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GROWTH KINETICS OF *Bacillus subtilis* IN LIGNOCELLULOSIC CARBON SOURCES

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Abstract- In this study, the growth kinetics of *Bacillus subtilis* in M9 minimal medium containing lignocellulosic components such as cellulose, lignin and D-xylose as a sole carbon source were determined. Cell growth in glucose, sucrose and starch were also studied. Studies were conducted in a 2.0 liter fermenter. The specific growth rates and maximum specific growth rates of *B. subtilis* in 0.1 % of the carbon sources mentioned were determined. The substrate utilization constant of *B. subtilis* was determined using a wide range of concentrations. The change in pO_2, acid and alkali additions during the growth in the fermenter was recorded. The study revealed that *B. subtilis* was grown in all of the carbon sources tested. The specific growth rate (h^{-1}), maximum specific growth rate (h^{-1}) and substrate utilization constant (mg/L) of *B. subtilis* in minimal medium containing glucose, sucrose, starch, cellulose, xylose and lignin were (0.481, 0.2, 1.3), (0.334, 0.1, 39.58), (0.074, 0.094, 67.32), (0.046, 0.049, 500), (0.077, 0.136, 132.2) and (0.034, 0.07,660) respectively. In all the carbon sources except cellulose, a decrease in pH was observed. The study concludes that *B. subtilis* can be efficiently grown using minimal medium containing lignocellulosic substrates as a sole carbon source.

Keywords- *Bacillus subtilis*, Growth, Lignocellulose, Minimal medium

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Introduction

In any fermentation process, the substrate plays vital role in the process yield and efficiency. One of the important factors which decide the commercial viability of the fermentation process is the substrate used [1]. Hence, availability of cost-effective substrates for producing biofuels and food products by fermentation is important [2]. Lignocellulosic substrates from plant materials are composed of lignin, cellulose and hemicelluloses. Annually, in excess of 1 billion tons of plant wastes are generated world-wide. These agro-wastes have potential as fuel, food, feed, fine chemicals and manure [3,4]. Cellulose is a polysaccharide of glucose units. Hemicellulose polysaccharide is built of five carbon sugars such as xylose. Though the composition between species varies, lignins are heteropolymers containing phenyl propane rings and many phenolic side chains. In plant systems, hemicellulose confers mechanical strength as a cementing agent between cellulose and lignin and serves to conduct water [5,6].

Microorganisms which are involved in biodegradation of lignocellulosic wastes are fungi, followed by actinomycetes and bacteria. The complex structure of lignocelluloses is degraded to metabolically useful monomers [7-9]. Fungi which are involved in lignocellulosic biodegradation include *Trichoderma* sp., *Ganoderma*, *Pleurotus* sp. and *Phanaerochaete chrysosporium. Streptomyces* sp., are a large genera of actinomycetes capable of biodegradation processes. Many soil bacteria such as *Bacillus* sp., *Pseudomonas* sp. and *Actinobacteria* sp. also exploit lignocellulose biodegradation [10,11]. Biodegradation of lignocelluloses occurs by extracellular enzymes. There are two types of extracellular enzymatic systems: (1) hydrolytic systems which produce hydrolases responsible for cellulose and hemicellulose degradation and (2) lignolytic systems which oxidatively degrade lignin [12].

Endoglucanases produced by microorganisms produces cellulosic chains with modified ends. These modified chains are substrates for cellobiohydrolases (CBH), otherwise called exo-β 1,4 gluconases. Hydrolysis results in cellobiose. β- glucosidase then catalyzes the hydrolysis of cellobiose, producing glucose which is then utilized by microbes [13]. Aerobic bacteria which utilize cellulose as a carbon source are species from the genera *Cellulomonas*, *Pseudomonas* and *Streptomyces*. Additionally, five to ten percent of cellulose is degraded in nature under anaerobic conditions; *Clostridium thermocellum* being an example [14,15].

Microbes can also biodegrade hemicelluloses to monomeric sugars and acetic acid. Xylan is main carbohydrate found in hemicelluloses. Degradation of xylan by endo-β-1,4-xylanase results in xylan oligosaccharides which is subsequently converted into the pentose xylose by β-1,4-xylosidase. Heat stable xylanases have been de-

scribed in Actinobacteria such as *Thermomonospora* and *Actinomadura* [16]. Xylanases active at alkaline pH have been isolated from *Bacillus* sp. and *Streptomyces viridosporus*. Xylose can be fermented into ethanol and xylitol (sweetner). A recent breakthrough is the development of improved strains of fermentative microorganisms capable of fermenting pentose and hexose sugars into ethanol [17,18].

The white rot fungi, *Phanerochaete chrysosporium,* is the most common and extensively studied organism for lignin degradation. Lignolytic enzymes grouped into peroxidises and laccases. Lignin peroxidases (LiPs) catalyze the oxidation of phenols, amines, aromatics and ethers. Manganese dependent peroxidases (MnPs) oxidize Mn(II) to Mn(III) which oxidizes phenolic compounds [19,20]. Laccases have been isolated from many fungi including *Aspergillus* and the thermophilic fungi *Myceliophora thermophila* and *Chaetomium thermophilum* [21].

Bacillus subtilis has been recognized as a generally safe agent for industrial enzyme production [22]. Numerous studies have indicated that certain strains of *Bacillus* sp. and *Bacillus subtilis* can be cultured in lignocellulosic substrates [23,24]. Understanding the growth characteristics of microorganisms in lignocellulosic substrates will help us to exploit microorganisms for bioconversion of abundant lignocellulosic substrates into useful products. The present study was undertaken to examine the growth kinetics of *Bacillus subtilis* in minimal medium containing different carbon sources including cellulose, lignin and xylose.

Materials and Methods

Microorganism and Culture Conditions

Bacillus subtilis was obtained from Carolina Biological supply company, Burlington, NC - 27215, USA. The cultures were grown in nutrient broth at 30ºC under shaking conditions. The culture was stored in nutrient agar slants at 4ºC.

Fermenter

A 2-liter Biostat A⁺Bioreactor (Sartorius-stedim) was used at a working volume of 1-liter. The fermenter was equipped for maintaining oxygen, pH and temperature of the media. The cell density was monitored online using an NIR density probe (Optek Danulat Inc., USA). The oxygen probe was calibrated for air by a standard sodium sulphite method. The pH probe was calibrated using standard buffers at pH 4.0 and 7.0. The fermenter was equipped with software for maintaining the fermentation conditions and recording data.

Minimal Medium

The composition of M9 medium was as follows. Mineral Salts (5X):Na_2HPO_4 - 6.4g; KH_2PO_4 – 1.5 g; NaCl – 0.25 g; NH_4Cl – 0.5 g; Deionized water (DI) – 100 mL. 1M; $MgSO_4$: 24.7 g of $MgSO_4$ was dissolved in 100 mL of DI; 1M $CaCl_2$: 1.0g in 100 mL in D.W. To prepare 100 mL of working solution, twenty ml of 5X mineral salts, 200µl of 1M $MgSO_4$ and 10µl of 1M $CaCl_2$ was added and made up to 100 ml. The individual carbon source was prepared for stock solution (10%). To make 0.1% individual carbon source, 1ml of stock solution was added in 100 mL medium.

Growth of *Bacillus subtilis* on Different Carbon Sources

Bacillus subtilis was streaked in minimal agar medium (M9) containing 0.1% of different carbon sources: glucose, sucrose, starch, cellulose, lignin and xylose. Glucose, sucrose, D-xylose and cellulose were purchased from Carolina Biological Supply Company, Burlington, NC, USA. Soluble starch was purchased from Fischer Scientific and lignin (dealkaline) from TCI (Tokyo Chemical Industry), Tokyo, Japan. The inoculated plates were kept at 30ºC for one week and observed for growth.

Specific Growth Rate of *B. subtilis* in Minimal Medium (M9) Containing Different Carbon Sources

Bacillus subtilis was grown in 1L M9 minimal medium broth containing different carbon sources: glucose, sucrose, starch, cellulose, lignin and xylose at 0.1% concentration in the bioreactor. The pH and dissolved oxygen concentration were maintained at 7.0 and 20% respectively. The cell density was measured and recorded over the growth period. The specific growth rate (µ) and generation time was determined by plotting time (h) versus cell density (CU) using the formula (1) and (2) [25]:

Specific growth rate (h^{-1}), $\mu = \ln (X_1 - X_0)/ (t_1 - t_0)$ (1)

X_1 and X_0 = CU at times t_1 and t_0 respectively.

Generation time (h) = 0.693/ µ (2)

Substrate Kinetics of *B. subtilis* in Minimal Medium (M9) Containing Different Carbon Sources

Kinetics experiments were conducted using shake flasks cultures. *B. subtilis* was grown in 100 ml of M9 minimal medium containing the described carbon sources. The concentrations were: glucose (2, 5, 10, 15, 20, 50, 100 and 150 mg/L); sucrose (5, 10, 25, 50 and 100 mg/L); xylose (100, 150, 200 and 250 mg/L); starch (100, 150 and 200 mg/L); cellulose (300, 400, 500 and 600 mg/L) and lignin (400, 500, 600, 700, 800 and 900 mg/L). All flasks were maintained at 30°C and 150 rpm. Changes in pH and oxygen were not monitored. Samples were aseptically taken at one- hour intervals and the cell densities were measured at 600 nm using spectrophotometer. The specific growth rate for all the carbon sources was calculated. The substrate utilization constant and maximum specific growth rate was calculated using equation (3) [25]:

$$\mu = -K_s (\mu/s) + \mu_{max} \quad (3)$$

where K_s(substrate utilization constant (mg/l)) and μ_{max} (maximum specific growth rate (h^{-1})

Results and Discussion

Specific Growth of *B. subtilis* in Minimal Medium Containing Different Carbon Sources

The growth characteristics of *B. subtilis* in M9 minimal agar medium containing different carbon sources were observed [Fig-1]. For all carbon sources tested, *B. subtilis* formed dry colonies with mat-like appearance, except in sucrose where it formed mucous colonies. The biomass of *B. subtilis* was found highest in sucrose and xylose (2mg/L). In other carbon sources, biomass was measured as 1 mg/L [Table-1].

A growth rate and generation time for each carbon source is depicted in [Table-1]. Pamphilis & Hanson [26] reported the maximum growth rate of *Bacillus subtilis* SB 168 under tryptophan and ammonium sulphate limitation to be 0.76 h^{-1}. The specific growth rate of *B.subtilis* grown in M9 medium containing glucose 0.1% was calculated as 0.481 h^{-1}. The Generation time was calculated to be 1.44 (h) [Table-1]. During the growth of *B. subtilis* in 0.1% glucose, base addition in the fermenter was observed which indicates metabolic

acid production. The pO_2 declined after 5 hrs. of growth during log phase [Fig-2](a). In a similar study, the maximum growth (dry cell mass) of *B. subtilis* KO strain ranged from 0.608 to 0.780 g/100 mL in molasses broth medium supplemented with 3%, w/v gelatin [27].

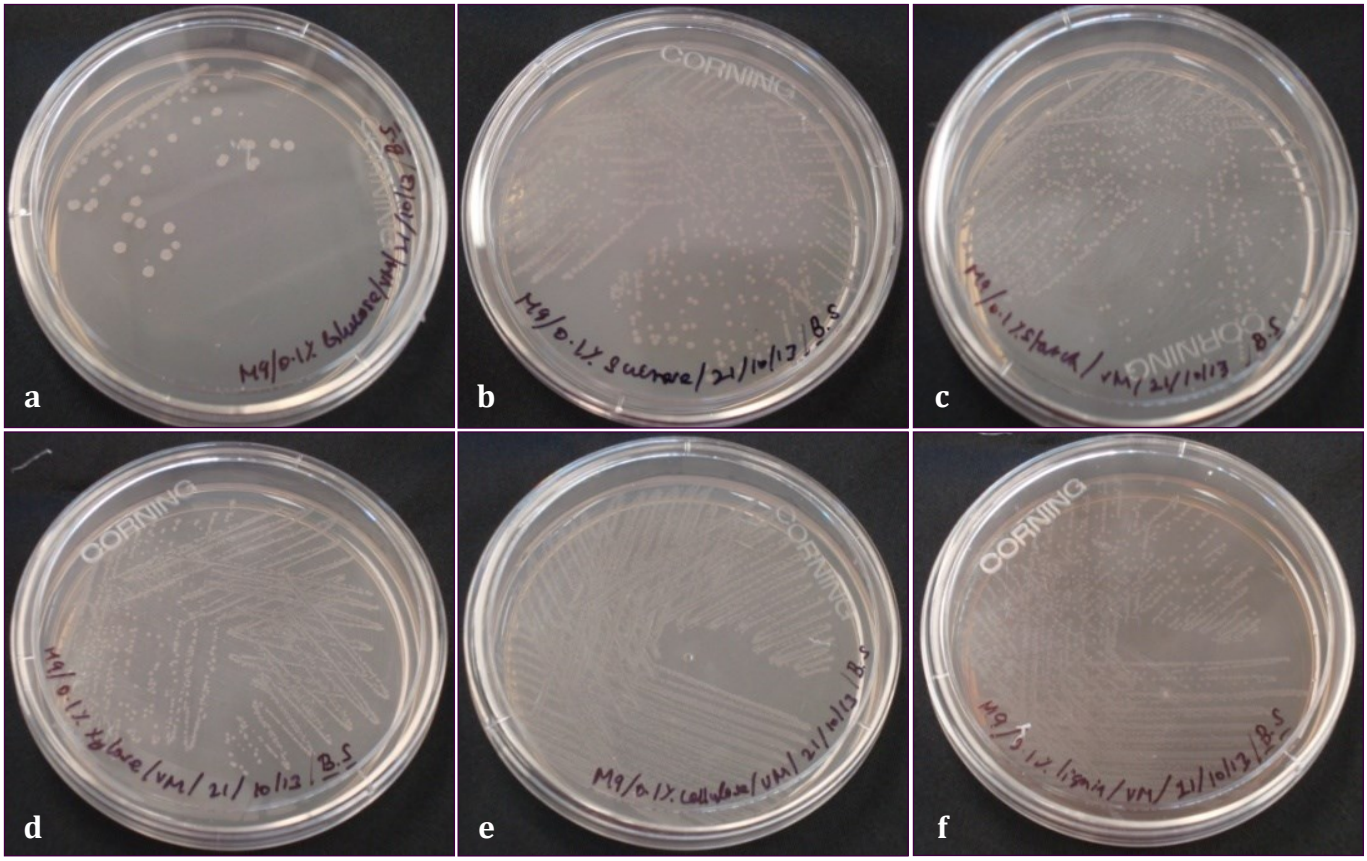

Fig. 1- Growth of *B. subtilis* in M9 minimal medium containing different carbon sources, **(a)** Growth in 0.1% glucose; **(b)** Growth in 0.1% sucrose; **(c)** Growth in 0.1% starch **(d)** Growth in 0.1% xylose; **(e)** Growth in 0.1% cellulose; **(f)** Growth in 0.1% lignin.

Table 1- Specific growth rate of *B. subtilis* in minimal medium (M9) containing different carbon sources @ 0.1%

Carbon source	Specific growth rate (h^{-1})	Generation time (h)	Biomass yield (mg/L)
Glucose	0.481	1.44	1
Sucrose	0.334	2.07	2
Starch	0.074	9.36	1
Cellulose	0.046	15.07	1
Xylose	0.077	9	2
Lignin	0.034	20.38	1

In our experiment, mucous formation in sucrose-containing medium was observed in both agar medium and the bioreactor. High levels of levan and exo-polymeric substances of *B. subtilis* have been observed in sucrose-containing medium [28]. The specific growth rate and generation time of *B. subtilis* in 0.1% sucrose were determined as 0.334 $h^{-1}$and 2.07 hrs. respectively [Table-1]. In sucrose, similar to the glucose experiment, there was base addition to the fermenter after 10 hrs. of growth. The pO_2 level decreased drastically during the log phase of growth after 10 hrs. [Fig-2](b). Takahashi, in a similar study, has demonstrated that *B. subtilis* produced 40-50 mg of levan mL^{-1} in medium containing 20% (w/w) sucrose [29].

In starch (0.1%) M9 medium, the specific growth rate and generation time of *B. subtilis* was measured as 0.094 h^{-1} and 7.37 (h) respectively [Table-1]. It was observed that *B. subtilis* grown in starch also produced acid during the fermentation process. The pO_2 level drastically decreased from 150% to 50% during the log phase of the growth [Fig-2](c). Amylase production by *B. subtilis* has been shown to be high in medium containing starches of barley, corn and maltose [30].

The specific growth rate and generation time of *B. subtilis* in M9 minimal medium containing 0.1% of cellulose was determined to be 0.046 (h^{-1}) and 15.07 (h) respectively [Table-1]. In cellulose (0.1%) M9 medium, after 40h, production of alkaline metabolites was indicated by auto-acid addition to the fermenter. Similarly after 40 hrs. of log growth, the pO_2 decreased [Fig-2](d). Shaheb, et al [31] reported maximum cellulase productivity of *B. subtilis* KO strain when molasses broth medium was supplemented with cellulose. Madhusudhan, et al [32] reported that among different substrates viz., soil, starch, rice juice, potato juice and arrow root powder, arrow root powder solution (0.5%) showed maximum growth of *Bacillus laterosporus.* Lignocellulosic wastes including raw palm kernel cake, defatted palm kernel cake and vegetable wastes have highest cellulase activity when inoculated with *Bacillus* sp. [33].

The specific growth rate and generation time of *B. subtilis* in D-xylose (0.1%) M9 medium was measured to be 0.077 (h^{-1}) and 9.0 (h) respectively [Table-1]. Similar to the other substrates, the pO_2 decreased during the log phase [Fig-2](e). Other studies have shown that a *Bacillus subtilis* mutant grew well on xylose [34]. A recombinant strain of *S. cerevisiae* TMB 3130 has been used to

ferment xylose to produce ethanol and arabitol under anaerobic conditions [35]. Similarly high *B. subtilis* xylanase activity was demonstrated in fermenting rice straw, wheat straw, wheat bran and kraft pulp [36].

The specific growth rate and generation time of *B. subtilis* in lignin (0.1%) M9 medium was observed as 0.034 (h^{-1}) and 20.38 (h) respectively [Table-1]. The auto-addition of base indicates the production of acidic products during fermentation. The pO_2 level fell after 2 hrs. incubation of growth [Fig-2](f). Others have demonstrated maximum lignin degradation by *Bacillus* sp. (EU978470) at pH 6.0 [23].

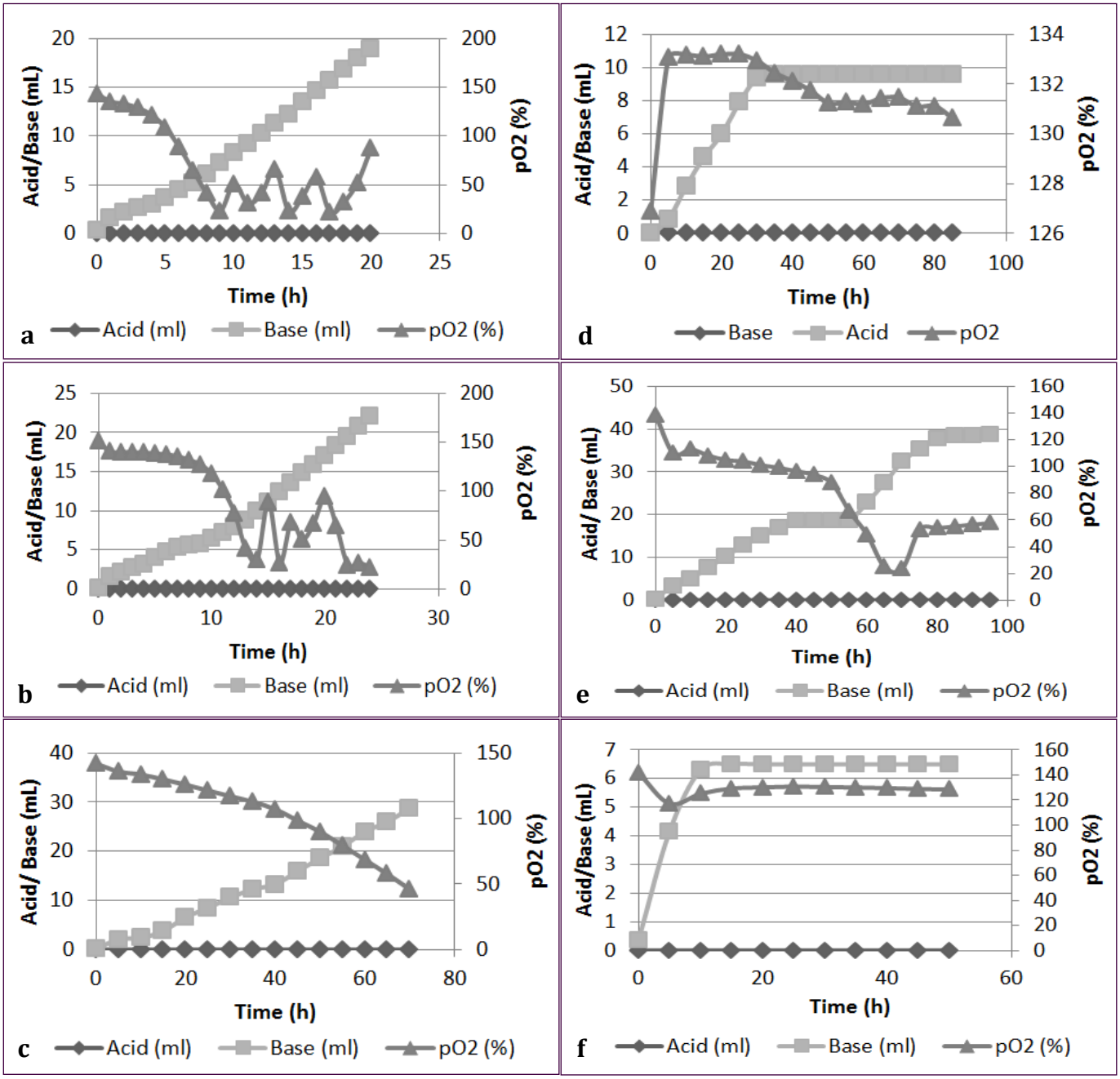

Fig. 2- Effect of growth of *B. subtilis* in different carbon sources on acid and base additions and pO_2 level, **(a)** 0.1% Glucose; **(b)** 0.1% Sucrose; **(c)** 0.1 % Starch; **(d)** 0.1 % Cellulose; **(e)** 0.1 % Xylose; **(f)** 0.1 % Lignin

Substrate Kinetics of *B. subtilis* in Minimal Medium (M9) Containing Different Carbon Sources

The K_s and μ_{max} of *B. subtilis* in minimal medium (M9) containing different carbon sources are presented in [Table-2]. The K_s and μ_{max} of *B. subtilis* for glucose are 1.3 (mg/L) and 0.2 (h^{-1}) respectively. The K_s of glucose is low compared to the other carbon sources tested in this study. Glucose is the preferred substrate. The K_s and μ_{max} of *B. subtilis* for sucrose are 39.58 (mg/L) and 0.987 (h^{-1}). The K_s and μ_{max} of starch are 67.32 (mg/L) and 0.074 (h^{-1}). The K_s and μ_{max} of *B. subtilis* for xylose are 133.2 (mg/L) and 0.136 (h^{-1}). The K_s and μ_{max} of *B. subtilis* for cellulose are 500 (mg/L) and 0.049 (h^{-1}). The K_s and μ_{max} of *B. subtilis* for lignin are 660 (mg/L) and 0.07(h^{-1}). In a similar study, aerobic biodegradation of 43 (mg/L) toluene by batch culture of *Pseudomonas putida* F1 at 30ºC showed K_s value of 13.8 ± 0.9 (mg/L), μ_{max} of 0.8 ± 0.01 (h^{-1}) and yield coefficient of 1.28 ± 0.13 (g/g). The same culture on aerobic degradation of phe-

nol in batch process showed K_s of 32.0 ± 2.4 (mg/L); μ_{max} of 0.11 ± 0.01 (h^{-1}) and yield coefficient of 0.80 ± 0.07 (g/g) [37]. Further explanations of microbial growth kinetics are described [38].

Table 2- Substrate utilization constant (Ks) and maximum specific growth rate (µmax) of *B. subtilis* in different carbon sources

Carbon source	Ks (Substrate utilization constant) (mg/L)	µ max (maximum specific growth rate) (h^{-1})
Glucose	1.3	0.2
Sucrose	39.58	0.1
Starch	67.32	0.094
Cellulose	500	0.049
Xylose	132.2	0.136
Lignin	660	0.07

Conclusions

In this study, the growth kinetics of *B. subtilis* in lignocellulosic carbon sources was revealed. It was observed that *B. subtilis* could grow in all of the carbon sources tested demonstrating the versatile nature of this organism. The results revealed *Bacillus subtilis* showed preference for carbon sources in the order of glucose > sucrose> starch> xylose> cellulose> lignin. This trait may be exploited for growing *B. subtilis* in medium containing ligno-cellulosic feed stocks for the production of industrially important products.

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Conflict of Interest: The authors confirm that there are no conflicts of interests or personal financial interests associated with the outcome of this work.

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