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# ENHANCEMENT OF CELLULOLYTIC ENZYME PRODUCTION OF LIGNOCELLULOSE BY DEGRADING BACILLUS CONSORTIUM DURING PRETREATMENT OF WHEAT BRAN IN SUBMERGED BASAL MEDIUM.

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## Introduction

Biological pretreatment plays the dominant role in bioconversion of lignocellulosic biomass, by modifying the recalcitrant structure of lignocellulosic biomass for saccharification downstream process. Among various approaches, utilization of microorganisms is highly appreciated than commercial enzymes which are costly and sensitive with harsh conditions. Among bacteria, Bacillus species can sufficiently secrete extracellular enzymes to modification lignocellulolytic structure, make it more accessible to the hydrolysis by enzyme in saccharification process.

To enhance the cellulolytic enzyme production during the pretreatment, various microbial communities were employed. The synergistic action of members in consortium have been proved in previous reports. The microbial consortium are more stable and able to perform complicated tasks than monocultures, present a potential frontier for effective bioconversion of lignocellulose biomass.

**AIM:**

The purpose of this study is to screening various Bacillus species and construction of their microbial consortium for the enhancement of extracellular cellulolytic enzyme for the efficient degradation of wheat bran in submerged medium.

Screening individual strains → Selecting → Identification efficient consortium

## Materials and Methods

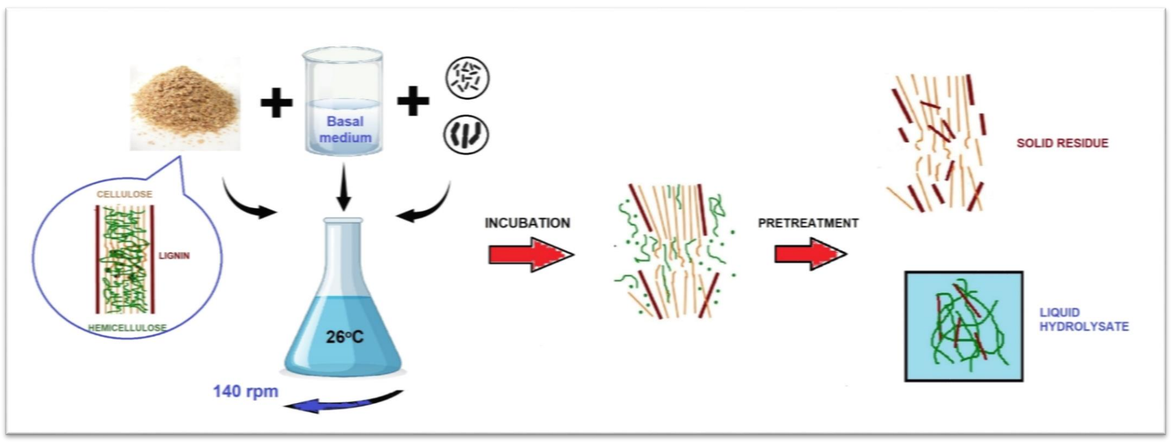
### MATERIALS AND METHODS

- Substrate: wheat bran
- Basal medium: ($gL^{-1}$) : lactose, 5.0; $NH_4NO_3$, 5.0; $KH_2PO_4$, 1.0; NaCl, 1.0; $MgSO_4.7H_20$, 0.6; $CaCl_2$, 0.1; $FeCl_3$, 0.01.
- The initial pH of the culture medium is adjusted with pH = 7, the initial inoculum of $10^5$CFU/ml and the incubation was proceeded at 30°C with agitation speed of 140rpm. The extract liquid was centrifugated to remove the solid residues before being analyzed. Sample was taken at 24 hours interval

**Table 1** : List of cellulolytic Bacillus species

| Genus | Species | NCAIM |
|---|---|---|
| *Bacillus* | *Bacillus subtilis* | B.01162 |
| | *Bacillus subtilis* | B.01212 |
| | *Bacillus licheniformis* | B.01223 |
| | *Bacillus licheniformis* | B.01231 |
| | *Bacillus cereus* | B.00076 |
| | *Bacillus cereus* | B.01718 |
| | *Bacillus coagulans* | B.01123 |
| | *Bacillus coagulans* | B.01139 |

### BIOPRETREATMENT PROCESS




**EVALUATION** →

- REDUCING SUGAR: Using Somogyi-Nelson method
- ENZYMATIC ACTIVITIES (Filter paper enzyme activity/total cellulase activity (FPase), carboxymethylcellulase/endo-glucanase (CMCase), xylanase) based on the activity of enzyme to hydrolyze proper substrates to reducing sugars and to p-nitrophenol in β-glucosidase assay

## Results

### ENZYMATIC PROFILE OF BACILLUS CONSORTIUM

The enzymatic profile of consortium AB was described in the Fig. 5 , the enzymatic activities including total cellulase (FPase), endo-glucanase (CMCase) and xylanase increased exponentially, obtaining 0.219; 0.791 and 2.187 U/ml, respectively. Longer pretreatment resulted in the slightly enhancement of total cellulase activity and xylanase, which could be regarded as the metabolic activity of Bacillus consortia in the presence of lignocellulose biomass (0.63 U/ml of total cellulase and 2.7 U/ml of xylanase). In turn, extracellular endo-glucanase activity dropped gradually during the time of pretreatment and remained at 0.47 U/ml in the last 3 days. The fluctuation of β-glucosidase during the pretreatment was observed, revealing the complexity of cellobiose cleavage action conducted by Bacillus strains in their co-cultures.

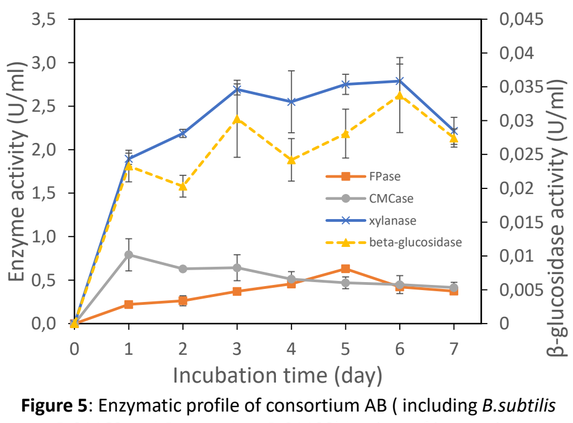



**Figure 5**: Enzymatic profile of consortium AB ( including *B.subtilis* B.01162 and *B.coagulans* B.01123) by time of incubation

## Conclusion

Among the Bacillus strains investigated, *B. cereus* and *B. coagulans* could degrade lignocellulosic biomass more efficiently than the remained strains in single cell cultivation. However, AB consortia, comprised of *B. subtilis* B.01162 and *B. coagulans* B.00076 are highly effective in pretreatment when the shorten optimal time of pretreatment and the enzyme activity of total cellulase and xylanase are improved, it was also highly adapted *B. licheniformis* strains. As the consequence, AB consortium is recommended to be applied in pretreatment process with optimized conditions, with the expectation for achieving higher glucose production for downstream process.

**References**

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[3] Poszytek, K., Ciezkowska, M., & Sklodowska, A. (2016). Microbial Consortium with High Cellulolytic Activity ( MCHCA ) for Enhanced Biogas Production, *7*(March), 1–11. https://doi.org/10.3389/fmicb.2016.00324

## Results

### Screening cellulase enzyme production

Screening of the cellulolytic bacteria was conducted using CMC agar plates with congo red straining method. The clear zones appeared on the surface of CMC agar flooded with 1% congo red were observed for all investigated *Bacillus* strains, thus preliminarily declaring the presence of the cellulase enzymes, specifically CMCase (endo-glucanases). Figure 1 illustrated the cellulolytic clear zone after staining in the CMC agar plates streaked by few *Bacillus* species.

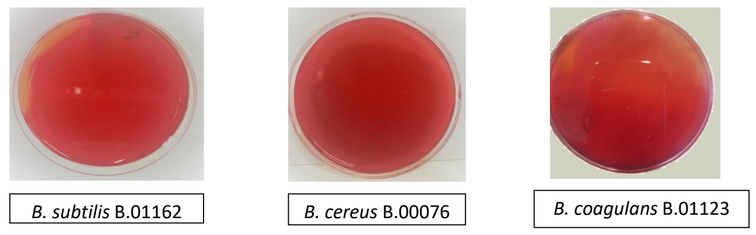



**Figure 1**: Cellulolytic clear zone appeared on CMC agar plates after Congo red staining

### SINGLE CELL PRETREATMENT

Reducing sugar was a one of the dominant factors to evaluate the lignocellulose degradation efficiency by catalytic enzymes secreted by microorganisms in submerged condition. It could be observed that *B. cereus*, *B. coagulans* successfully degraded wheat bran with overall higher reducing sugar contents than the other two strains (Fig.2). The maximal yield ranged from 240-254 mg/gds reached after 72 hours of cultivation by above strains. *B. subtilis* B.01162 placed at the second top among efficient strains with the highest reducing sugar obtained at 48 hours cultivation, value of 150mg / gds.

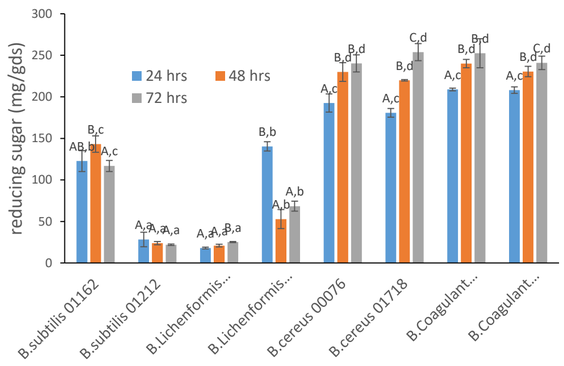
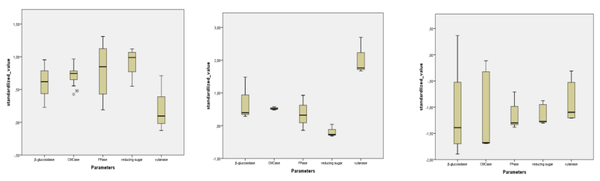



**Figure 2**: Hydrolysis profile of *Bacillus* strains in different incubation time. Data were presented as mean ± SD of three replicates

Meanwhile, extracted hydrolysate by *B. licheniformis* contained a sugar yield approximately of 140 mg/gds after 24 hours of incubation and it dramatically decreased within next day to 52 mg/gds. The remaining strains including *B. subtilis* B.01212 and *B. licheniformis* B.01223 were ineffective in pretreatment of lignocellulose biomass in shake flask cultivation.

### SELECTION OF STRAINS FOR CONSTRUCTION OF MICROBIAL CONSORTIUM




The total of 24 variances were classified into 3 clusters (Fig.3), including cluster A (*B. cereus* and *B. coagulans*), cluster B (*B. subtilis* B.01162) and cluster C (*B. licheniformis* and *B. subtilis* B.0112). Cluster A showed the highest enzyme activities among all investigated strains. These strains also performed the promising lignocellulolytic biomass degradation according to the high yield of fermented sugar measured by Somogyi-Nelson method. Xylanase activities of strains in cluster B varied less and remained at high level, however it may not significantly affect to the degradation process with low reducing sugar in pretreatment hydrolysate. Meanwhile, it was obvious that *B.licheniformis* and *B.subtilis* B.0112 which belong to cluster C were not as effective as other strains in single cell cultivation.

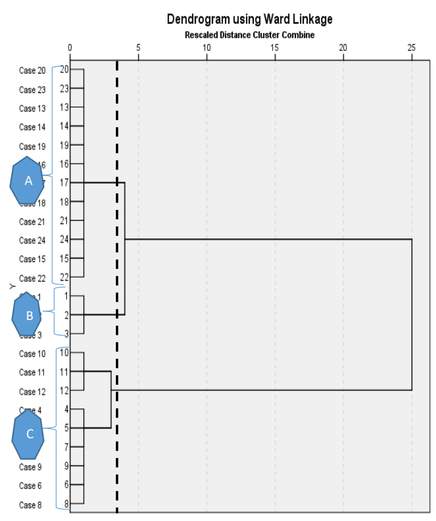



**Figure 3**: Classification of different strains of Bacillus based on hydrolytic enzymes.

### IDENTIFICATION OF HIGH ENZYME PRODUCTION CONSORTIUM

**Table 2:** Description of the cellulolytic consortium. All synthetic microbial communities were at least 3 replicates. Color cell indicated strains which were inoculated at 24hrs after other strains cultivation.

| Type of Mix | Synthetic community | Bacillus strains | | | | |
|---|---|---|---|---|---|---|
| 5 selected strains | denoted | A | B | C | D | E |
| 2-member consortium | AB | *B.subtilis* B.01162 | *B.coagulans* B.01123 | | | |
| | AC | *B.subtilis* B.01162 | | *B.cereus* B.00076 | | |
| | BC | | *B. coagulans* B.01123 | *B.cereus* B.00076 | | |
| 3-member consortium | ABC | *B.subtilis* 01162 | *B.coagulans* B.01123 | *B.cereus* B.00076 | | |
| | AB-D | *B.subtilis* B.01162 | *B.coagulans* B.01123 | | *B.Lichenformis* B.01223 | |
| | AB-E | *B. subtilis* B.01162 | *B.coagulans* B.01123 | | | *B.Lichenformis* B.01231 |
| | BC-D | | *B.coagulans* B.01123 | *B.cereus* B.00076 | *B.Lichenformis* B.01223 | |
| | BC-E | | *B.coagulans* B.01123 | *B.cereus* B.00076 | | *B.Lichenformis* B.01231 |
| | E-AB | *B. subtilis* B.01162 | *B.coagulans* B.01123 | | | *B.Lichenformis* B.01231 |
| | E-AC | *B. subtilis* B.01162 | | *B.cereus* B.00076 | | *B.Lichenformis* B.01231 |
| | E-BC | | *B.coagulans* B.01123 | *B.cereus* B.00076 | | *B.Lichenformis* B.01231 |

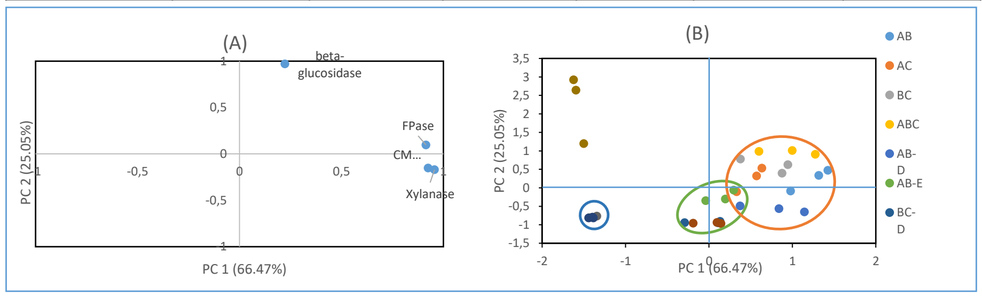



**Figure 4:** Principal component analysis (PCA) plot (factor loadings: component plot in rotated space; B: plot of regression factor on the first and second axes from PCA of 11 *Bacillus* consortia)

As shown in the Fig.4, dominant components were extracted by Principal component analysis (Fig.) PC 1 accounted for the 66.47% and PC2 for an additional 25.05% of the variability. FPase, CMCase, Xylanase were positively explained by the PC1 while PC2 loaded with β-glucosidase activity. The 2-member consortia including AB, AC, CD and 3 member consortia denoted as ABC, AB-D located at the right quadrant with high scores of cellulolytic enzyme activities, while the remain 6 consortia took place in other quadrants with lowest intensities of hydrolytic enzymes.

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