contains potassium tetrathionate and ferrous sulphate which areutilized as energy sources. The iron sulphur precipitate formation due to acidic pH was also reduced due to the buffering effect of this medium. In the spectrometric method, there is a possibility of enumerating both viable and non-viable cells. Moreover, the precipitate formed may also interfere with the results. In the case of cell counts, this limitation can be overcome by differentiating between viable and non-viable cells. Therefore, in the present study, cell count enumeration was also performed, even though it is a tedious process, by using the microscopic technique-Neubauer cell counter. (Table 2) In the microscopic method, trypan staining was carried out to differentiate viable cells from non-viable cells. Viable cells do not take up the stain and thus appear colourless, whereas dead cells take up the stain and appear blue in colour.

Table 2. Cell count determination by hemocytometeric and spectrophotometeric method

Sample name	Cell count by hemocytometer	Optical density at 478 nm	Cell viability (%)
NY-1	2.80×10^6	1.816	75.3

3.3 Assay of RuBisCo by ion exchange chromatography

After partial purification by dialysis overnight at 4°C the sample was loaded in Ion exchange chromatography column. This method was followed as it is a simple and cheaper method used to separate RuBisCo from the sample (Robinson et al., 1988). The equilibrium buffer $(NH_4)_2SO_4$ are generally used in ion exchange chromatography as it is capable of removing the endogenous inhibitors. In this chromatographic technique large volume protein samples can be used compared to other methods. Hence in the present study ion exchange chromatography was followed using ammonium sulphate buffer after dialysis of the crude extract.

The fractions were collected at regular time interval and absorbance was read at 280 nm. About 4 peaks were observed at 5th, 9th, 12th and 14th fraction. The curve obtained from ion exchange chromatography was compared with the RuBisCo obtained from spinach leaves. Out of these a highest peak was seen at the 9th fraction which was found to be RuBisCo on comparison with the standard. This was eluted at about 0.1 M of $(NH_4)_2SO_4$ with an A_{280} of 0.154 (Figure 3). The ratio of the absorbance at 280 nm and 260 nm was found to be 2.40 confirming the presence of proteins.

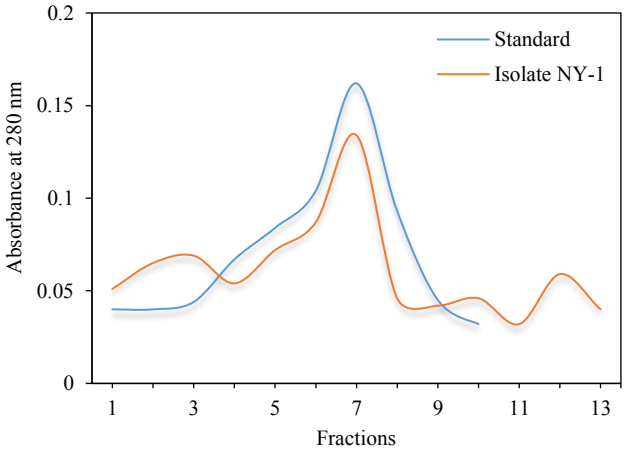

Figure 3. Ion exchange chromatogram of standard RuBisCo obtained from spinach and from the *Sulfobacillus acidophilus* strain NY-1

4. CONCLUSION

Due to global warming, the atmospheric temperature is constantly increasing, which is contributed by the rapid increase in carbon dioxide level. Scientists have been working to limit the increase in global temperature by not only diminishing the amount of carbon released into the environment, but also by disposing it. In this aspect, new findings such as carbon capture and sequestration have been followed. Carbon capture is a simple method in which the carbon emissions generated by many industries are trapped underground. The major drawback of this method is the chance of leakage in carbon pipes that may cause lethal damage to the environment. Carbon sequestration in biological systems is therefore becoming a suitable alternative. RuBisCo is an effective enzyme that sequesters carbon dioxide, but has low efficiency and is a heat labile protein. This protein is to a great degree a slow catalyst whose activity is mainly dependent on temperature. Therefore, in the present study, RuBisCo was extracted from the wild strain *Sulfobacillus acidophilus* NY-1 which has heat stable properties. Only a few reports on the presence of RuBisCo in gram positive acidophiles are available, hence this study may establish a framework for future studies to improve RuBisCo carbon sequestering efficiency.

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