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Production, Purification and Characterization of Cellulase from *Bacillus subtilis* Isolated from Soil

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Abstract
Cellulose is a long chain polymer, made up of repeating units of glucose, a simple sugar, joined together with β-1, 4glycosidic linkages. Cellulases cause hydrolysis of the individual cellulose fibers to break it into smaller sugars units & finally producing glucose molecules. The purpose of the current investigation was to screen Bacillus species isolated from soil in order to study the isolation, purification and characterization of exo-cellular cellulase enzyme. The soil samples were obtained (10gm) from Bhaktapur. Bacterial colonies were grown over CMC-Agar medium [1]. Maximum cellulase production was obtained after 24-48 hrs of incubation at 37 °C in medium containing 1% carboxymethyl cellulose (CMC) as substrate. The optimum pH for the enzyme was found to be ranging between 6.5 and 7.5 at which it was found to be most stable. Bacteriological studies indicated *Bacillus subtilis* to be the most frequent cellulolytic bacteria to be found in the agricultural fields.

Keywords: Cellulose, Cellulases, CMC-Agar, *Bacillus Species*

1. Introduction
Cellulose are highly stable polymer consisting of β-1, 4-linked glycosyl residues, along with other polysaccharides [2]. The biodegradation of cellulose has been studied for many years, and a number of cellulolytic enzymes, especially, cellulases produced by fungi and bacteria, have been isolated and characterized [3].
These enzymes belonging to glycosyl hydrolases, cleave the β-1,4 bond of cellulose. Based on the mechanism of cellulose degradation, cellulases are subdivided into either non-processive cellulases (endocellulases) or processive cellulases(including different exocellulases and some new processive cellulases) [4, 5]. Endocellulases can randomly cleave cellulose at exposed positions and producing non-reducing ends , however, the processive cellulases remain attached to the chain and release mainly cellobiose or cellotetraose units from one end of the chain [6]. During the degradation of cellulose, non processive cellulases and processive cellulases have been found to
Cellulase is commonly used in many industrial applications, especially in animal feed, textile, waste water treatment, brewing and wine-making. [12, 13] Production of bio-ethanol from lignocellulosic biomass [14] has been increasing worldwide, with the increasing shortage of petroleum fuels, increase of Greenhouse gases and air pollution due to incomplete combustion of fossil fuels.
Moreover, Cellulase is used for commercial food processing in coffee. It performs hydrolysis of cellulose during drying of beans. Furthermore, cellulases are widely used in textile industry and in laundry detergents. They have also been used in the pulp and paper industry for various purposes, and they are even used for pharmaceutical applications. Cellulase is used in the fermentation of biomass into biofuels, although this process is relatively experimental at present. Cellulase is used as a treatment for phytobezoars, a form of cellulose bezoars found in the human stomach. Bacteria which have high growth rate as compared to fungi have good potential to be used in cellulase production. However, the application of bacteria in producing cellulase is not widely used. Among the bacteria, *Bacillus* can produce large numbers of extracellular polysaccharide hydrolyzing enzyme. [25] However these, carboxymethyl cellulase cannot hydrolyze crystalline cellulose [26].
Bacillus subtilis was one of the first bacteria studied by scientists. It was originally named *Vibrio subtilis* in 1835 by Christian Gottfried Ehrenberg and later renamed *Bacillus subtilis* by Ferdinand Cohn in 1872 [18, 19].
Bacillus subtilis produces a natural surfactant and posses the ability to biodegrade

hydrocarbons. *B. subtilis* is a facultative aerobe; spores can survive extreme heat [18, 19]. It stimulates the gene expression of natural defense mechanisms in plants and animals. [20, 21, 22] It suppresses pathogenic microorganisms in soil through competitive inhibition and the production of natural antibiotics [23].

A cellulase producing *Bacillus* strains have been isolated from soil and optimized the culture conditions for enzyme production [27]. In this study, the cellulase was purified and characterized from *Bacillus subtilis*.

2. Materials and Methods

2.1 Sample collection

In this study, *Bacillus subtilis* sample was collected from Microbiology Lab of Tribhuwan University. The sample bacteria were selectively isolated by streak plate technique on NA+CMC agar plate, which was then incubated for 24 hrs at 37 °C. Finally, various Biochemical tests were performed for *Bacillus subtilis*, as explained below:

2.2 Identification of *Bacillus subtilis*

2.2.1 Cultural characterization

The isolates were observed under microscope. The colony morphology was noted with respect to color, shape, size, nature of colony and pigmentation [33].

2.2.2 Microscopic observation

The bacterial isolation was gram stained and observed under a high power magnifying lens in light microscope.

2.2.3 Biochemical characterization

The bacterial isolates were characterized biochemically by

a. Catalase Test:
b. Methyl-Red Test:
c. Vogues-Proskauer Test:
d. Sugar-Fermentation Test:

2.2.4 Cellulase production

After the confirmatory biochemical tests of bacteria, bacteria were used for the production ofcellulase. [37] For this, the bacteria was inoculated into the culture medium containing 2% rice bran, 1% casein, 1% soy tone and 0.1% NaCl. The resulting culture medium was placed over shaking incubator at 37 °C for 36-48 hrs, at about 150 rpm. The medium was then centrifuged at 8000×g for 20 minutes at 4 °C. Pellet was discarded and supernatant was further partially purified for the detail study [37].

2.3 Partial purification of enzyme:

Partial purification of cellulase enzyme was achieved by ammonium sulfate precipitation followed by dialysis.

a. Ammonium sulfate precipitation:

In this process, about 70 ml of supernatant solution was saturated with ammonium sulfate to 80% saturation at 4 °C for 1 hr and the solution was centrifuged at 8000×g at 4 °C for 15 minutes. The pellet thus obtained was dissolved in 4 ml of phosphate buffer pH 8(0.02M) in 1:4 ratios [38].

b. Activation of dialysis tube:

An appropriate length of dialysis tube was cut into fragment and the boiled on 100 ml of 2%(w/v) sodium bicarbonate solution in 1mM EDTA solution of pH 8 for 5 minutes, then, after ringing dialysis tube with d/w, it was again boiled in 1mM EDTA solution of pH 8 for 10 minutes. The dialysis tube was allowed to cool and used for dialysis. Before using dialysis tube was washed with clean D/W and all procedure was handled with gloves. [39]

c. Dialysis of salt precipitated enzyme solution:

The pellet dissolved in buffer solution was now, transferred in a dialysis bag and immersed in phosphate buffer pH 8(0.02M) at 4 °C for about 24hrs with continuous stirring using magnetic stirrer. Buffer was changed during the process in order to obtain proper purified enzyme product. [40]

Thus dialyzed enzyme solution was then, used to study various characteristics of cellulose

2.4 Cellulase Activity Assay

Cellulase activity was determined by:

Test Sample: Incubation of 900µL of 1% CMC in 0.02M phosphate buffer pH 8, with 100µL of dialyzed enzyme at 37 °C. After 30 minutes reaction, 3 mL of DNS was added and placed in boiling water bath for about 5 minutes to stop the reaction, DNS method [41].

Control: Incubation of 900 µL of 1% CMC in 0.02M phosphate buffer pH 8, at 37 °C, while 100 µL of dialyzed enzyme was added after DNS was added. After 30 minutes reaction, 3 mL of DNS was added and 100µL of enzyme was now added and placed in boiling water bath for about 5 minutes to stop the reaction, DNS method [41].

The resulted samples were then cooled to room temperature and measured at the absorbance of 540 nm. One unit of cellulase activity was defined as the amount of enzyme that could hydrolyze CMC and release 1µg of glucose within 1 minute at 37 °C.

2.5 Estimation of protein content

The protein concentration was measured by Bradford method. Working solution of BSA was made by diluting standard BSA solution of 1mg/ml to 0.1mg/ml. 5 test tubes were labeled as:- 200 µL, 400 µL, 600 µL, 800 µL and 1mL of BSA working solution was taken respectively and then the volume was maintained to 1 mL by adding d/w. A blank was set with only distilled water, now all samples were used for Bradford assay. 5 ml of Bradford reagent was added in all test tubes and after 5 minutes of incubation, absorbance was taken with UV-ray spectrophotometer at 595 nm [42].

2.6 Characterization of partially purified cellulase:

Effect of pH on cellulase activity:

The effect of pH on the activity of cellulase was measured by incubating 0.1ml of diluted enzyme and 0.9ml of phosphate buffer ranging from 2 to 9, containing 1% CMC starch. [37]

Effect of temperature on cellulase activity:

The effect of temperature on the enzyme activity was determined by performing the standard assay procedure within a temperature range of 0 °C, 20 °C, 40 °C, 60 °C, and 80 °C [37].

Effect of incubation time on cellulase activity

The effect of incubation time on the enzyme activity was determined by incubating enzyme at different incubation

period ranging from 0 mins, 15 mins, 30 mins, 60 mins and 90 mins [37].

Effect of metal ions on cellulase activity
The effect of metal ions on cellulase activity was studied by preparing 10 mM solution of K^+, Na^+, NH_4^+, Fe^{2+} and Mn^{2+} and using them to study the change in the activity of cellulase [37].

Effect of inhibitors and other reagents on cellulase activity
Inhibitors and other reagents were also used to study their effect on cellulase activity. SDS, EDTA, β-Me and IAA were used as reagents. These reagents were incubated along with enzyme 25 °C and 30 mins [37].

2.7 Effect of different carbon sources
The effect of various carbon sources such as starch, glucose, maltose, lactose, and fructose at the concentration of 1 to 5% was examined in the production medium.

2.8 Effect of different Nitrogen Sources
Various nitrogen sources like yeast extract, peptone, urea, and ammonium sulphate were examined for their effect on enzyme production by replacing 0.5% peptone in the production medium.

3. Results and Discussion

3.1 Identification of the bacterial isolate

Various cultures of *Bacillus* species were isolated, and screened for the production of cellulase in 1% CMC. However, *Bacillus subtilis* was isolated which shows the following physical and biochemical characteristics:

Table 1: Identification of the bacterial isolate

Characters	*Bacillus subtilis*
Shape	Irregular
Size	Large
Color	White
Consistency	Dry
Margin	Filamentous
Elevation	Flats
Gram-Staining	+, rods
Voges-Proskauer test	+
Methyl-Red	-
Catalase	+
Glucose	+
Mannitol	+

From the above observation table, bacteria were found to be Gram positive, MR-negative, VP-positive, Catalase-positive and possessed the ability to ferment sugar. On the basis of this characterization the bacterial isolate has been identified as *Bacillus* species.

3.2 Cellulase activity in different fractions

Cellulase activity in different fraction is shown in figure no. 2. The enzyme activity in crude, precipitated and dialyzed fraction was measured by considering the Glucose Calibration Curve. Dialyzed fraction was found to have highest enzyme activity.

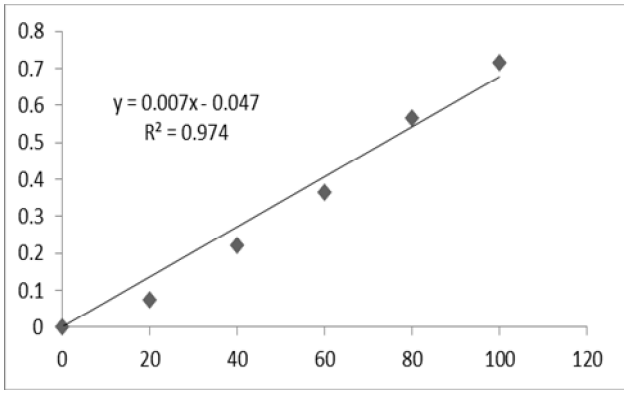

Fig 1: Glucose Calibration Curve

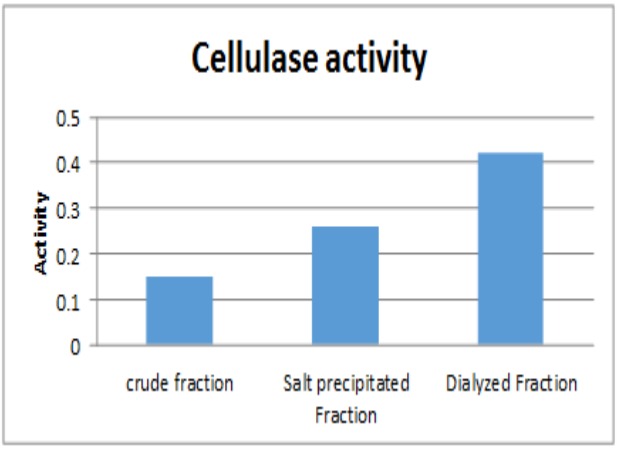

Fig 2: Cellulase activity in different fraction

3.3 Protein concentration in different fraction:

Concentration of protein in crude, precipitated and dialyzed fraction was measured by Bradford method, using Bovine Serum Albumin as a standard and the amount of protein in dialyzed fraction was found to be 2.467 mg/ml, whereas, previously, in case of crude fraction and salt precipitated fraction, it has been noted to be 0.4mg/ml and 4.4 mg/ml respectively. Figure, shows the standard curve of BSA.

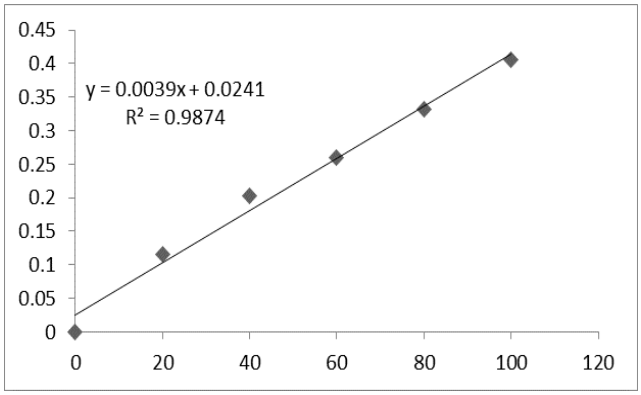

Fig 3: Standard curve for Bradford assay.

Table 3: Protein concentration in crude, salt precipitated and dialyzed fractions of enzyme.

Sample	Enzyme (ml)	Distilled water (ml)	Concentration (mg/ml)
a. Crude fraction	0.010	0.990	0.4
b. Salt precipitated fraction	0.010	0.990	4.4
c. Dialyzed fraction	0.010	0.990	2.467

3.4 Characterization of cellulase enzyme

The activity of cellulase enzyme was calculated by using following equation:

$$\text{Enzyme activity} = \frac{\text{Amount of product formed}}{\text{Molecular weight of CMC} \times \text{Vol. of enzyme (ml)} \times \text{Incubation time (mins.)}}$$

Note: Molecular weight of CMC is 263.19 g/mole.

3.5 Cellulase activity at different pH

The effect of pH on cellulase activity as a function of pH is shown in figure 4. Optimum pH was found to be 6 where the cellulase activity was found to be 1.03µmole/ml/min, however, the activity is just slightly less at pH 7, which indicates that, the cellulase activity is optimum ranging from pH 6-7. This shows that the activity of cellulase is fairly higher in alkaline pH. And, according to previous studies, the optimal pH for the *Bacillus* strain was 5-6.5.

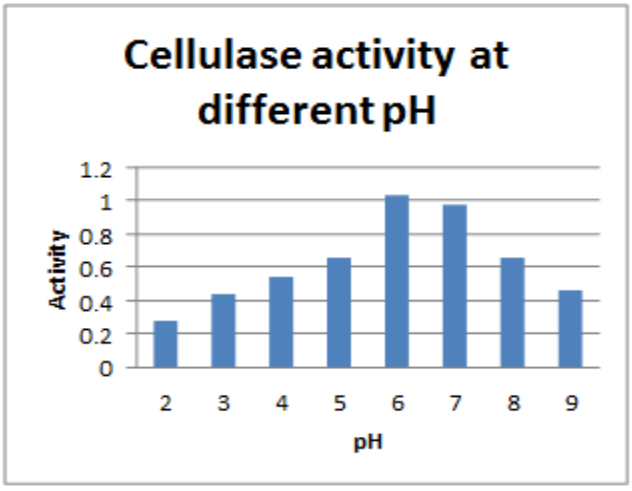

Fig 4: Effect of pH on cellulase activity.

4.6 Cellulase activity at different temperatures

The effect of temperature on cellulase activity has been shown in figure 5. The optimum temperature was found to be 60 °C with an activity of 0.9 µmole/ml/min. The cellulase activity goes increasing with rise in temperature and become maximum at 60 °C and drastically decreases at 80°C. The main reason may be, due to the denaturation of cellulase enzyme with an increase in temperature.

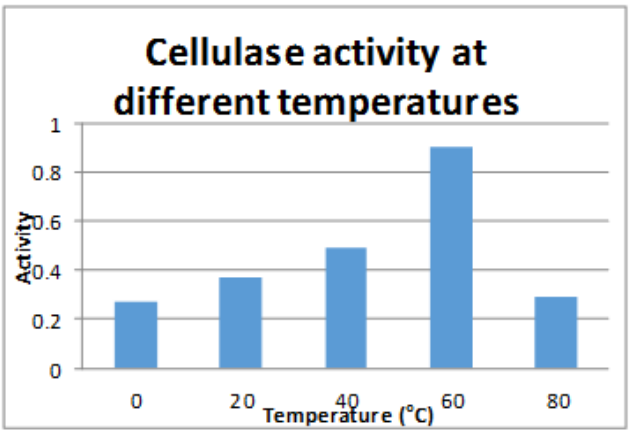

Fig 5: Effect of temperature on cellulase activity

4.7 Cellulase activity at different incubation time:

The effect of incubation time on cellulase activity has been shown in figure it can be observed that the cellulase activity is maximum at 30 minutes incubation time, whereas, with the increase in time of incubation, the activity of enzyme goes on decreasing. The main reason for the decrease in cellulase activity may be due to the denaturation of enzyme in increase in time of incubation.

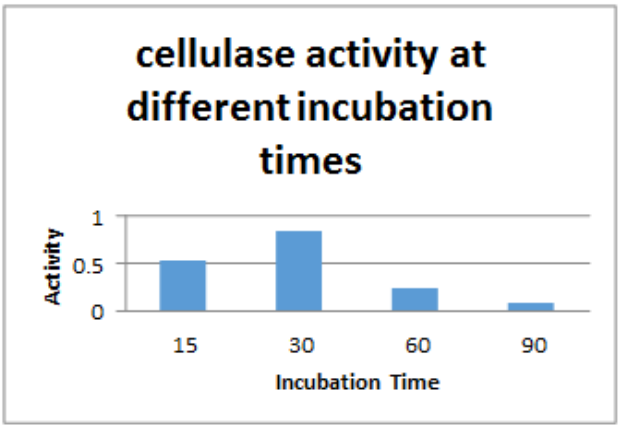

Fig 6: Effect of incubation time on cellulase activity

4.8 Cellulase activity on different metal ions

The effect of different metal ions on the enzyme activity has been shown It shows that, the effect of Mn^{++}leads to higher expression of enzyme activity i.e. greatly activate the purified cellulase, whereas, other metal ions such as K^{+}, Na^{+}, andNH_4^{+}express low enzyme activity, andFe^{++} shows the least enzymatic activity. This result confirmed that the active site of purified cellulase contain –SH group [43].

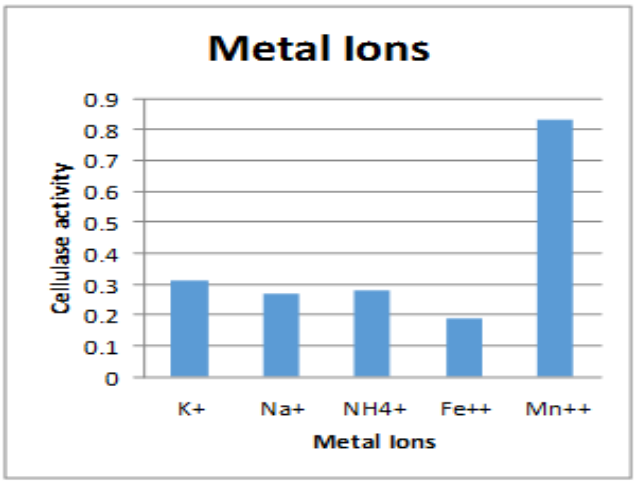

Fig 7: Effect of metal ions on cellulase activity

4.9 Cellulase activity on different Inhibitors and Reagent:
The effect of different inhibitors and reagents are shown It shows that the activity of enzyme is high in case of β-Me with an activity of 0.8 µmole/ml/min, whereas EDTA and IAA shows low enzyme and SDS seems to inhibit enzyme activity to a great extent.
It is because, IAA can bind with –SH group [43] with different degree interaction and subsequently inhibit the activity of enzyme. However, β-Me can reduce the disulfide bonds and renature the enzyme activity.

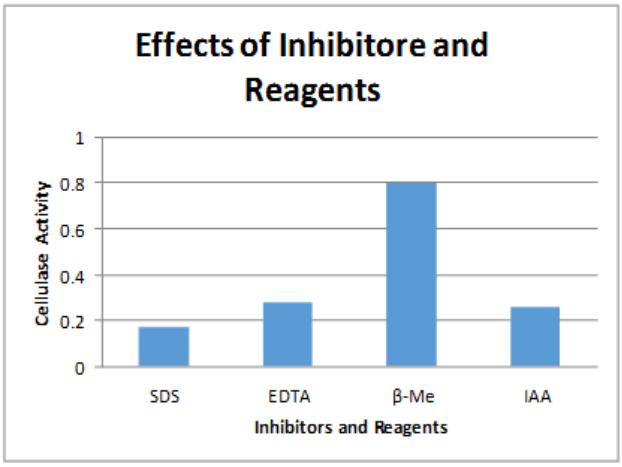

Fig 8: Effect of inhibitors and reagents on cellulase activity.

4.10 Effect of Nitrogen Sources
Production of extracellular cellulase has been shown to be sensitive to repression by different carbohydrate and nitrogen sources. The effect of nitrogen sources was studied in the growth medium, where peptone was replaced by ammonium sulphate, urea, and yeast extract. Among the various nitrogen sources tested, ammonium sulphate was found to be the best nitrogen source for cellulase production (Figure 9). Nitrogen is one of the major cell proteins and stimulation of cellulase activity by ammonium sulphate salt might be due to their direct entry in protein synthesis

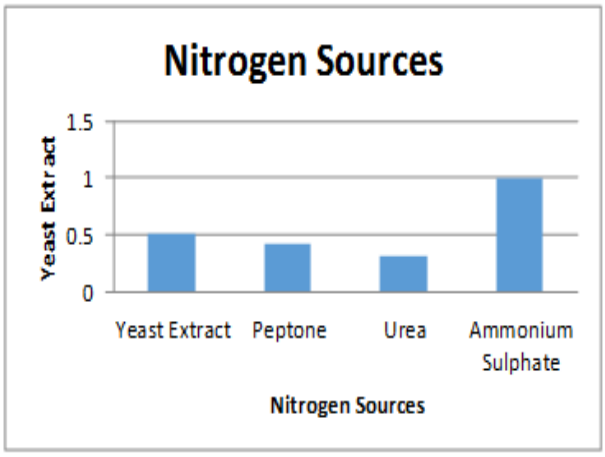

Fig 9: Effects of Nitrogen sources

4.11 **Effects of Various Sources of Carbon**
Various sources of carbon such as starch, fructose, maltose, and sucrose were used to replace glucose which was the original carbon source in growth media. Results obtained showed that glucose brought the highest cellulase production compared to other carbon sources at 24 h incubation. Lactose and fructose also showed high cellulase production at 24 h of incubation. Hence, glucose was found to be the best source for cellulase production (Figure 9). Glycerol is the best substrate for cellulase production with the efficiency of 28.7% on the added substrate weight.

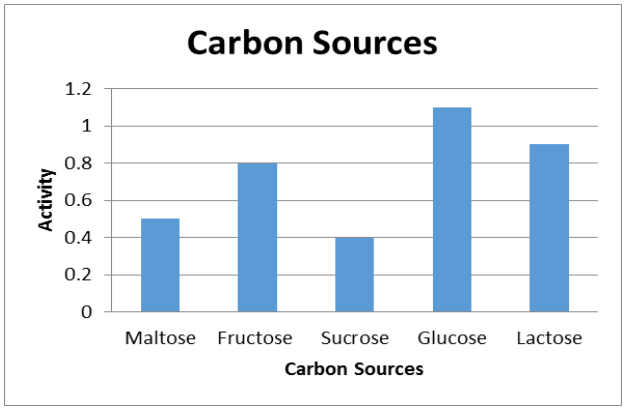

Fig 5: Effect of temperature on cellulase activity

5. Conclusion
Cellulase is the most stable polymer, which is of great significance in both industrial and natural wide applications. Our study was based on the isolation of cellulase isolated from *Bacillus subtilis*, and it was reported that, the enzymatic activity was higher at pH 6 and at the temperature of 60 °C with the activity of 1.03µmole/ml/min and 0.9 µmole/ml/min respectively. Similarly, 30 mins. Incubation times express higher enzyme activity of 0.83 µmole/ml/min and Mn^{++} metal ions and β-Me activated cellulase activity with 0.83 µmole/ml/min and 0.8 µmole/ml/min respectively.
Since my study was fully based on the isolation of cellulase from *Bacillus subtilis,* and it is well known that cellulase has a wide range of applications, its production can be commercialized after optimization for enzyme production, so that its uses can be broadened.

6. Acknowledgements
The authors are sincerely grateful to the Management of Universal Science College, Chakupat, Lalitpur Nepal, for encouragement and support.

7. Results
Isolation of the Microorganisms
A total of 6 isolates were isolated from 12 different sources. These isolates were cultured and maintained on YPD agar medium for further investigation. The isolates were mainly obtained from juice samples and soil.

8. Antimicrobial Activity
The antimicrobial activity for each of the isolate was checked against *B. subtilis, Pseudomonas, E.coli, Klebseilla, Salmonella, Serratia, Staphyloccous, Streptococcus.* Isolate 1 and 5 showed antimicrobial activity against most of the pathogens and had a greater zone of inhibition. *Klebseilla* was inhibited by all the isolates proving that *S. cerevasiea* can be used as of the antimicrobial agent against *klebseilla*.

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List of abbreviations

α	Alpha
+	Positive
-	Negative
℃	Degree Celsius
mg	Milligram
g	Gram
nm	Nanogram
ml	Milliliter
l	Liter
µl	Micro liter
NA	Nutrient Agar
MR	Methyl Red
VP	Voges-Proskauer
%	Percentage
hrs	Hours
mins	Minutes
sec	Second
rpm	Revolution per minute
UV	Ultraviolet
BSA	Bovine Serum Albumin
P.B.	Phosphate Buffer
ppt.	Precipitate
CMC	Carboxy Methyl Cellulose
EDTA	Ethylene Diamine Tetra-Acetate
IAA	Iodo-Acetic Acid
SDS	Sodium Do-decylSulphate
β-Me	β-Mercaptoethanol