Optimizing biofuel production from fruit waste biomass by combination of yeast and cellulose degrading bacteria from vermicompost

Telphy Kuriakose*, Preetha Nair**

Abstract

Ethanol is a potential biofuel that can be produced from comparatively cheaper sources of raw materials such as fruit peels, pulp, molasses etc. Here, we used the peels of fruits such as papaya and pomegranate because of their year-round availability, and their waste accumulation, which needs environmental related attention. The dried fruit peels were subjected to acid hydrolysis, and the total carbohydrate, specifically cellulose content was estimated before and after the pre-treatment. Pre-treatment effectively removes the lignin content which is supposed to be a barrier to the cellulolytic enzymes. Cellulose Degrading Bacteria (CDB) was isolated from the vermicompost and its biochemical characterization was done. A standard CDB strain was obtained from a company. The CDB strains and yeast were inoculated into the pre-treated samples and allowed to ferment for alcohol production by subjecting the samples to different optimization parameters like temperature and pH. The purpose of this study was to compare the effect of CDB alone and the combined effect of CDB and yeast on the fermentation process. The growth kinetics of the CDB was also measured. The ethanol produced was spectroscopically analysed on the 7th, 14th and 21st day of incubation. The result indicated that alcohol can be produced from fruit peels.

Keywords:
Biofuel;
Fruit waste;
Cellulose Degrading Bacteria;
Vermicompost.

Author correspondence:

Telphy Kuriakose,
Research Scholar
Department of Biotechnology,
Bharathiar University,
Coimbatore, Tamilnadu, India- -641046,
Email: telphyk@gmail.com

1. INTRODUCTION

Alternative energy resources are non-traditional sources of energy that have low environmental impact. Ethanol is usually the most common product used to make biofuels and is a form of pseudo-renewable energy source that can be produced from agronomic feedstock [1].

The production of ethanol from comparatively cheaper sources of raw materials through efficient microbial fermentation processes is necessary to address demand for bioethanol in the present situation of energy crisis [2]. Peels are the major by-products obtained from various fruits, but these are mostly discarded as waste which causes real environmental problems. These could be used as potential feedstock for bioethanol production and could therefore be a viable alternate for disposal of these residues. Papaya and pomegranate peels have been chosen for our study due to their year-round availability; the peels are easily removable and are usually discarded by individuals. The cellulose content present in the substrates is an

*Department of Biotechnology, Bharathiar University, Coimbatore, India-641046
** Department of Biotechnology ,Mount Carmel College, No.58, Palace Road, Bengaluru, India-560052

important factor in ethanol production from lignocellulosic biomasses, which can then be hydrolysed by a mixture of enzymes that convert it into glucose [3]. Pre-treatment, whether through physical or chemical means or a combination of both is essential to enable the break-down of the polymers present in the cell walls of peels and convert them into monomers such as glucose, xylose etc. which can then be further fermented to ethanol [4]. If no pre-treatment process is applied before enzymatic hydrolysis the glucose yield cannot be higher than 20% of the maximum glucose yield possible from breakdown of cellulose that it theoretically possible, even under a high level of enzyme concentration or by incubating the reaction for a longer period of time[5]. Therefore, to enhance this yield, lignin containing biomasses need some sort of pre-treatment to alter this association to enhance the access of the enzyme to cellulose for its conversion to glucose monomers. There are several pre-treatment methods which can be used to remove the lignin content. acid pre-treatment, alkaline pre-treatment, etc. [6].

The main purpose of our research efforts is to improve and optimise the bioconversion process, which could create a sustainable method for the production of bioethanol from fruit wastes. This study focuses on the production of bioethanol using the peels of fruits such as papaya and pomegranate as the substrates by subjecting them to acid pre-treatment, enzymatic saccharification by microbial enzymes and further ethanol fermentation of the obtained pre-treated hydrolysates from papaya and pomegranate biomass by the combined action of cellulose degrading bacteria that is isolated from vermicompost, and yeast, to carry out the characterization of the cellulose degrading bacteria, to optimize the growth conditions of the cellulose degrading bacteria and yeast, as well as the bio-fermentation process.

2. RESEARCH METHOD

2.1Isolation and screening:

1 gram of vermicompost was dissolved in 10ml of 0.85% NaCl, and then homogenized for 5 minutes. Serial dilution of the sample (10-1-10-7) with triplicates, were plated on nutrient agar (NA) plates using the spread plate method and incubated at 37°C for 24 hours. The selected colonies isolated from NA plates were transferred to carboxy methyl cellulose (CMC) agar plates and incubated at 37°C for 48-72 hours. Colonies form CMC agar plates were sub-cultured and CMC agar slants were stored at 4°C for further experiments.

After 72 hours of CMC agar plate incubation, the plates were flooded with 1% Congo red for 20 minutes. A clear zone of lysis around the colonies, on washing the plates with 1M NaCl solution indicates the presence of cellulose degrading bacteria. The colonies showing the zone of lysis were selected and used for further studies. [7].

2.2 Cell growth assay:

The culture inoculum of both isolated and standard CDB was prepared at 1:20 ratio in sterile nutrient medium for cell growth assay. The samples were immediately drawn into a cuvette and the absorbance was read at 600nm, which was taken as the zeroth value. Samples were incubated on a shaker incubator at 37°C at 120rpm and absorbance was taken every 25 minutes. The absorbance values so obtained were compiled and plotted on a graph to obtain the growth curve.

2.3 Biochemical characterization:

The bacterial isolates were characterized by means of 16s rRNA sequencing by Eurofins Genomics India Pvt. Ltd.

2.4 Measurement of cellulolytic activity:

Isolated and standard colonies were inoculated in liquid LB medium at 37°C and grown in a shaker incubator at 100 rpm for 24h. Then, the extract then centrifuged at 5000 rpm for 15 min, and the clear supernatant was examined for enzyme assays under optimum reaction conditions for screening of highly cellulolytic bacterial strains.

2.4.1Endoglucanase test:

To 0.2 ml of this supernatant 1.8ml of 0.5% cellulose in 50Mm Sodium Phosphate buffer was added and incubated at 45°C for 30 minutes. The above tube was added with 3 ml Di-nitro Salicylic acid reagent and was kept in boiling water bath for 5 minutes. It was then cooled immediately to room temperature to stop the reaction. The absorbance was measured at 540 nm. The endoglucanase activity was measured as the amount of enzyme required to release the reducing sugar from the substrate in terms of glucose equivalent min/g of the enzyme sample.

Enzyme activity (U/ml) = conc. of glucose × 1000 / 0.18 x 0.2 x 30

2.4.2 Exoglucanase test:

0.5 ml of enzyme supernatant and 1 ml of sodium citrate buffer with a pH of 5.8 along with 1.0 x 6.0 cm

FPU/ml units= mg glucose released x 0.185. [7]

(~ 50mg) of Whatman no.1 filter paper was incubated in a water bath at 50˚C for 1 hour. After incubation 3 ml of Di-nitro Salicylic acid was added and kept in boiling water bath for 5-10 minutes. The optical densities were measured at 540nm. Exoglucanase unit was calculated in terms of filter paper units using the formula,

2.5 Optimization of cellulase production:

2.5.1Effect of Temperature:

To determine the effect of temperature on cellulase production, nutrient broth was inoculated with the respective CDB strain, and incubated at 35°C and 45°C (24 hours at 100rpm), which was found to be the average optimum temperature and Q10 temperature of the strains respectively.

2.5.2 Effect of pH:

To determine the optimum pH for the cellulose production, nutrient broth was adjusted using 1N sodium hydroxide (NaOH) and 1N hydrochloric acid (HCl) to pH 5, 7,9, 12 and 14 respectively, and then inoculated with the respective CDB strain, followed by incubation at 37°C for 24 hours at 100rpm.

2.5.3 Effect of Metal Ions:

To determine the metal ions required for optimum cellulase production, nutrient broth was supplemented with the following metal ions: Magnesium Sulphate, Manganous Sulphate, Potassium Dihydrogen Phosphate and Ferrous Sulphate in separate flasks, and each flask was inoculated with the respective CDB strain, followed by incubation at 37°C for 24 hours at 100 rpm.

2.6 Substrate preparation:

Peels of Papaya and Pomegranate were used as the biomass or substrate for the process of bio-fermentation. The peels were collected and dried in the sun for a few days before being powdered in a blender. The powdered peel, were stored at room temperature in zip-lock bags at until further use.

2.7 Acid Hydrolysis Pre-Treatment:

2.5 g of dried and powdered fruit peel residues were placed in anaerobic bottles containing 50 ml of 75% 0.2M sulphuric acid (H_2SO_4) and allowed to soak for 24 hours. Following the pre-treatment, the bottles were inoculated with the CDB and yeast strains. [8].

2.8 Inoculation:

All the samples pre-treated with sulphuric acid, were inoculated with the cellulose degrading bacteria isolated from vermicompost and the standard cellulose degrading bacteria respectively. One set of bottles had only the cellulose degrading bacteria, and into another set of bottles, yeast was also inoculated. The bottles were incubated as per the pre-determined parameters for a total of 21 days with periodic estimation of their glucose and alcohol content at set intervals of 7, 14 and 21 days.

Optimization of bio fermentation:

2.9.1 Effect of innoculum used:

The standard and isolated strains were inoculated into the respective bottles. *Saccharomyces cerevisiae* (the chosen yeast strain) was inoculated into one set of bottles containing CDB. The distribution of the bottles inoculated with CDB and yeast is given in Table 1.

Inoculum	Parameters	Papaya						Pomogranate					
Isolated CDB	Temperature	RT (250C)		350C		450C		RT (250C)		350C		450C	
	pH	4	5	7	9	12	14	4	5	7	9	12	14
Isolated CDB + *Saccharomyces cerevisiae*	Temperature	RT (250C)		350C		450C		RT (250C)		350C		450C	
	pH	4	5	7	9	12	14	4	5	7	9	12	14
Standard CDB	Temperature	RT (250C)		350C		450C		RT (250C)		350C		450C	
	pH	4	5	7	9	12	14	4	5	7	9	12	14
Standard CDB + *Saccharomyces cerevisiae*	Temperature	RT (250C)		350C		450C		RT (250C)		350C		450C	
	pH	4	5	7	9	12	14	4	5	7	9	12	14

2.9.2 Effect of temperature:

To determine the effect of temperature on the bio-fermentation process, a set of samples inoculated with only the respective CDB strain, and a set of samples inoculated with the respective CDB strain along with yeast, were incubated at room temperature (25°C), 35°C and 45°C. 35°C and 45°C are the average optimum temperature and Q10 temperature of the strains respectively.

2.9.3 Effect of pH:

To determine the optimum pH for the bio-fermentation process, the acid hydrolyzed pre-treated samples were adjusted using 1N sodium hydroxide (NaOH) to pH 4, 5, 7,9, 12 and 14 respectively. A set of samples inoculated with only the respective CDB strain, and a set of samples inoculated with the respective CDB strain along with yeast, were incubated at room temperature (25°C).

2.10 Estimation of total carbohydrate content:

The total carbohydrate content of the peels was measured both before and after pretreatment. Before pretreatment, 0.1 g of the sample, and after pretreatment, 1 ml of the sample was taken into a boiling tube, which was then hydrolyzed by incubating it for a span of 3 hours in a boiling water bath along with 5.0 ml of 2.5 N HCl and subsequently cooled to room temperature. It was then neutralized with solid sodium carbonate until the effervescence ceased and then the volume was made up to 100 ml followed by centrifugation. 0.2-1ml of the supernatant was collected and taken for analysis, by the addition of 4ml of anthrone reagent. This was followed by incubation for eight minutes in a boiling water bath, rapid cooling the transition from green to dark green colour was read at a wavelength of 630 nm. A standard graph to determine the concentration was obtained using a glucose working standard. [9].

2.11 Estimation of cellulose content:

This test was performed both before and after pre-treatment. Before pre-treatment, 0.5g of the sample and after pre-treatment, 1 ml of the sample was taken in a test tube. 3ml acetic/nitric reagent was added to the sample taken in the test tube and mixed well, followed by incubation in a water bath at 100°C for 30minutes. The contents were cooled and then centrifuged for 15-20minutes. The supernatant was discarded and the pellet was re-suspended in 10ml of 67% sulphuric acid and allowed to stand for 1hour. 0.1ml of the above solution was diluted to 10 ml, and to 0.5 ml of this solution, 5ml of anthrone reagent was added and mixed well, followed by incubation in a boiling water bath for 10 minutes. The solution was cooled to stop the reaction and the absorbance was measured at 630nm. Calculate the concentration of cellulose in the samples using a standard curve. [10]

2.12 Estimation of reducing sugar by DNS:

This test is performed on the 7th, 14th and 21st day after inoculation. For the estimation of reducing sugar in the samples, 1 ml of sample was taken periodically at intervals of 7, 14 and 21 days, and 1ml of Di-nitro Salicylic reagent was added to it, followed by incubation in a boiling water bath for 10 minutes. A blank was also set with 1ml distilled water instead of the sample. After incubation, the tubes were cooled to stop the reaction and the optical density was measured at 540 nm. Concentrations of the samples were determined by extrapolating the absorbance values on a standard curve. [11].

3. Results and Analysis

3.1 Isolation and screening:

At the end of the incubation period, growth was observed in all plates but the number of colonies decreased with increasing dilution. Three different colonies of bacteria were observed growing on the plates, one with a circular morphology and another with irregular morphology and one with a translucent colour showed in figure Ia.

After incubating the isolated colonies in CMC media, the plates were screened for cellulose degrading activity. As shown in figure Ib, one bacterial strain showed a zone of clearance on flooding the plates with 0.1% Congo red and was used for further studies.

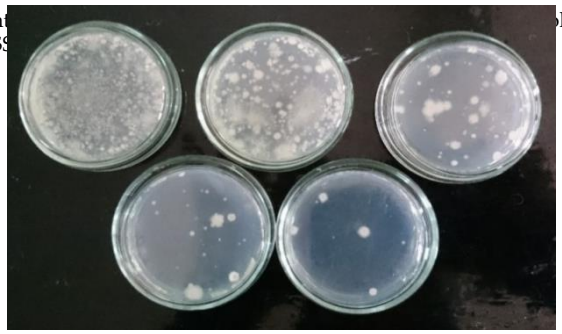

Fig: Ia: Serial dilution of bacteria isolated from vermicompost

Fig: Ib: Screening for CDB using Congo red

3.2 Cell growth assay:

The growth curve of the CDB isolated from vermicompost (later identified as *Bacillus licheniformis*), and the standard CDB (*Paenibacillus borealis* (MTCC No: 8085) is graphically represented in figure II. It was observed that both the cultures started off with a similar (overlapping) initial phase; there after we can further notice that there is a slight increase in the measured absorbance for the standard CDB strain when compared to that of the CDB strain isolated from vermicompost. It was observed that both the cultures displayed the phases of a typical bacterial growth curve. The lag phase, log phase, stationary phase and death phase were observed.

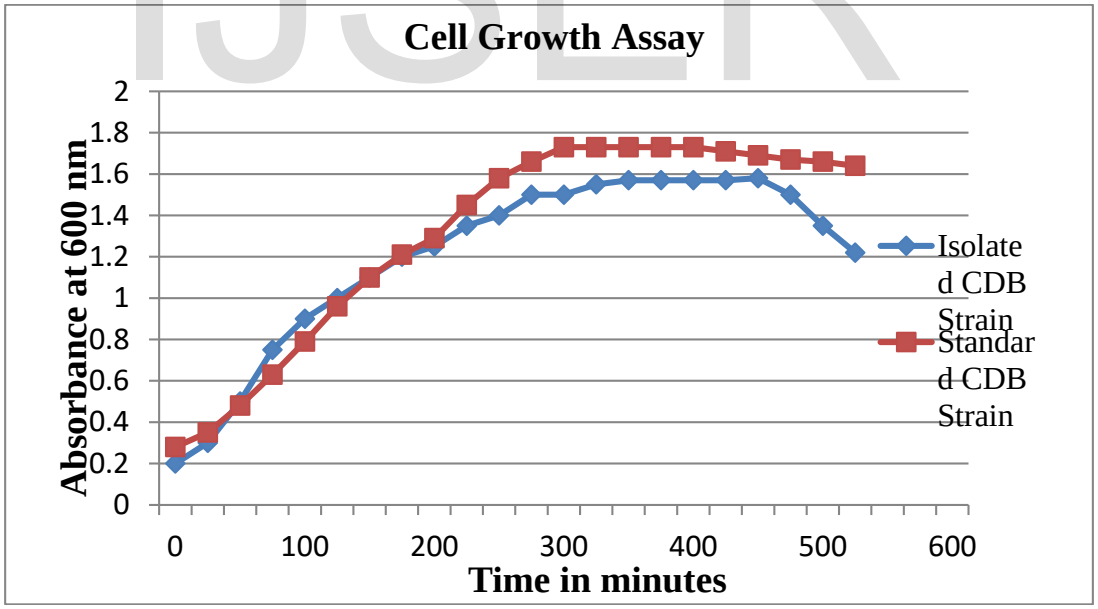

Fig: II: Bacterial growth curve in respect to time

3.3 Characterization by 16s rRNA sequencing:

The isolated CDB strain was characterized by means of 16s rRNA sequencing by Eurofins Genomics India Pvt. Ltd., and the result showed high similarity with *Bacillus licheniformis* based on nucleotide homology and phylogenetic analysis

3.4 Measurement of cellulolytic activity:

The endoglucanase and exoglucanase activity were measured using DNS reagent and it was observed that the standard strain P. borealis gave a higher absorbance than the CDB strain *B. licheniformis* that was isolated from the vermicompost. The obtained absorbance readings were used to find out the concentration of glucose. The glucose concentration was used to find out the endoglucanase and exoglucanase activity using the respective formulae. The endoglucanase and exoglucanase activity were plotted on the Y-axis with the CDB strain on the X-axis. Fig: III showed that endoglucanase activity was found to be higher than the exoglucanase activity.

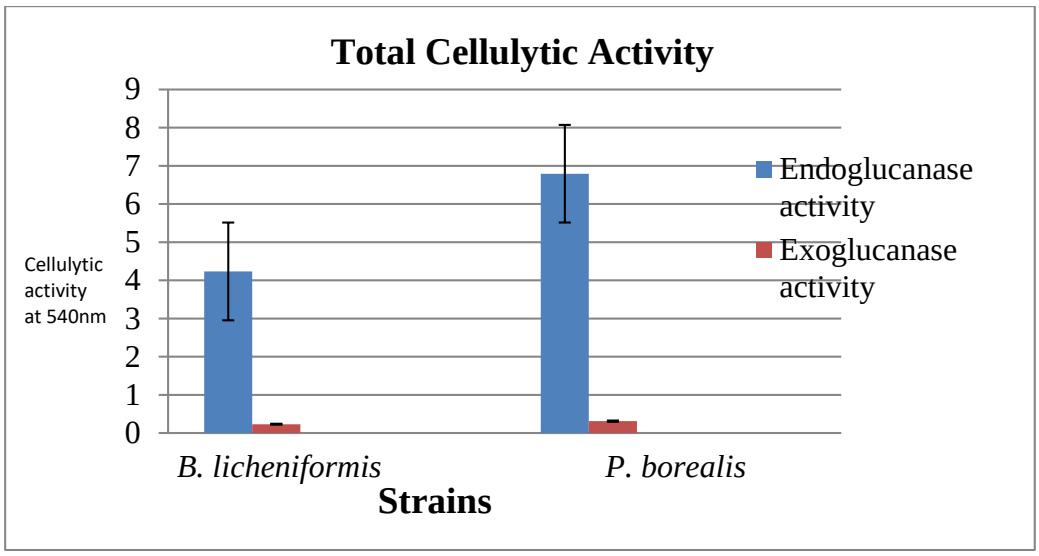

Fig: III: Endoglucanase activity of CDB strains *B. licheniformis* and *P. borealis*

3.5 Optimization of cellulytic activity:

3.5.1: Effect of Temperature on Endoglucanase activity of Cellulose Degrading Bacteria:

The endoglucanase activity was measured using DNS reagent. It was observed that the absorbance was higher at 35°C than at 45°C.The obtained absorbance readings were used to find out the concentration of glucose. The glucose concentration was used to find out the endoglucanase activity using the formula. The endoglucanase activity was plotted on the Y-axis with the CDB strains on the X-axis. Figure IVa showed that endoglucanase activity of *P. borealis* was found to be higher than that of *B. licheniformis*.

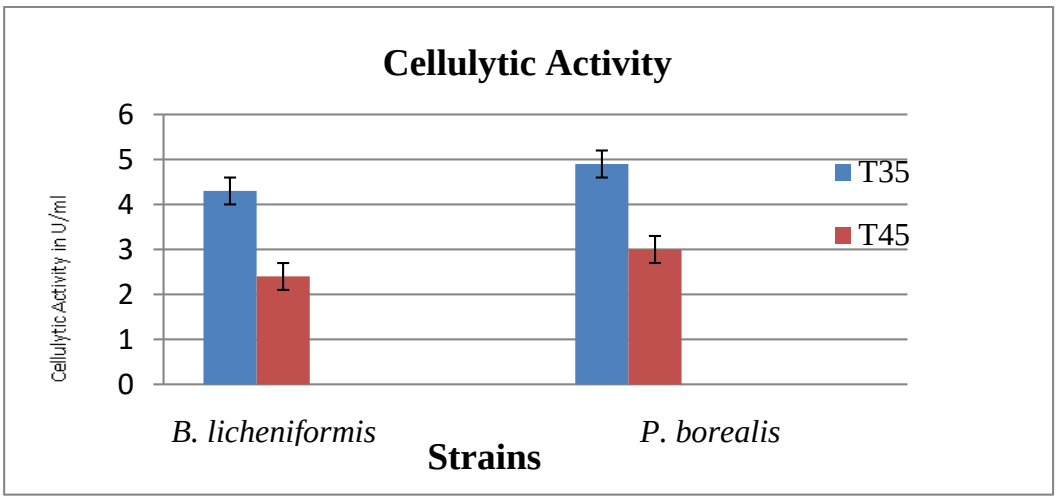

Figure IVa: Effect of temperature on Endoglucanase activity of CDB strains *B. licheniformis* and *P. borealis*

3.5.2 Effect of pH on Endoglucanase activity of Cellulose Degrading Bacteria:

The endoglucanase activity was measured using DNS reagent. It was observed that the absorbance was highest at pH 7, followed by pH 5, pH 9, pH 12 and pH 14.The obtained absorbance readings were used to find out the concentration of glucose. The glucose concentration was used to find out the endoglucanase

activity using the formula. The endoglucanase activity was plotted on the Y-axis with the CDB strains on the X-axis. Figure IVb showed that endoglucanase activity of P. borealis was found to be higher than that of *B. licheniformis*.

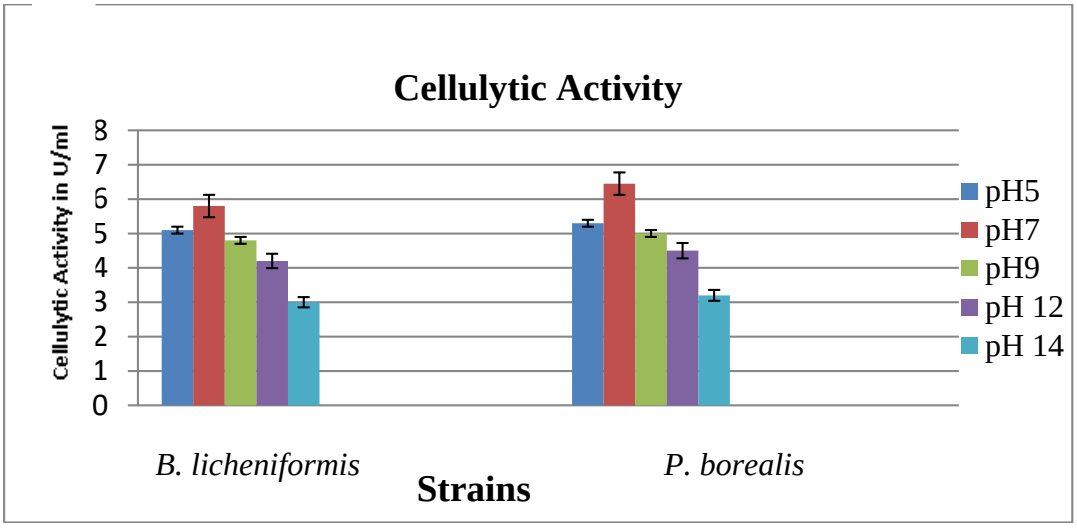

Figure IVb: Effect of pH on Endoglucanase activity of CDB strains *B. licheniformis* and *P. borealis*

3.5.3 Effect of metal ions:

3.5.3.1 Effect of Metal Ions (0.1g/l) on Endoglucanase Activity of CDB:

The endoglucanase activity was measured using DNS reagent. It was observed that the absorbance was highest in the presence of KH2PO4, followed by MnSO4, FeSO4 and MgSO4 when present at a concentration of 0.1g/l. The obtained absorbance readings were used to find out the concentration of glucose. The glucose concentration was used to find out the endoglucanase activity using the formula. The endoglucanase activity was plotted on the Y-axis with the CDB strains on the X-axis. Figure IVc1 showed that endoglucanase activity of *P. borealis* was found to be higher than that of *B. licheniformis*.

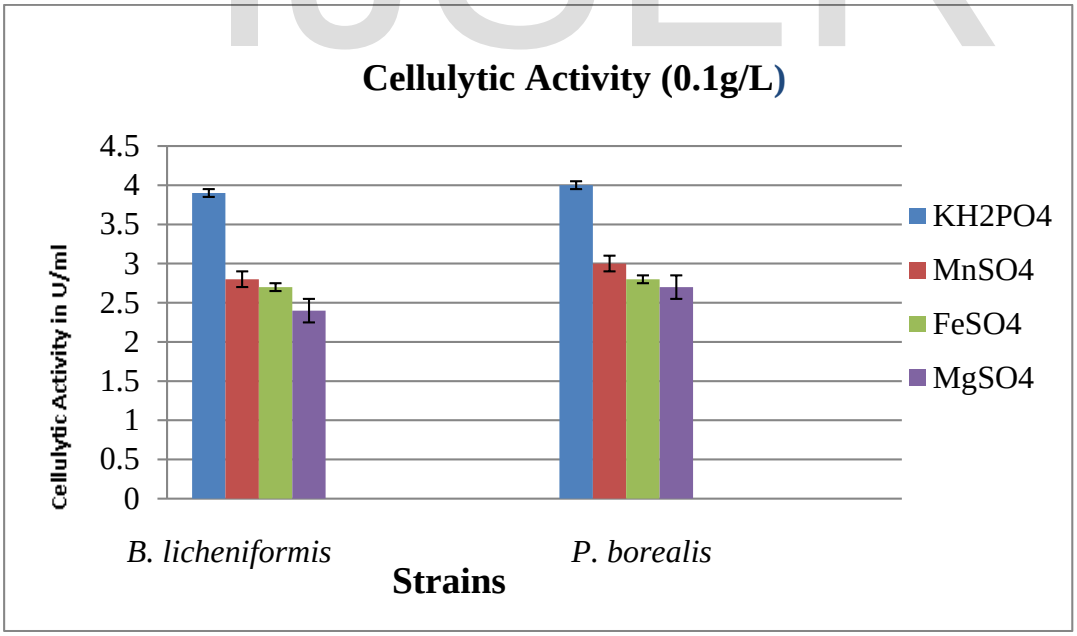

Figure IV c1: Effect of Metal ions (0.1g/l) on Endoglucanase activity of CDB strains *B. licheniformis* and *P. borealis*

3.5.3.2 Effect of Metal Ions (1.0g/l) on Endoglucanase Activity of CDB:

The endoglucanase activity was measured using DNS reagent. It was observed that the absorbance was highest in the presence of MnSO4 followed by FeSO4, KH2PO4and MgSO4 when present at a concentration of 1g/l. The obtained absorbance readings were used to find out the concentration of glucose. The glucose concentration was used to find out the endoglucanase activity using the formula. The

endoglucanase activity was plotted on the Y-axis with the CDB strains on the X-axis. Figure IVc2 showed that endoglucanase activity of *P. borealis* was found to be higher than that of *B. licheniformis*.

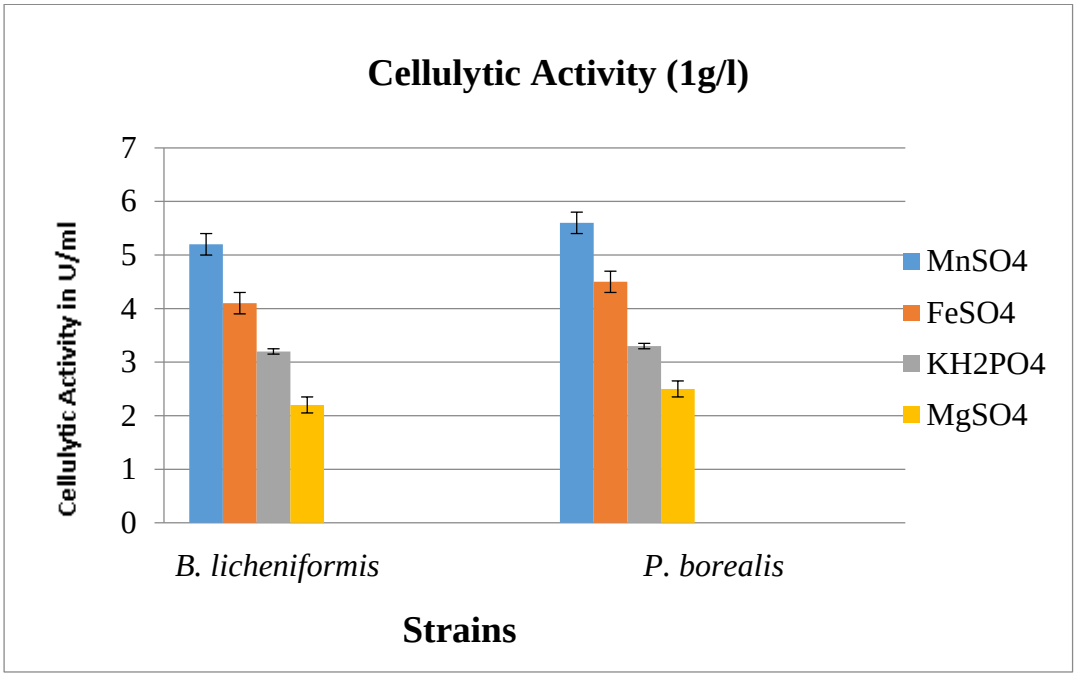

Figure IVc2: Effect of Metal ions (1.0g/l) on Endoglucanase activity of CDB strains *B. licheniformis* and *P. borealis*

3.6 Pre treatment and bio fermentation:

3.6.1 Total carbohydrate content:

The Total carbohydrate content was measured by the Anthrone method. Absorbance readings obtained were used to obtain concentration which was plotted on the Y-axis with the respective fruit peels on the X-axis. The carbohydrate content increased after the pre-treatment for both the peels. From figure Va, it can be concluded that pomegranate yielded the highest carbohydrate, both before and after pre-treatment, followed by papaya.

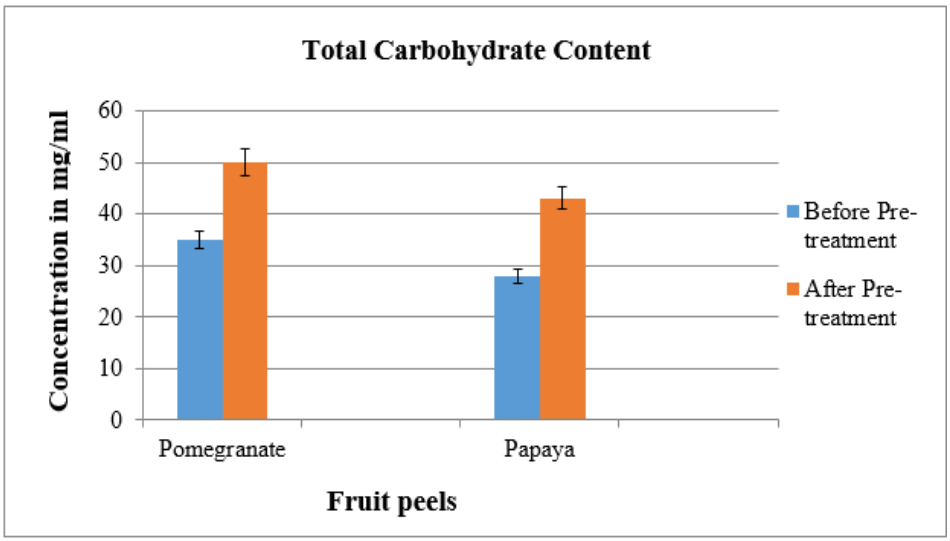

Figure Va: Total Carbohydrate Content before and after Pre-Treatment

3.6.2 Cellulose content:

The Cellulose content was measured by the Updegraff method. Absorbance readings obtained were used to obtain concentration, which was plotted on the Y-axis with the respective fruit peels on the X-axis. The

cellulose content increased after the pre-treatment for both the peels. From figure Vb, it can be concluded that pomegranate yielded the highest cellulose, both before and after pre-treatment, followed by papaya.

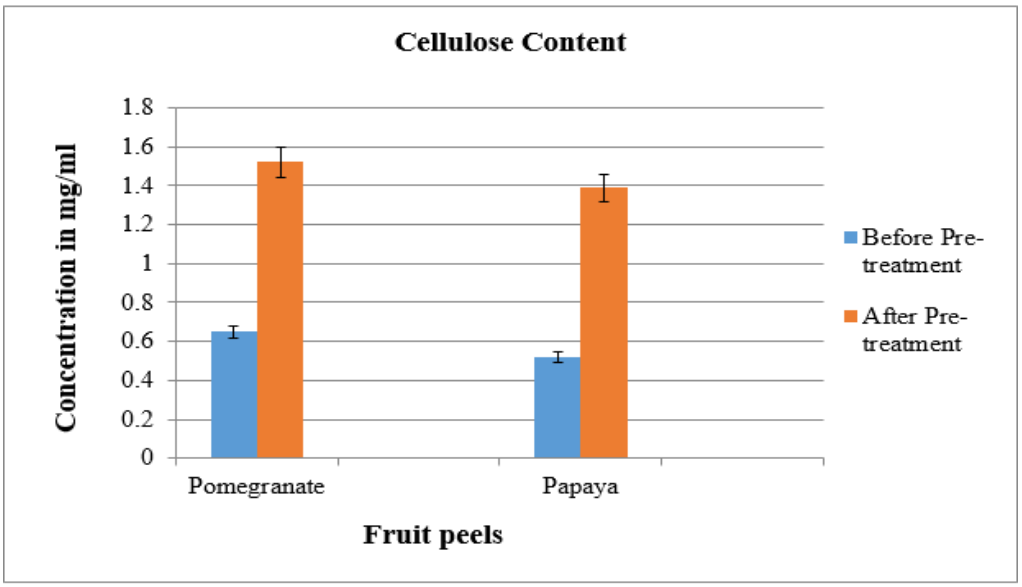

Figure Vb: Total Cellulose Content before and after Pre-Treatment

3.7 Optimisation of Biofermentation Process:

3.7.1Effect of Temperature on Glucose Concentration:

3.7.1.1Effect of Temperature on Glucose Concentration after Pre-treatment and Inoculation with CDB strain *B. licheniformis:*

The glucose concentration was measured using DNS reagent. From figure Via, it was observed that when only *B. licheniformis* was used, highest reducing sugar concentration was observed at the temperature of 45°C, followed by room temperature (25°C) and 35°C. Papaya showed a higher glucose concentration than pomegranate. The glucose concentration decreased as the days went by.

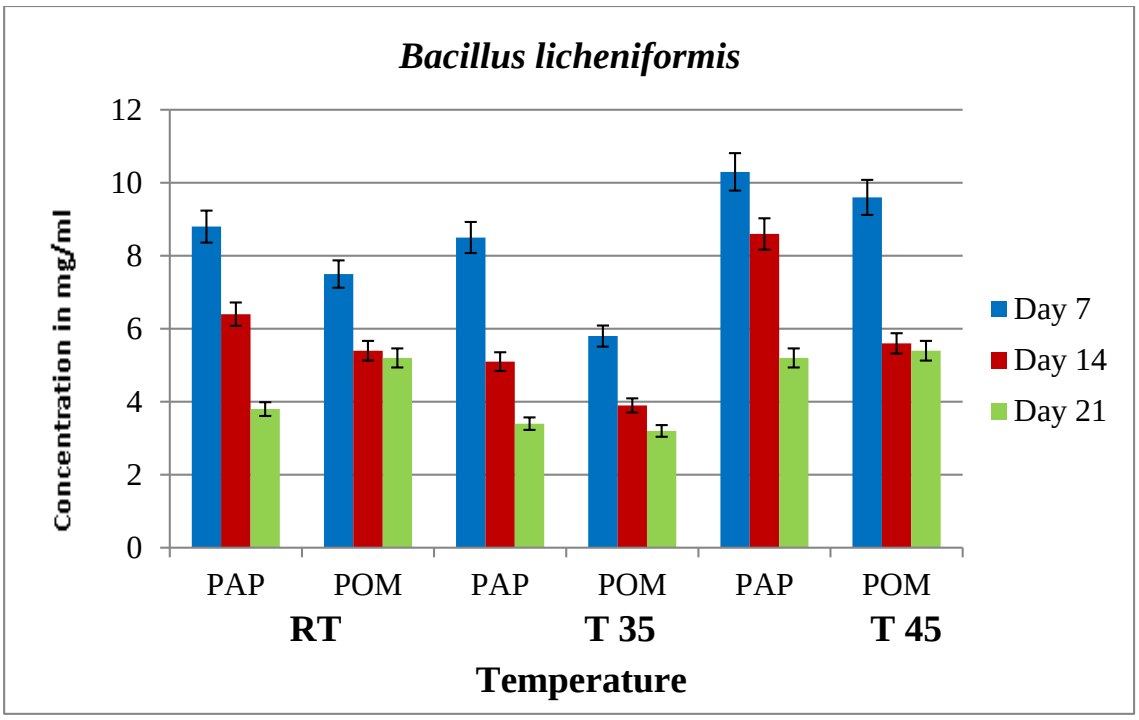

Figure VIa: Effect of Temperature on Glucose Concentration after Pre-treatment and Inoculation with CDB strain *B. licheniformis*

3.7.1.2 Effect of Temperature on Glucose Concentration after Pre-treatment and Inoculation with CDB strain *B. licheniformis* and Yeast strain *S. cerevisiae*:

Figure VIb showed that when both *B. licheniformis* and *S. cerevisiae* were used, highest reducing sugar concentration was observed at the temperature of 45°C, followed by room temperature (25°C) and 35°C. Papaya showed a higher glucose concentration than pomegranate. The glucose concentration decreased as the days went by. The reduction in the glucose concentration was observed to be higher when yeast was added.

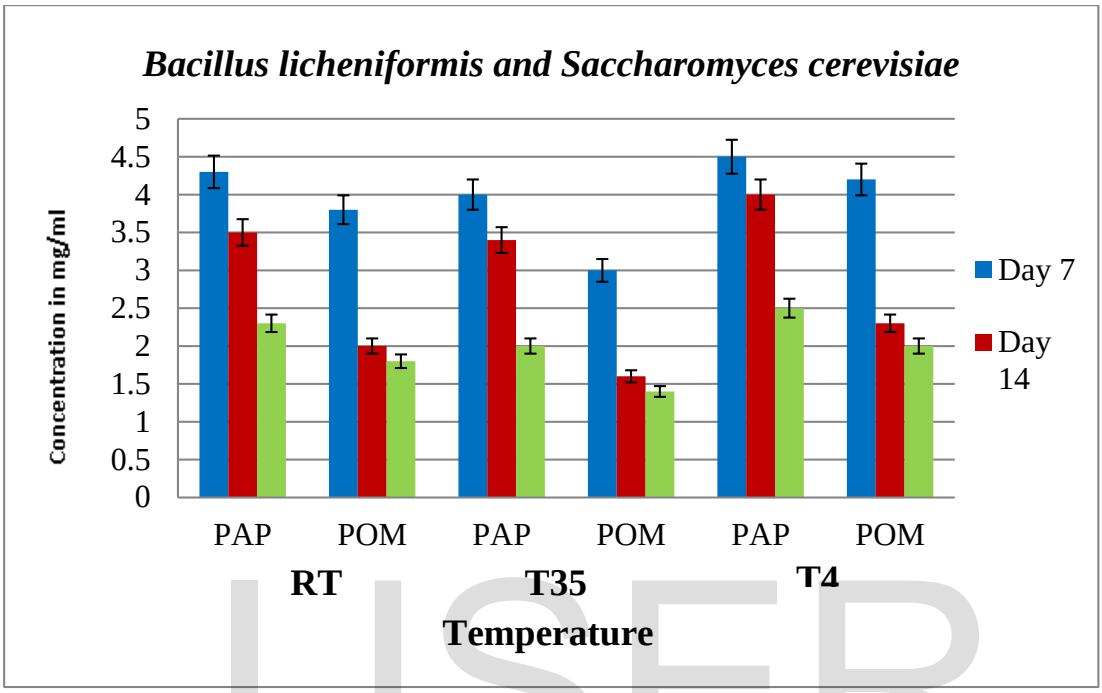

Figure VIb: Effect of Temperature on Glucose Concentration after Pre-treatment and Inoculation with CDB strain *B. licheniformis* and Yeast strain *S. cerevisiae*

3.7.1.3 Effect of Temperature on Glucose Concentration after Pre-treatment and Inoculation with standard CDB strain *P. borealis*:

Figure VIc showed that when only *P. borealis* was used, highest reducing sugar concentration was observed at the temperature of 45°C, followed by room temperature (25°C) and 35°C. Papaya showed a higher glucose concentration than pomegranate. The glucose concentration decreased as the days went by.

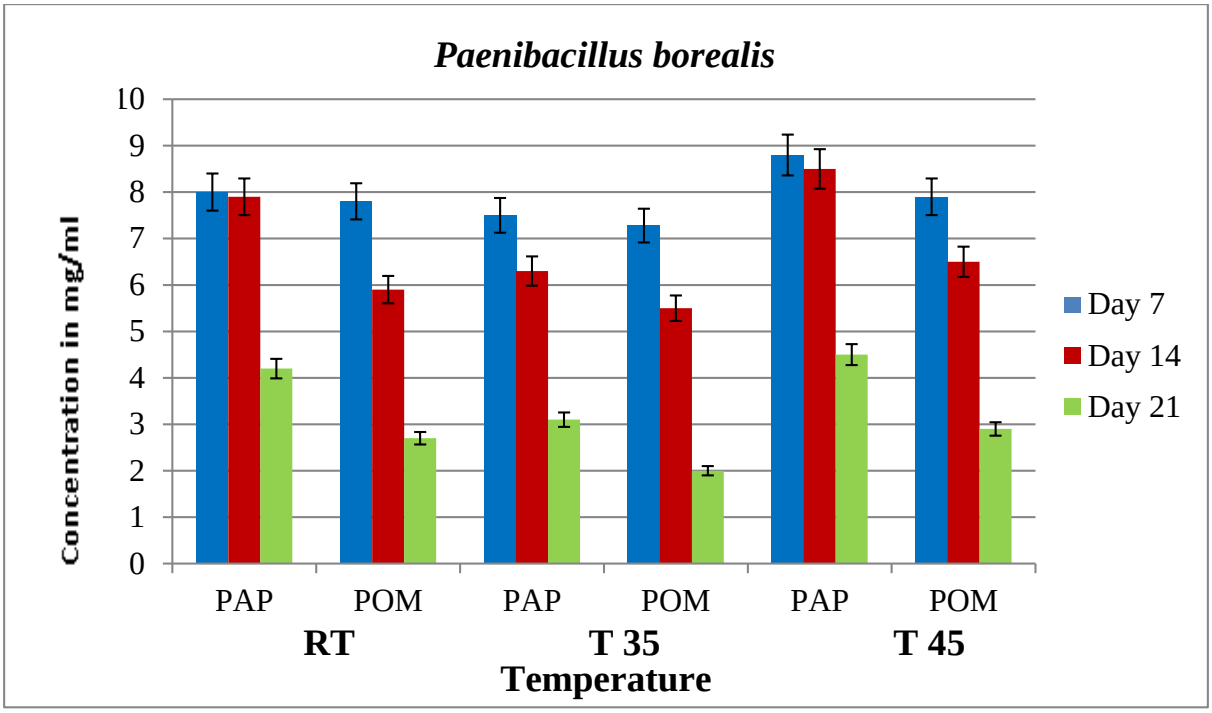

Figure VIc: Effect of Temperature on Glucose Concentration after Pre-treatment and Inoculation with CDB strain *P. borealis*

3.7.1.4 Effect of Temperature on Glucose Concentration after Pre-treatment and Inoculation with standard CDB strain *P. borealis* and Yeast strain *S. cerevisiae*:

From figure VId, it was observed that when both *P. borealis* and *S. cerevisiae* were used, highest reducing sugar concentration was observed at the temperature of 45°C, followed by room temperature (25°C) and 35°C. Papaya showed a higher glucose concentration than pomegranate. The glucose concentration decreased as the days went by. The reduction in the glucose concentration was observed to be higher when yeast was added.

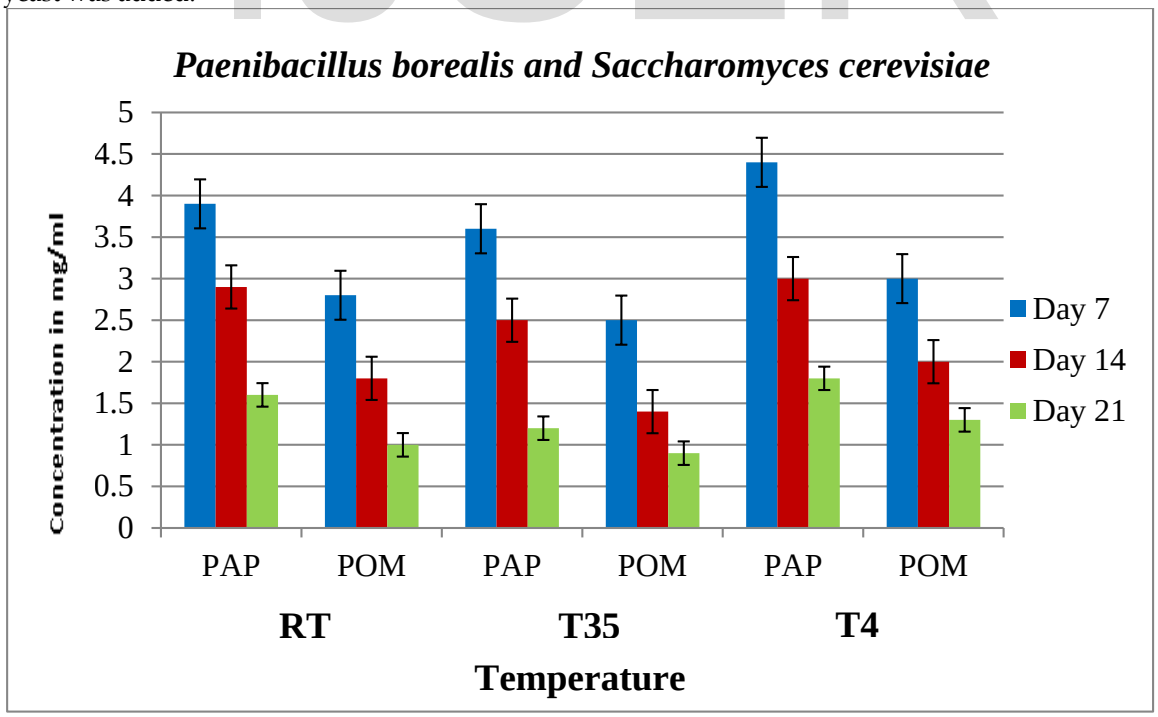

Figure VId: Effect of Temperature on Glucose Concentration after Pre-treatment and Inoculation with CDB strain *P. borealis* and Yeast strain *S. cerevisiae.*

3.7.2 Effect of pH on Glucose Concentration:

3.7.2.1Effect of pH on Glucose Concentration after Pre-treatment and Inoculation with CDB strain *B. licheniformis*:

Figure VIe showed that when only *B. licheniformis* was used, highest reducing sugar concentration was observed at pH 14, followed by pH 12, pH 9, pH 5, pH 4 and pH7. Papaya showed a higher glucose concentration than pomegranate. The glucose concentration decreased as the days went by.

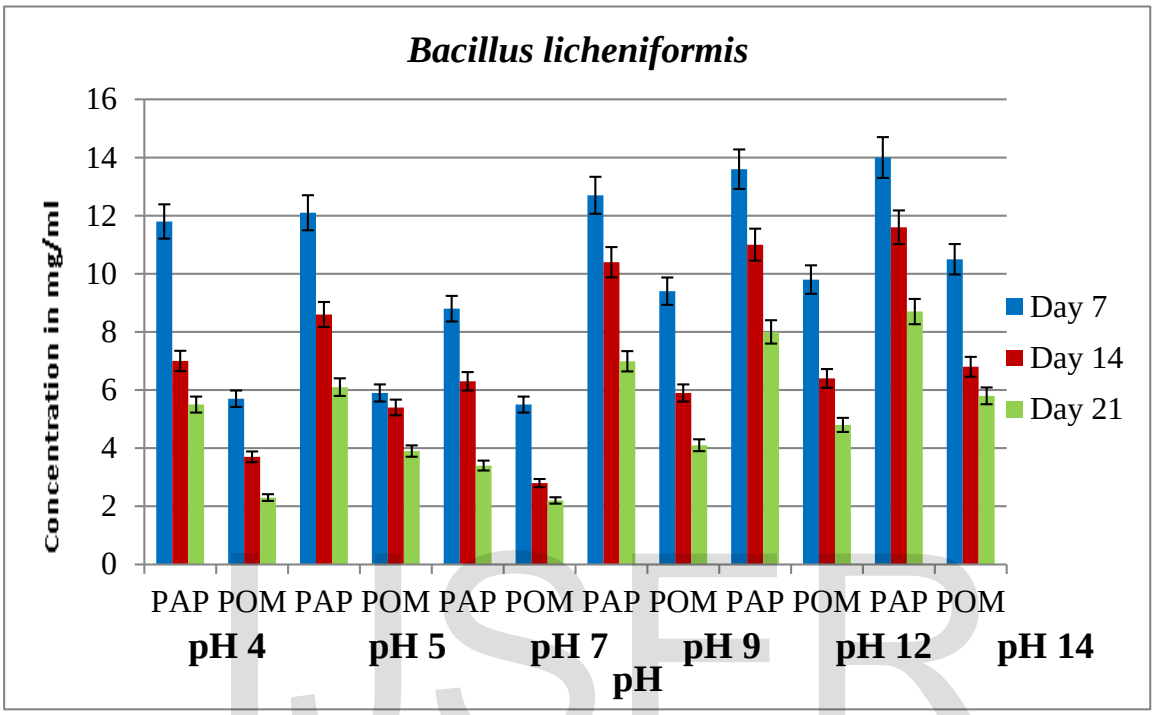

Figure VIe: Effect of pH on Glucose Concentration after Pre-treatment and Inoculation with CDB strain *B. licheniformis*

3.7.2.2 Effect of pH on Glucose Concentration after Pre-treatment and Inoculation with CDB strain *B. licheniformis* and Yeast strain *S. cerevisiae*:

Figure VIf showed that when both *B. licheniformis* and *S. cerevisiae* were used, highest reducing sugar concentration was observed at pH 14, followed by pH 12, pH 9, pH 5, pH 4 and pH 7. Papaya showed a higher glucose concentration than pomegranate. The glucose concentration decreased as the days went by. The reduction in the glucose concentration was observed to be higher when yeast was added.

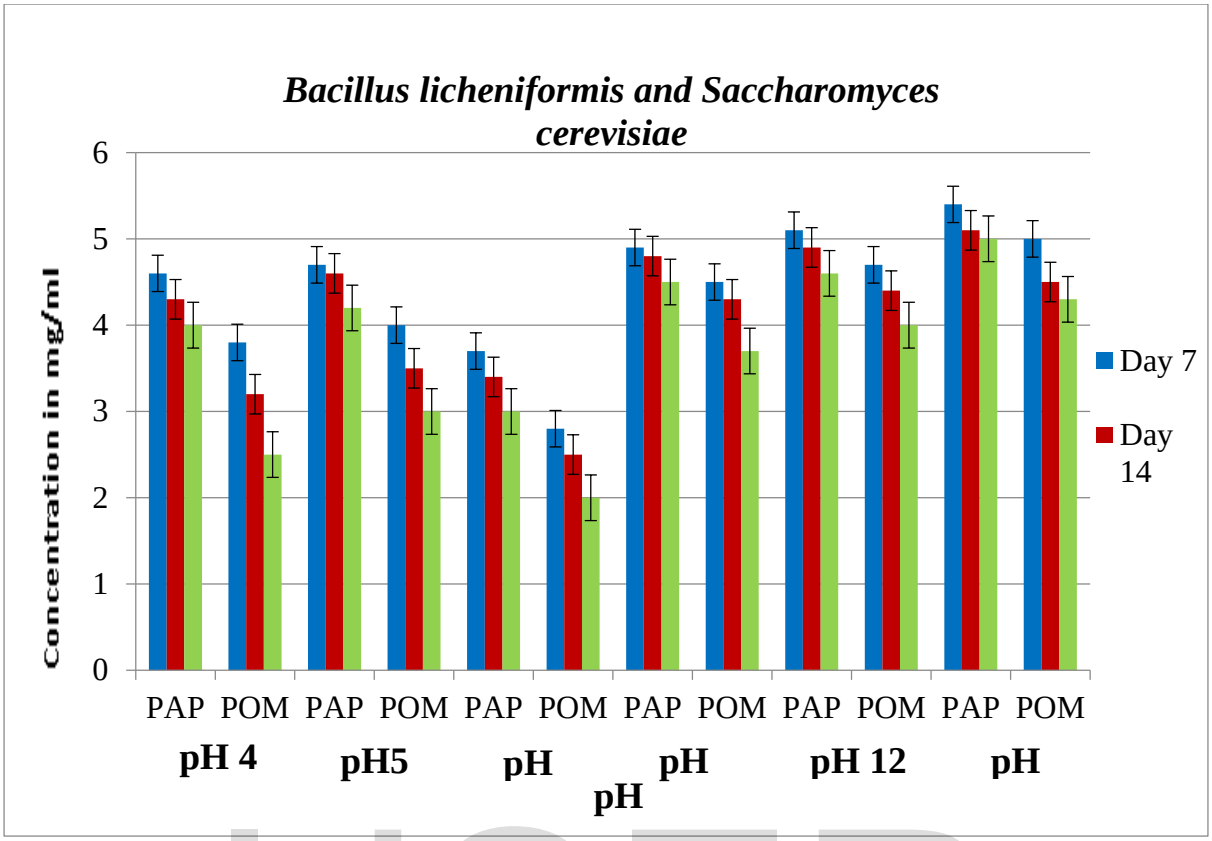

Figure VIf: Effect of pH on Glucose Concentration after Pre-treatment and Inoculation with CDB strain *B. licheniformis* and Yeast strain *S. cerevisiae*

3.7.2.3 Effect of pH on Glucose Concentration after Pre-treatment and Inoculation with standard CDB strain *P. borealis*:

From figure VIg, it was observed that when only *P. borealis* was used, highest reducing sugar concentration was observed at pH 14, followed by pH 12, pH 9, pH 5, pH 4 and pH 7. Papaya showed a higher glucose concentration than pomegranate. The glucose concentration decreased as the days went by.

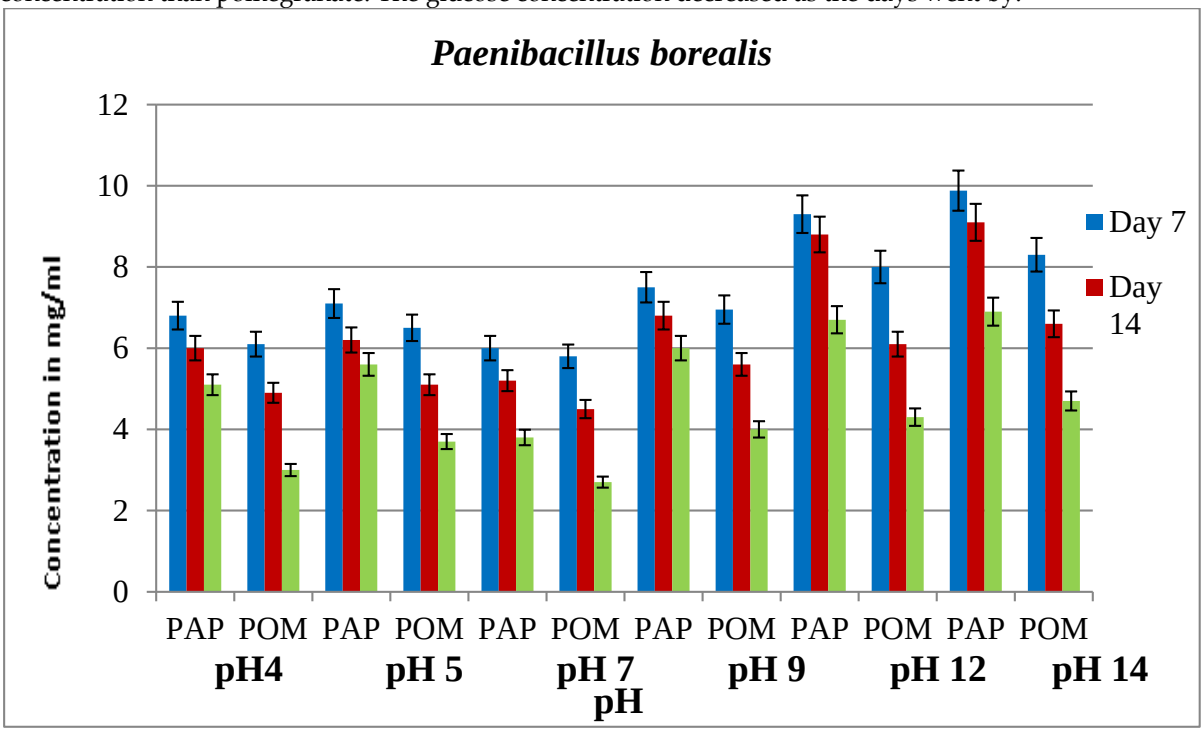

Figure VIg: Effect of pH on Glucose Concentration after Pre-treatment and Inoculation with standard CDB strain *P. borealis*

3.7.2.4 Effect of pH on Glucose Concentration after Pre-treatment and Inoculation with standard CDB strain *P. borealis* and yeast strain *S. cerevisiae*:

From figure VIh, it was observed that when both *P. borealis* and *S. cerevisiae* were used, highest reducing sugar concentration was observed at pH 14, followed by pH 12, pH 9, pH 5, pH 4 and pH 7. Papaya showed a higher glucose concentration than pomegranate. The glucose concentration decreased as the days went by. The reduction in the glucose concentration was observed to be higher when yeast was added.

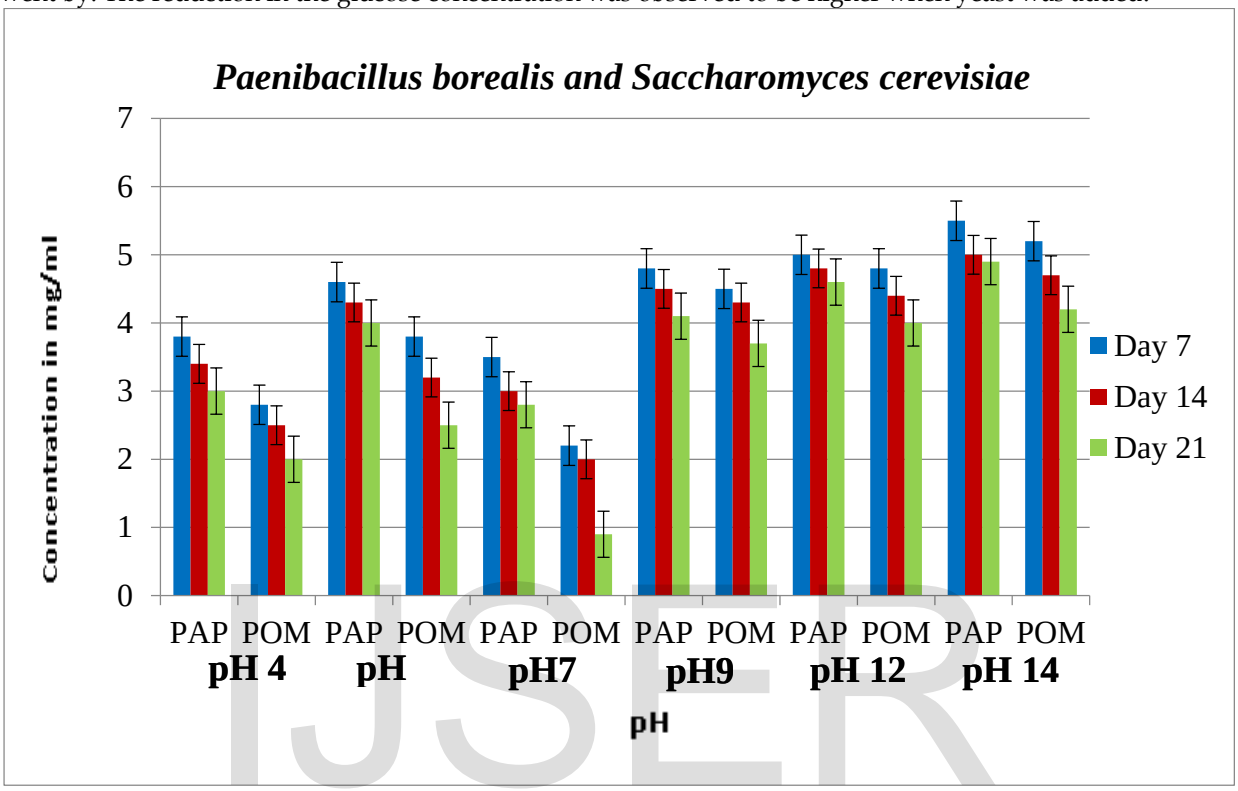

Figure VIh: Effect of pH on Glucose Concentration after Pre-treatment and Inoculation with standard CDB strain *P. borealis* and yeast strain *S. cerevisiae*

3.7.3 Effect of Temperature on Alcohol Concentration

3.7.3.1 Effect of Temperature on Percentage of Alcohol after pretreatment and Inoculation with CDB Strain *B. licheniformis*:

The alcohol concentration was measured by the potassium dichromate titration method. The titre values obtained were used to calculate the alcohol percentage and plotted on the Y-axis with the fruit peels and temperature on the X-axis. Figure VIIa showed that when only *B. licheniformis* was used, highest alcohol percentage was observed at the temperature of 35°C, followed by room temperature (25°C) and 45°C. Pomegranate showed a higher alcohol percentage than papaya. The alcohol percentage increased as the days went by.

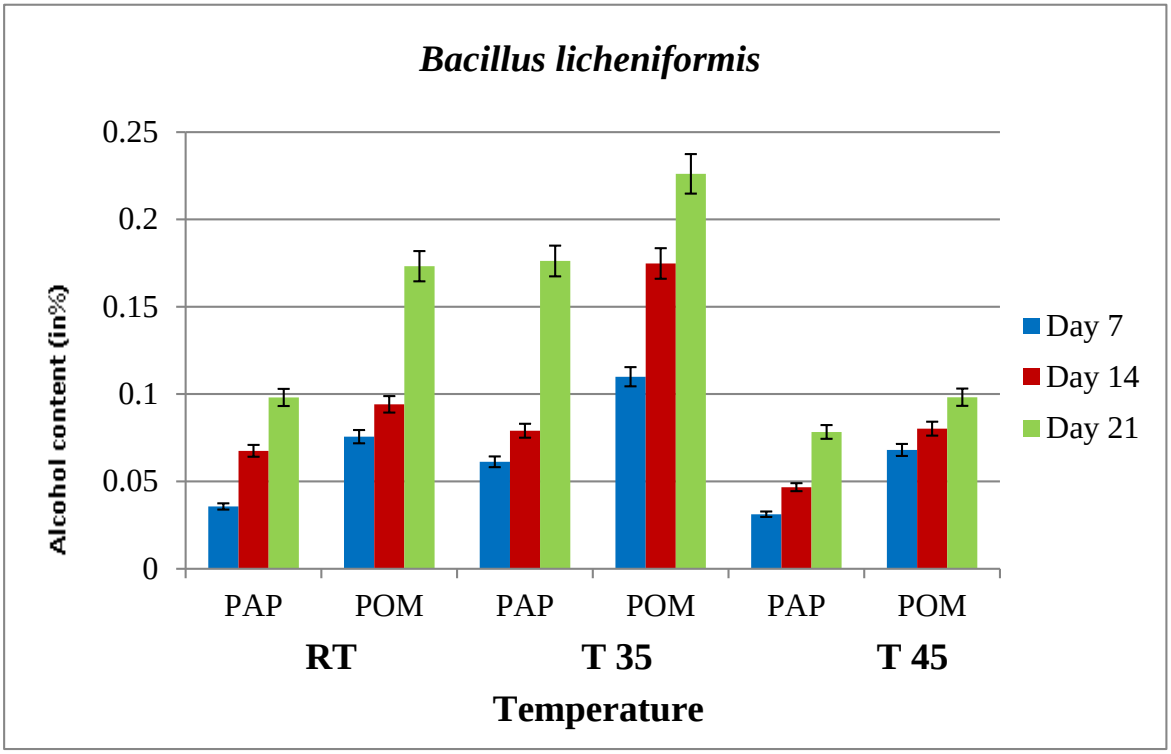

Figure VIIa: Effect of Temperature on Percentage of Alcohol after Pretreatment and Inoculation with CDB Strain *B. licheniformis*

3.7.3.2 Effect of Temperature on Percentage of Alcohol after Pretreatment and Inoculation with CDB Strain *B. licheniformis* and Yeast strain *S. cerevisiae*:

From figure VIIb, it was observed that when both *B. licheniformis* and *S. cerevisiae* were used, highest alcohol percentage was observed at the temperature of 35°C, followed by room temperature (25°C) and 45°C. Pomegranate showed a higher alcohol percentage than papaya. The alcohol percentage increased as the days went by. The increase in the alcohol percentage was observed to be higher when yeast was added.

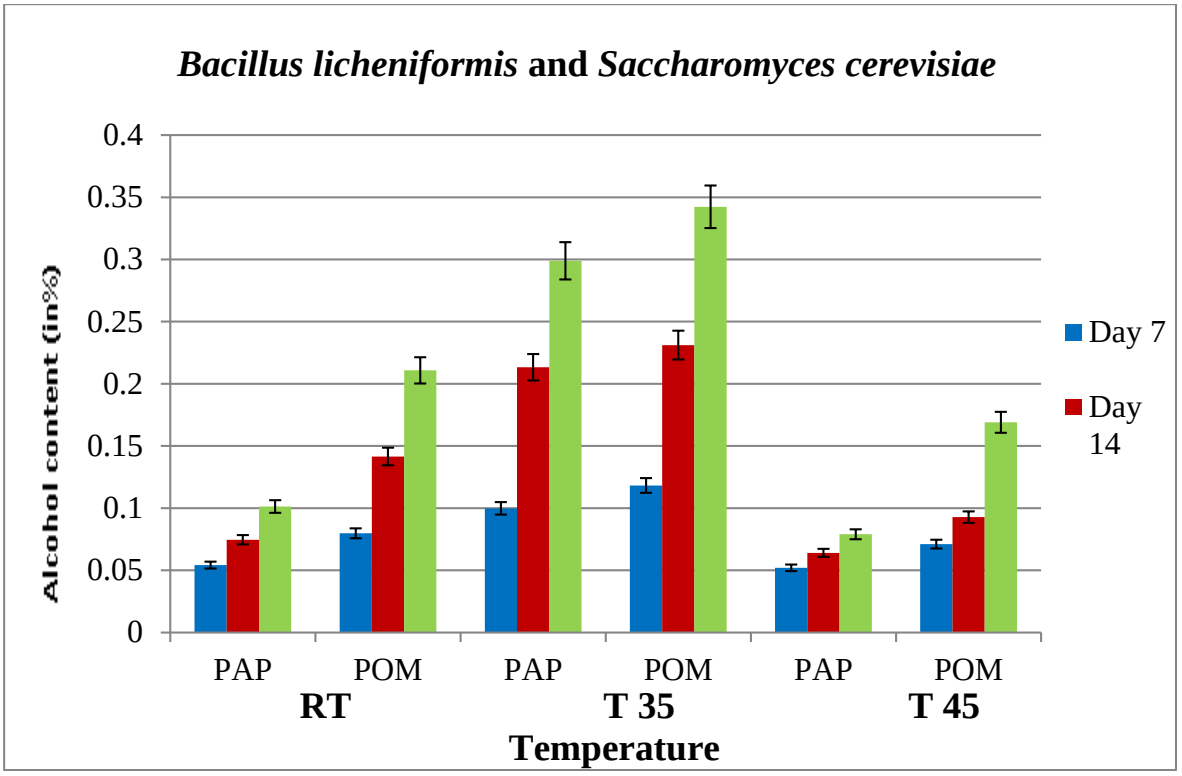

Figure VIIb: Effect of Temperature on Percentage of Alcohol after Pretreatment and Inoculation with CDB Strain *B. licheniformis* and Yeast strain *S. cerevisiae*

3.7.3.3 Effect of Temperature on Percentage of Alcohol after pretreatment and Inoculation with standard CDB Strain *P. borealis*:

Figure VIIc showed that when only *P. borealis* was used, highest alcohol percentage was observed at the temperature of 35°C, followed by room temperature (25°C) and 45°C. Pomegranate showed a higher alcohol percentage than papaya. The alcohol percentage increased as the days went by.

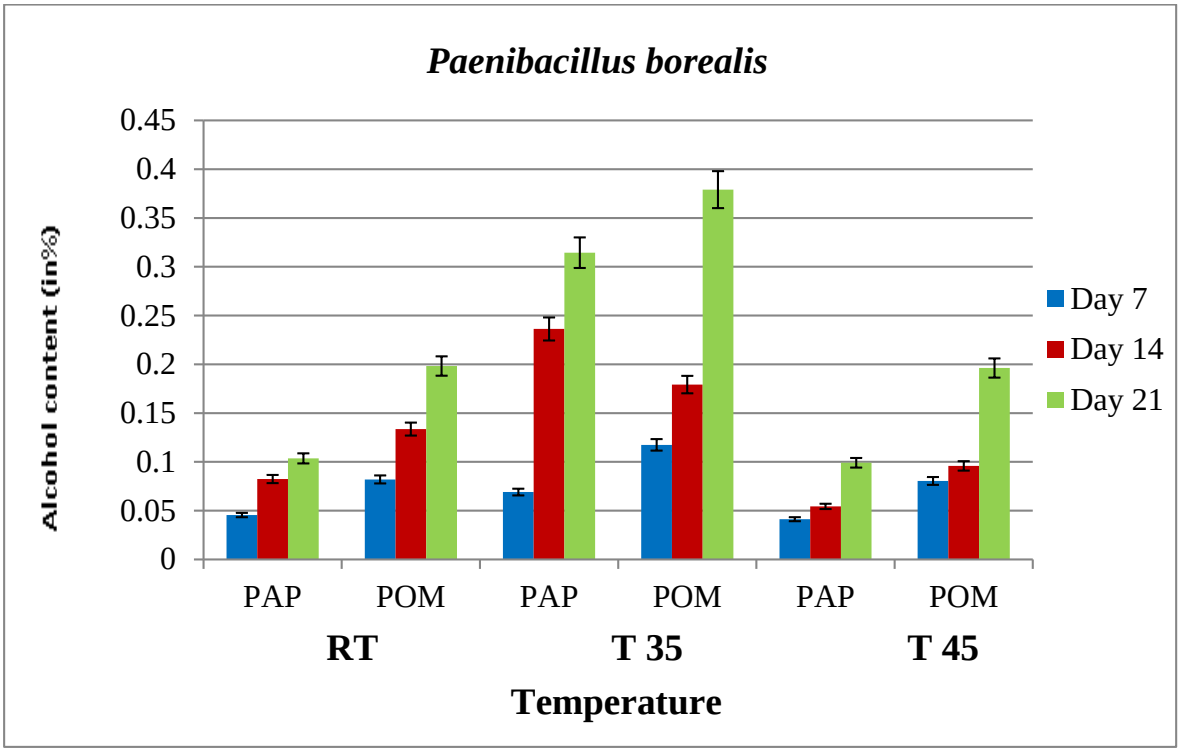

Figure VIIc: Effect of Temperature on Percentage of Alcohol after pretreatment and Inoculation with standard CDB Strain *P. borealis*

3.7.3.4 Effect of Temperature on Percentage of Alcohol after pretreatment and Inoculation with standard CDB Strain *P. borealis* and Yeast strain *S. cerevisiae*:

Figure VIId showed that when both *P. borealis* and *S. cerevisiae* were used, highest alcohol percentage was observed at the temperature of 35°C, followed by room temperature (25°C) and 45°C. Pomegranate showed a higher alcohol percentage than papaya. The alcohol percentage increased as the days went by. The increase in the alcohol percentage was observed to be higher when yeast was added.

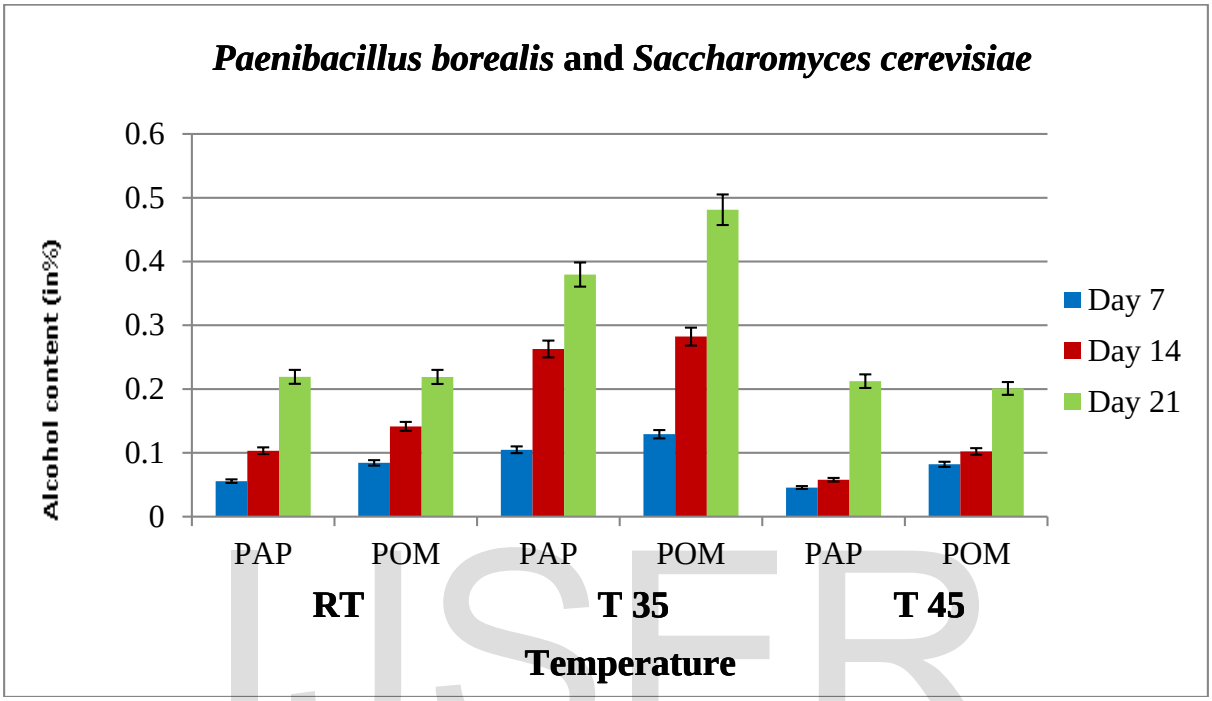

Figure VIId: Effect of Temperature on Percentage of Alcohol after pretreatment and Inoculation with standard CDB Strain *P. borealis* and Yeast strain *S. cerevisiae*

3.7.4 Effect of pH on Alcohol Concentration:

3.7.4.1 Effect of pH on Percentage of Alcohol after pretreatment and Inoculation with CDB Strain *B. licheniformis*:

Figure VIIe showed that when only *B. licheniformis* was used, highest alcohol percentage was observed at pH 7, followed by pH 4, pH 5, pH 9, pH 12 and pH 14. Pomegranate showed a higher alcohol percentage than papaya. The alcohol percentage increased as the days went by.

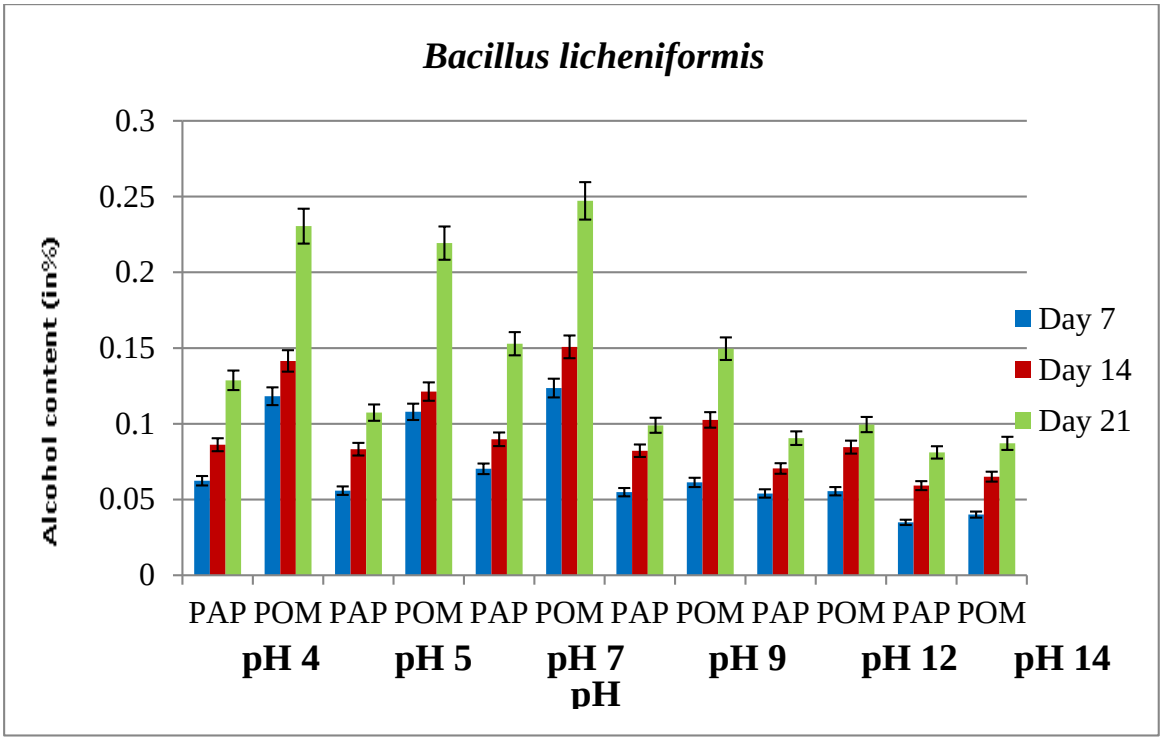

Figure VIIe: Effect of pH on Percentage of Alcohol after pretreatment and Inoculation with CDB Strain *B. licheniformis*

3.7.4.2 Effect of pH on Percentage of Alcohol after pretreatment and Inoculation with CDB Strain *B. licheniformis* and yeast strain *S. cerevisiae*:

From figure VIIf, it was observed that when both *B. licheniformis* and *S. cerevisiae* were used, highest alcohol percentage was observed at pH 7, followed by pH 4, pH 5, pH 9, pH 12 and pH 14. Pomegranate showed a higher alcohol percentage than papaya. The alcohol percentage increased as the days went by. The increase in the alcohol percentage was observed to be higher when yeast was added.

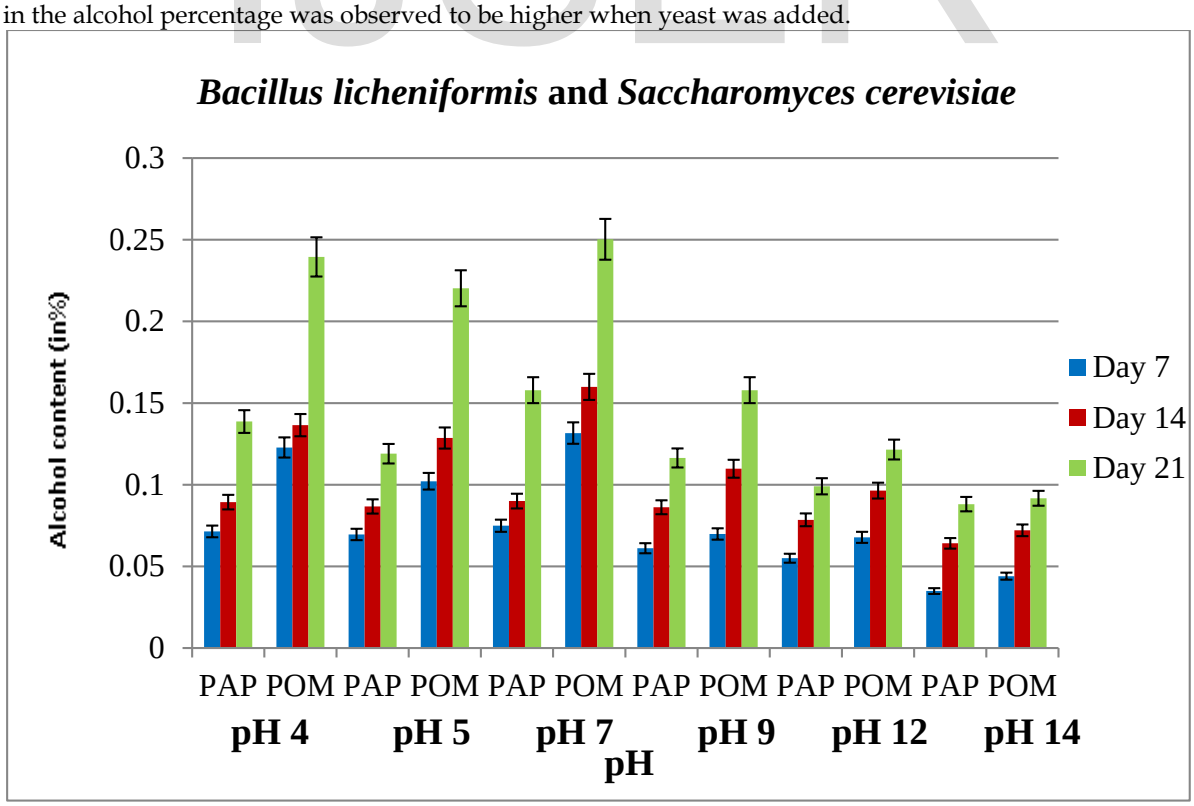

Figure VIIf: Effect of pH on Percentage of Alcohol after pretreatment and Inoculation with CDB Strain *B. licheniformis* and yeast strain *S. cerevisiae*

3.7.4.3 Effect of pH on Percentage of Alcohol after pretreatment and Inoculation with standard CDB Strain *P. borealis*:

Figure VIIf showed that when only *P. borealis* was used, highest alcohol percentage was observed at pH 7, followed by pH 4, pH 5, pH 9, pH 12 and pH 14. Pomegranate showed a higher alcohol percentage than papaya. The alcohol percentage increased as the days went by.

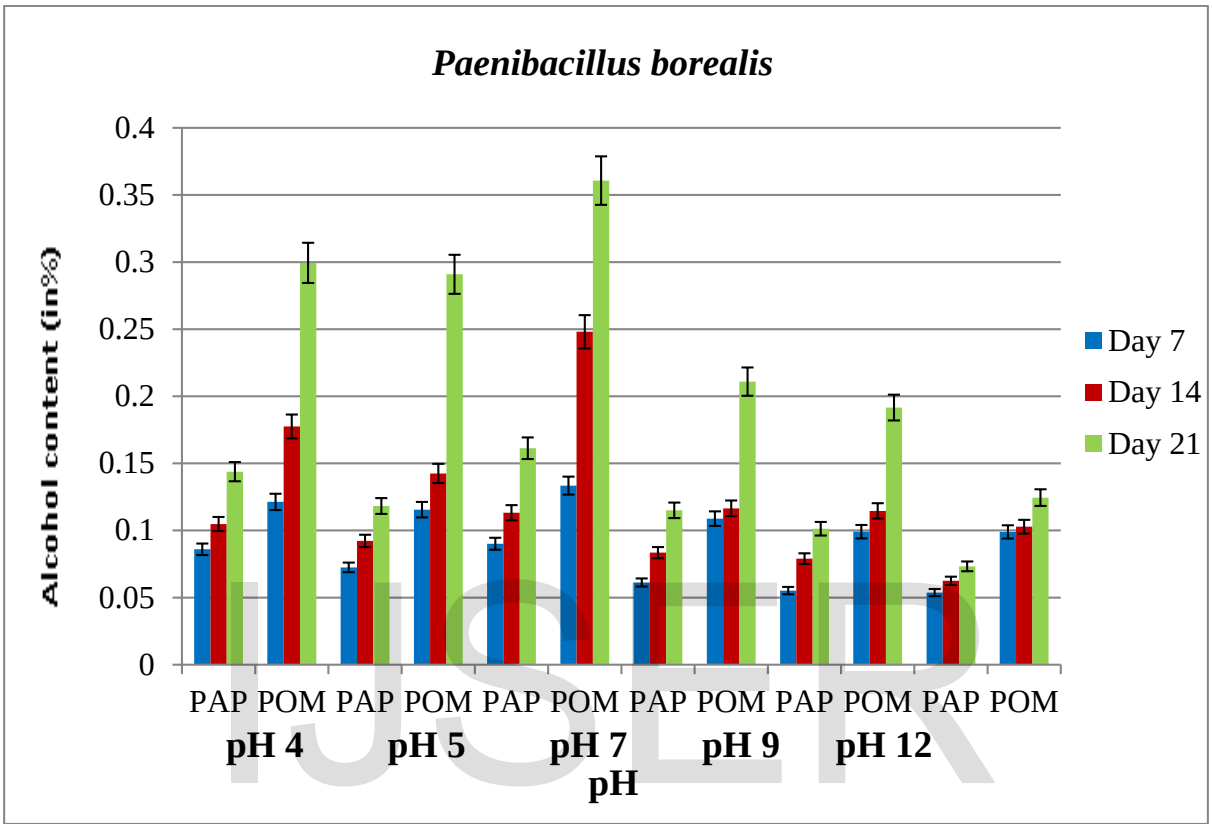

Figure VIIg: Effect of pH on Percentage of Alcohol *after* Pretreatment and Inoculation with standard CDB Strain *P. borealis*

3.7.4.4 Effect of pH on Percentage of Alcohol after Pretreatment and Inoculation with standard CDB Strain *P. borealis* and yeast strain *S. cerevisiae*:

Figure VIIh showed that when both *P. borealis* and *S. cerevisiae* were used, highest alcohol percentage was observed at pH 7 followed by pH 4, pH 5, pH 9, pH 12 and pH 14. Pomegranate showed a higher alcohol percentage than papaya. The alcohol percentage increased as the days went by. The increase in the alcohol percentage was observed to be higher when yeast was added.

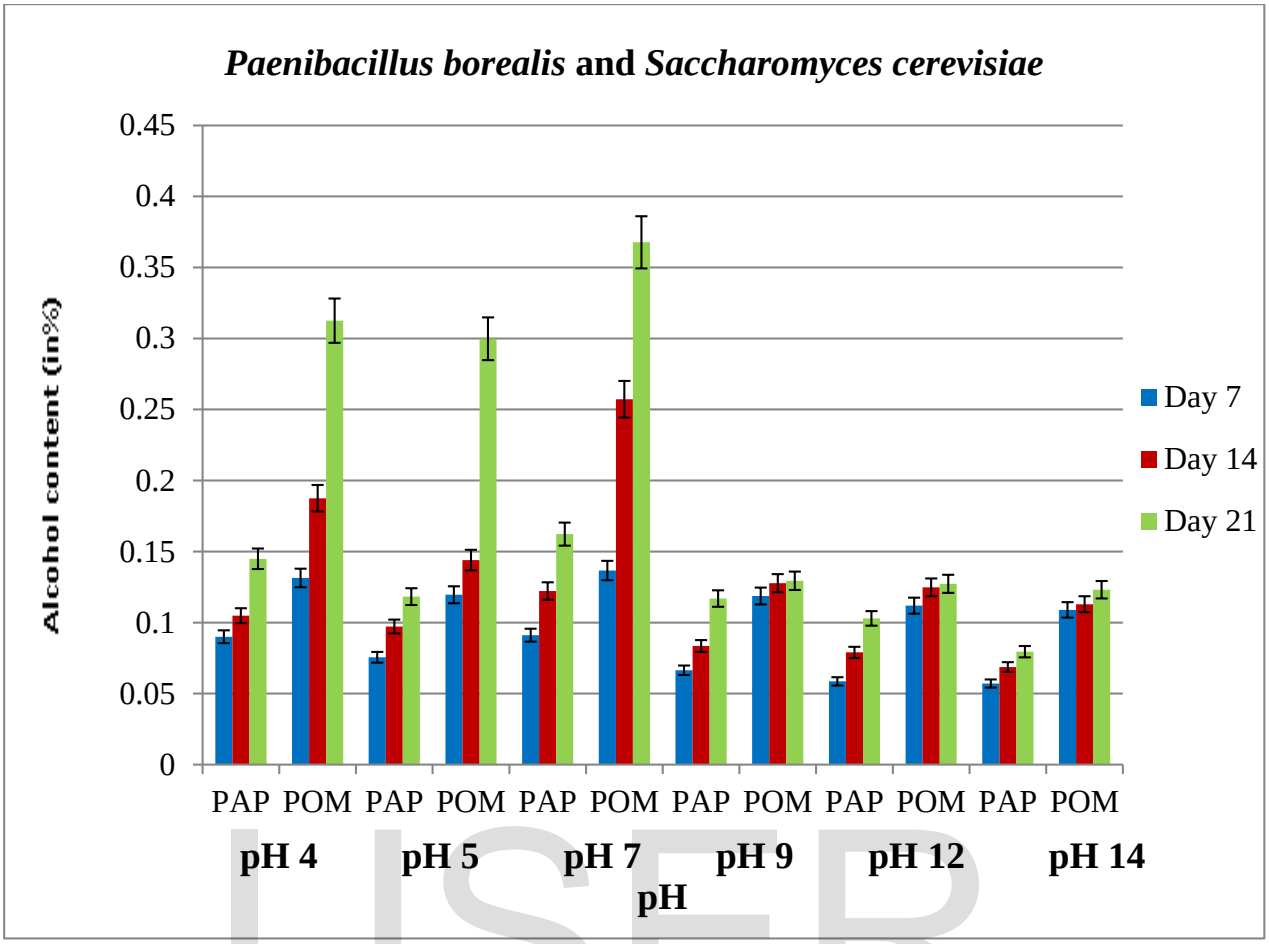

Figure VIIh: Effect of pH on Percentage of Alcohol after pretreatment and Inoculation with standard CDB Strain *P. borealis* and yeast strain *S. cerevisiae*

4. Conclusions

In the cell growth assay, it was observed that both the cultures (CDB isolated from vermicompost, *B. licheniformis* and the standard CDB, *P. borealis*) shared a similar (overlapping) initial phase; thereafter , a slight increase in the measured absorbance for the standard CDB strain when compared to that of the CDB strain isolated from vermicompost was observed. It was observed that both the cultures displayed the phases of comparable bacterial growth curve. The lag phase, log phase, stationary phase and death phase were observed.

The effect of temperature on the cellulolytic activity was analysed at temperatures 35°C and 45°C. For both the stains, i.e., *B. licheniformis* and *P. borealis*, the endoglucanase activity was higher at 35°C than at 45°C.This shows that 35°C is the optimum temperature for microbes. Henceforth the cellulytic activity decreases with increase in temperature. At both the temperatures, the endoglucanase activity of P. borealis was found to be slightly higher than that of *B. licheniformis*. The effect of pH on the cellulytic activity shows that pH 7 is the optimum temperature for microbes. Henceforth the cellulytic activity increases at neutral pH. In all the above-mentioned pH conditions, the endoglucanase activity of P. borealis was found to be slightly higher than that of *B. licheniformis*.

At the concentration range of 0.1g/l of different metal ions, *B. licheniformis* and P. borealis showed maximum activity with KH2PO4, and at the concentration range of 1g/L, both the strains showed maximum cellulytic activity with MnSO4,. In the presence of all the above-mentioned metal ions (at a concentration of 0.1g/l and 1g/l), the endoglucanase activity of P. borealis was found to be higher than that of *B. licheniformis*.

The carbohydrate content was measured prior to all experimental manipulations and found that before pre-treatment, pomegranate showed a higher carbohydrate content than papaya. After pre-treatment too, pomegranate showed a higher carbohydrate content then papaya. Assessing the cellulose content, we observed that the amount of cellulose in pomegranate is higher than in papaya before pre-

treatment, and it increases in both papaya and pomegranate, after pre-treatment, in the same ratio. This indicates that pomegranate has higher amount of fermentable sugar then papaya.

After pre-treatment, when the cultures where subjected to bio fermentation process with different temperature and pH ranges at different incubation periods. And it was observed that there was a gradual reduction in the amount of glucose and increase in the production of ethanol. When effects of temperature and pH on the glucose activity of the two fruit peel sources were compared, it was observed that pomegranate showed the maximum reduction in the amount of glucose, and more alcohol production. And in papaya, reduction in the glucose concentration, and corresponding less alcohol production were observed. Temperature 35°C and pH 7 were observed to be the optimum culture conditions for alcohol production.

Inoculation with a combination of *B. licheniformis* and *S. cerevisiae* gave better results, when compared to inoculation with only *B. licheniformis.* Similarly, inoculation with both P. borealis and *S. cerevisiae* gave better results, when compared to inoculation with only P. borealis. Commercially available CDB (P. borealis) is showing better results in alcohol conversion of fermentable sugar in fruit waste , but comparable results obtained with the CDB isolated from vermicompost shows that it is an ideal candidate easily available and can be subjected to experimental manipulations to obtain maximum alcohol production.

Therefore, the overall output of this study was that, the alcohol production was found to be higher with the use of a combination of cellulose degrading bacteria and yeast; and that pomegranate is better than papaya, when considering alcohol production.

5. ACKNOWLEDGEMENTS

The authors acknowledge MCC management, students and Commercial CDB provider - The Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh for the successful completion of this work.

REFERENCES

[1] Balat, M., "Global bio-fuel processing and production trends", *Energy Explore Exploit*, pp.195-218, 2007.
[2] Pramanik, K., & Rao, D.E., "Kinetic Study on Ethanol Fermentation of Grape Waste using Saccharomyces cerevisiae Yeast Isolated from Toddy", *Journal- Institution of Engineers India part Ch Chemical Engineering Division* 85 , 2 ; 53-58 ; 2005.
[3] Curreli, N. et al., "Complete and efficient enzymic hydrolysis of pretreated wheat straw", *Proc. Biochem*, pp. 937-941, 2002.
[4] Grohmann, K., Cameron, R. & Buslig, B., "Fractionation and pretreatment of orange peel by dilute acid hydrolysis", *Bioresour. Technol*., pp. 129-141, 1995.
[5] Kim, T. & Lee, Y., "Pretreatment of corn stover by soaking in aqueous ammonia at moderate temperatures", *Appl Biochem Biotechnol*, pp. 81-92, 2007.
[6] Mosier, N. et al., "Features of promising technologies for pretreatment of lignocellulosic biomass", *Biores Techno*l, pp. 673-686, 2005.
[7] Jacob, K. M. et al., "Isolation and Screening of Cellulose Degrading Microorganisms from the Gut of Composting Earthworms and its Industrial Applications", *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 5(3), pp. 501-507, 2014.
[8] Lalitha, G. & Rajeshwari, S., "Use of Fruit Biomass Peel Residue for Ethanol Production", *Internation Journal of Pharma and BioSciences*, 2(2), pp. 15-23, 2011.
[9] DuBois, M. et al., "Colorimetric Method for Determination of Sugars and Related Substances". *Analytical Chemistry*, 28(3), pp. 350-356, 1956.
[10] Updegraff, D., "Semimicro determination of cellulose in biological materials", *Analytical Biochemistry*, 32(3), pp. 420-424, 1969.
[11] Miller, G., "Use of Dinitrosaycilic acid Reagent for Determination of Reducing Sugar", *Analytical Chemistry*, 31(3), pp. 426-428, 1959.