

# Co-cultivation of *T. asperellum* GDFS1009 and *B. amyloliquefaciens* 1841: Strategy to regulate the production of ligno-cellulolytic enzymes for the lignocellulose biomass degradation

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## ABSTRACT

The influence of fossil fuels on the environment focused on the development of new technology on biofuels. In this situation, lignocellulolytic hydrolysis enzymes such as Cellobiohydrolase,  $\beta$ -Glucosidase, Endoglucanase, cellulase and xylanase have broad applications in the biofuel production. The *Trichoderma* have used for the production of cellulase and xylanase to hydrolyze the lignocellulose. Hence, in the present study, co-culture has been employed to induce the production of polysaccharide hydrolyzing enzymes under both induction and repression conditions. The enzyme activity and its gene expression were induced by the co-culture of *T. asperellum* and *B. amyloliquefaciens* compared to the monoculture. Further, the co-culture upregulated the transcription regulatory genes and downregulated the repressor genes under both repressor and inducer conditions, respectively. The crude enzyme produced by the co-culture and monocultures using the optimized medium containing molasses, cornmeal and rice bran were further used to hydrolyze the pretreated corn Stover, rice straw, and wheat straw. These results indicate that the co-culture of *T. asperellum* and *B. amyloliquefaciens* is a promising and inexpensive method to advance the innovation on the continuous production of cellulase and xylanase under different circumstances for the bioconversion of lignocellulosic biomass into glucose for the biofuels.

## 1. Introduction

Consistent consumption of petroleum products affects the environmental conditions and demands the development of new technologies. The growing global population requires more energy consumption. The requirement for eco-friendly fuel such as biofuels from the agricultural biomass can reduce the utilization of petroleum products (Okolie et al., 2021). Hence, the research on developing the sustainable energy resources is in high need. The bioprospection of agricultural biomass specifically from crop residues can be an alternative source for the development of biofuels (Setiawan and Chiang, 2021). Dumping of agricultural biomass in the field is a frequent source of field contamination and reduces the subsequent crop yield, hence it can be used as a source of biofuels (Angulo-Mosquera et al., 2021). The polysaccharides

from these biomasses can be utilized for biofuel with the help of polysaccharide degrading micro-organisms (Bhujbal et al., 2021).

Cellulose and hemicellulose are the important ingredients of agricultural biomass and it has been utilized for biofuel preparation through enzymatic transformation. Highly effective enzymatic hydrolysis of cellulose is essential for the production of biofuels. Hence, the synthesis of effective cellulolytic degrading enzymes such as cellulase, xylanase, etc., has considerable attention on the lignocellulose degradation (Zhang et al., 2019). The majority of cellulase producing fungus is belonging to the genus *Trichoderma*, *Aspergillus*, *Mucor*, *Rhizopus*, and *Penicillium* etc. However, in bacteria, three different groups such as anaerobic cellulose-degrading bacteria, thermophilic cellulolytic bacteria, and alkalophilic cellulose-degrading bacteria are involved in the lignocellulose degradation (Behera et al., 2017; Saranraj et al., 2012;

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Singh et al., 2019).

In general, *Trichoderma* has been the most extensively studied fungus for the lignocellulolytic biomass degradation (Fitzpatrick et al., 2014; Karuppiyah et al., 2021; Xin and Geng, 2010). The regulations of hydrolyzing enzymes are depending upon the energy requirement of the fungus, i.e., the fungus produces these enzymes during the absence of glucose to convert the polysaccharide into glucose for its energy. Either the addition of glucose in the medium or the glucose as a product from the polysaccharides in the medium could inhibit the production of enzymes. In addition, expression of the cellulolytic hydrolyzing enzyme is also controlled by the different regulatory genes such as xylanase regulator 1 (*xyl1*), cellulase-related TFs (*ace1*, *ace2* and *ace3*), putative methyltransferase (*lae1*), Velvet A (*vel1*), beta-glucosidase regulator (*bglr*), *hap2*, *hap3*, and *hap5* complex, transcription repressor (*rce1*), and carbon catabolite repressor 1 (*cre1*) (de Paula et al., 2018; Zhang et al., 2018). The application of cellulosic biomass degradation through *Trichoderma reesei* has been widely attempted for the preparation of bio-ethanol (Zhang et al., 2019). The *T. reesei* Rut C-30 at higher inoculation conditions produced more soluble protein and higher filter paper activity (Domingues et al., 2000). Cellulase produced by *T. reesei* ZU-02 in corncob residue was higher than the cellulase produced from cellulose. Further, it increased the hydrolysis of corn cob for the biofuel production (Liming and Xueliang, 2004). *T. reesei* RUT C30 produced maximum cellulase on wheat bran for the ethanol production at 37.56% moisture and 30 °C temperature (Singhania et al., 2007). Continuous feeding of lingo-cellulosic biomass led to highest filter paper activity (FPAase) (19.85 FPU/ml) than batch and fed-batch fermentation by *T. reesei* RUT C30 (Xiang et al., 2021). Ligno-cellulosic biomass present in the natural environment is hydrolyzed by a group of microorganisms. The interactions between these microorganisms enhance the biomass degradation with in the short period. Further, The regulation on the continuous production of enzymes without inhibition by the end-product is required for the industrial application and bioenergy conversion (da Silva et al., 2020). In this scenario, the co-cultivation could be employed to induce the enzyme activity throughout the cultivation period without any inhibition by the end product (Marmann et al., 2014).

Co-culture achieves higher production of enzymes than the single culture. Additionally, the lignocellulolytic enzymes produced from the co-cultivation are more competitive to improve the lignocellulose hydrolysis (Srivastava et al., 2021; Zhao et al., 2021). Co-cultivation of *Trichoderma* with other bacteria was attempted less to effectively induce the production of cellulolytic hydrolyzing enzymes, particularly little work was carried out to understand the significance on co-culture of *Trichoderma* and *Bacillus* for biofuel preparation, even though the combined use of both microbes has been widely used for the plant disease biocontrol. In our previous study, the co-cultivation of *T. asperellum* and *B. amyloliquefaciens* increased the production of chitinase,  $\beta$  1–3glucanase, cellulase, and protease in the YMC medium (Karuppiyah et al., 2019a, 2019b). Therefore, it is important to induce the enzymatic complex containing endoglucanase, exoglucanase, like cellobiohydrolase, xylanase and  $\beta$ -glucosidase to hydrolyze the lignocellulolytic biomass. However, there is no study dealing with cellulolytic hydrolyzing enzyme production through the co-cultivation of *T. asperellum* and *B. amyloliquefaciens*. The strategy of co-culture could increase microbial productivity and enzymatic systems, including the degradation of lignocellulosic biomass for substrate competition, which provokes an accelerated synthesis of the target enzymes under both induction and repression conditions. In particular, no study report so far is available on the continuous production of enzymes under both induction and repression conditions using the co-culture of *T. asperellum* and *B. amyloliquefaciens*.

The main aim of the present study was used to evaluate the potential of lignocellulolytic enzymes production from the co-culture of *T. asperellum* and *B. amyloliquefaciens* to hydrolyze the pretreated corn stover, rice straw and, wheat straw with the following objectives. (i)

Continuous production of lignocellulolytic enzymes complex such as Filter paper activity (FPAase), endoglucanase (CMCase), cellobiohydrolase (pNPCase), and  $\beta$ -glucosidase (pNPGase) under different types of inducers (avicel and lactose) and repressors (glucose). (ii) The expression of genes encoding cellulase and xylanase and the regulation of transcription regulatory genes under different regulatory conditions. (iii) Optimize the enzyme production medium with the low-cost and easily available agro-industrial waste. (iv) Specify the cocktail of crude enzyme of the optimized medium to increase lignocellulose hydroxylation.

## 2. Materials and methods

### 2.1. Strains and growth conditions

The *T. asperellum* GDFS1009 (CGMCC NO. 9512) and *B. amyloliquefaciens* 1841 (CGMCC NO. 15465) were obtained from the China General Microbiological Culture Collection Center (CGMCC), Beijing. *T. asperellum* GDFS1009 was cultured on Potato Dextrose agar at 28 °C, and *B. amyloliquefaciens* was cultured in Luria-Bertani medium at 37 °C.

### 2.2. Production of lingo-cellulolytic enzymes and enzyme assay

*T. asperellum* ( $10^6$ /mL spores) was added into 100 mL of minimal medium supplemented with either 2% glucose, lactose, or avicel as described by Zhang et al. (2018). Consequently, 100  $\mu$ l of *B. amyloliquefaciens* 1841 was inoculated into the 24th and 48th hour *T. asperellum* pre-culture medium and named as TB1 and TB2 respectively. Further, it was incubated at 180 rpm at 28 °C until 72 h s. Filter paper activity (FPAase), endoglucanase (CMCase), cellobiohydrolase (pNPCase), and  $\beta$ -glucosidase (pNPGase) were estimated as explained by Zhang et al. (2018). The activity of Xylanase I and II was estimated at pH 3.7 and 5.0, respectively (Stricker et al., 2008). The concentration of secreted protein was estimated according to the Lowry et al. (1951). All experiments were performed in biological triplicates.

### 2.3. RNA extraction, cDNA synthesis, and RT-qPCR analysis

Total RNA from the co-culture and monoculture were isolated as suggested by Karuppiyah et al. (2019a). Followed by, the cDNA was synthesized using the Prime Script RT reagent kit with a DNA eraser (Takara). The expression of cellulase-related genes, xylanase genes, accessory protein gene, positive transcription regulators, and repressors were estimated using RT-qPCR and normalized with the elongation factor 1 gene through the  $2^{-\Delta\Delta Ct}$  method. The primers used are shown in Table S1. The cycling condition includes 95 °C for 5 min, 35 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. All experiments were carried out in three independent experiments in triplicate.

### 2.4. Optimization of lingo-cellulolytic enzyme production using the response surface methodology

The central composite design (CCD) was used to optimize the fermentation medium using the commercial substrate to produce the lingo-cellulolytic hydrolyzing enzymes using the mono and co-culture. The CMCase activity was used as a common test to optimize the medium. Three factors such as molasses, corn gluten meal and rice bran at five levels in the range of 0–4% (Table 2). 20 different experiments were performed to optimize the fermentation medium based on CMCase activity (Table 2). The results of this model were evaluated using ANOVA (Minitab-14) to define the fitness and interaction between different variables. The CMCase production was optimized based on the polynomial equation as suggested by Karuppiyah et al. (2019a).

## 2.5. Pretreatment and enzymatic hydrolysis of lignocellulose biomass

Corn Stover, rice straw, and wheat straw were pretreated as described by Tsegaye et al. (2019). The crude enzymes produced by the *T. asperellum* in both mono-culture and co-cultures of the optimized medium were used to hydrolyze the lignocellulose biomass with the particle size of 2–5 mm according to the method of Zhang et al. (2018). Hydrolysis of corn Stover, rice straw, and wheat straw was carried out in a flask comprising 10% (w/v) of each biomass with crude enzyme (15 FPIU/ml) at 37 °C and pH 6 for 48 h. The glucose concentration was measured by HPLC with RI detection (Agilent 1100 RI, CA, USA) using the Aminex HPX-87H column (Bio-Rad, Copenhagen, Denmark). The concentration of glucose in the hydrolysis sample was calculated using the peak area of the standard glucose. The column temperature was set at 60 °C and 5 mM H<sub>2</sub>SO<sub>4</sub> was used as eluent with a flow rate of 0.6 ml/min.

## 2.6. Statistical analysis

All experiments were carried out in triplicates. The graphs were plotted using Microsoft office excel and origin 6.0. Results shown were mean of triplicate values through standard error. The student's T-test was carried out to observe the changes between the gene expressions through the SPSS 20. P < 0.05 was considered as significant. The optimization of the lingo-cellulolytic enzyme production was statistically carried out using Minitab 18 software.

## 3. Results

### 3.1. Co-cultivation stimulates lignocellulolytic enzyme production under the presence of both inducers and repressors

The induction medium (avicel and lactose) was used to examine the production of lignocellulolytic enzyme by the co-culture of *T. asperellum* and *B. amyloliquefaciens* (Table 1 and Table S2). Noticeably, the activity

of FPase, pNPCase, pNPGase, and CMCase of TB2 and TB1 in lactose and avicel were higher than that of monocultures (Table 1 and Table S2). Few studies have reported that lactose is an inducer for cellulases production (Dhillon et al., 2012). Hence, in the present study, lactose was used as an inducer to assess its influence on the production of the cellulolytic enzyme. Three samples, including the mono-culture (T) and co-culture (TB1 and TB2), were tested to recognize the induction of FPAase, CMCase, PNPCase, PNPCase, xylanase I and xylanase II by the lactose. The monoculture of *T. asperellum* showed 2.96, 0.91, 26.68, 2.1, 39.76, and 39 IU/ml activity of FPAase, CMCase, PNPCase, PNPCase, xylanase I and xylanase II, respectively (Table 1). TB2 showed 4.81, 1.92, 37.45, 2.8, 51.23, and 51 IU/ml of FPAase, CMCase, PNPCase, PNPCase, xylanase I, and xylanase II at 120 h, respectively, which was higher than the monoculture (T) and co-culture TB1. Furthermore, the TB2 showed enhanced activity in avicel compared to lactose. The FPase activity in TB2 (9.85 U/ml) was superior compared to the monoculture (Table 1). Further, pNPCase (3.93 U/ml) and CMCase (53.43 U/ml) of TB2 were also better than monoculture. Similarly, the pNPGase, xylanase I, and II activities also exhibited significant changes between co-culture and monoculture (Table 1). The enzyme activity was not detected in the monoculture of *B. amyloliquefaciens*. 7.2 mg/ml and 6.52 mg/ml secreted protein was identified in the TB2 grown on avicel and lactose-containing media respectively, which was higher than that of the monoculture of *T. asperellum* (Table 1). It was noticeable that co-culture achieved the highest enzyme activities at 96 h than monoculture. The rapid and highest lignocellulolytic enzyme activity by co-culture was not reported previously. Hence, these highest enzyme productions could be efficiently utilized for biomass degradation.

Cellulase and xylanase production of *Trichoderma* is always depend on the carbon source availability. Hence, glucose was used as the metabolite repressor to examine the effects of co-culture on the continuous production of an enzyme (Table 1). In TB2, FPase, pNPCase, CMCase, and pNPGase activities were increased than that of monocultures (Table 1). TB2 showed the highest production of cellulase and xylanase under glucose repression conditions than that of the

**Table 1**

Comparison of cellulase and xylanase production by monoculture (T) and the co-culture (TB1 and TB2) at different time intervals in the media containing 2% avicel, lactose, and glucose.

| Fermentation time         | Minimal medium supplemented with 2% avicel |              |              | Minimal medium supplemented with 2% lactose |              |              | Minimal medium supplemented with 2% glucose |             |             |
|---------------------------|--------------------------------------------|--------------|--------------|---------------------------------------------|--------------|--------------|---------------------------------------------|-------------|-------------|
|                           | TA                                         | TB1          | TB2          | TA                                          | TB1          | TB2          | TA                                          | TB1         | TB2         |
| FPase (U/ml)              |                                            |              |              |                                             |              |              |                                             |             |             |
| 72                        | 6.34 ± 0.21                                | 7.26 ± 0.21  | 7.83 ± 0.11  | 2.23 ± 0.32                                 | 2.86 ± 0.48  | 3.12 ± 0.54  | 0.12 ± 0.02                                 | 0.62 ± 0.02 | 1.82 ± 0.01 |
| 96                        | 7.92 ± 0.51                                | 8.55 ± 0.51  | 8.67 ± 0.51  | 2.45 ± 0.34                                 | 3.15 ± 0.52  | 3.94 ± 0.62  | 0.34 ± 0.05                                 | 0.98 ± 0.05 | 2.12 ± 0.05 |
| 120                       | 8.23 ± 0.41                                | 9.15 ± 0.31  | 9.85 ± 0.41  | 2.96 ± 0.25                                 | 4.22 ± 0.35  | 4.81 ± 0.24  | 0.65 ± 0.04                                 | 1.51 ± 0.03 | 3.25 ± 0.04 |
| PNPCase (U/ml)            |                                            |              |              |                                             |              |              |                                             |             |             |
| 72                        | 0.83 ± 0.13                                | 1.43 ± 0.21  | 1.83 ± 0.23  | 0.44 ± 0.21                                 | 0.83 ± 0.46  | 0.92 ± 0.21  | 0.13 ± 0.013                                | 0.64 ± 0.02 | 0.92 ± 0.02 |
| 96                        | 1.65 ± 0.51                                | 2.67 ± 0.34  | 3.14 ± 0.45  | 0.8 ± 0.32                                  | 1.23 ± 0.23  | 1.54 ± 0.39  | 0.23 ± 0.05                                 | 0.91 ± 0.03 | 1.2 ± 0.04  |
| 120                       | 2.13 ± 0.21                                | 3.25 ± 0.56  | 3.93 ± 0.31  | 0.91 ± 0.51                                 | 1.74 ± 0.5   | 1.92 ± 0.53  | 0.65 ± 0.02                                 | 1.41 ± 0.05 | 1.7 ± 0.03  |
| CMCase (U/ml)             |                                            |              |              |                                             |              |              |                                             |             |             |
| 72                        | 30.23 ± 0.12                               | 41.45 ± 0.26 | 45.34 ± 0.48 | 18.27 ± 0.23                                | 22.36 ± 0.32 | 26.57 ± 0.23 | 1.6 ± 0.01                                  | 4.78 ± 0.02 | 5.8 ± 0.04  |
| 96                        | 38.65 ± 0.32                               | 47.87 ± 0.25 | 51.76 ± 0.51 | 21.89 ± 0.51                                | 29.56 ± 0.42 | 32.46 ± 0.25 | 2.8 ± 0.03                                  | 5.91 ± 0.02 | 7.25 ± 0.05 |
| 120                       | 38.76 ± 0.15                               | 50.45 ± 0.34 | 53.43 ± 0.34 | 26.68 ± 0.34                                | 32.78 ± 0.31 | 37.45 ± 0.16 | 5.7 ± 0.01                                  | 7.5 ± 0.03  | 8.15 ± 0.03 |
| PNPGase (U/ml)            |                                            |              |              |                                             |              |              |                                             |             |             |
| 72                        | 1.01 ± 0.35                                | 1.56 ± 0.45  | 1.87 ± 0.51  | 0.8 ± 0.29                                  | 1.2 ± 0.23   | 1.7 ± 0.25   | 0.09 ± 0.03                                 | 0.23 ± 0.04 | 0.51 ± 0.05 |
| 96                        | 1.24 ± 0.45                                | 2.74 ± 0.32  | 2.94 ± 0.26  | 1.9 ± 0.6                                   | 2.1 ± 0.19   | 2.7 ± 0.35   | 0.19 ± 0.04                                 | 0.49 ± 0.03 | 0.75 ± 0.02 |
| 120                       | 1.59 ± 0.23                                | 3.13 ± 0.25  | 3.52 ± 0.39  | 2.1 ± 0.42                                  | 2.2 ± 0.35   | 2.8 ± 0.9    | 0.32 ± 0.02                                 | 0.51 ± 0.02 | 0.92 ± 0.03 |
| Xylanase 1 (U/ml)         |                                            |              |              |                                             |              |              |                                             |             |             |
| 72                        | 41.34 ± 0.23                               | 43.63 ± 0.16 | 48.65 ± 0.42 | 18.45 ± 0.31                                | 19.55 ± 0.42 | 21.45 ± 0.16 | 1.25 ± 0.02                                 | 1.95 ± 0.01 | 2.56 ± 0.04 |
| 96                        | 45.67 ± 0.12                               | 47.45 ± 0.28 | 51.64 ± 0.39 | 35.54 ± 0.53                                | 42.87 ± 0.51 | 43.76 ± 0.24 | 2.6 ± 0.01                                  | 3.85 ± 0.02 | 4.15 ± 0.03 |
| 120                       | 49.26 ± 0.32                               | 52.64 ± 0.34 | 55.53 ± 0.21 | 39.76 ± 0.23                                | 49.94 ± 0.32 | 51.23 ± 0.35 | 4.51 ± 0.03                                 | 5.61 ± 0.03 | 6.24 ± 0.02 |
| Xylanase 2 (U/ml)         |                                            |              |              |                                             |              |              |                                             |             |             |
| 72                        | 42.43 ± 0.61                               | 43.78 ± 0.51 | 47.45 ± 0.49 | 18 ± 0.34                                   | 19.5 ± 0.33  | 21.4 ± 0.32  | 1.53 ± 0.06                                 | 1.56 ± 0.05 | 2.48 ± 0.04 |
| 96                        | 47.76 ± 0.59                               | 48.97 ± 0.26 | 51.87 ± 0.26 | 35 ± 0.36                                   | 42 ± 0.31    | 43 ± 0.41    | 2.96 ± 0.05                                 | 3.58 ± 0.02 | 4.26 ± 0.02 |
| 120                       | 50.35 ± 0.26                               | 52.36 ± 0.38 | 56.93 ± 0.34 | 39 ± 0.25                                   | 49 ± 0.32    | 51 ± 0.12    | 5.12 ± 0.02                                 | 5.92 ± 0.03 | 6.34 ± 0.03 |
| Secreted proteins (mg/ml) |                                            |              |              |                                             |              |              |                                             |             |             |
| 72                        | 3.12 ± 0.25                                | 4.75 ± 0.42  | 5.12 ± 0.51  | 2.82 ± 0.31                                 | 3.98 ± 0.25  | 4.88 ± 0.45  | 2.56 ± 0.02                                 | 3.21 ± 0.04 | 4.26 ± 0.05 |
| 96                        | 4.57 ± 0.36                                | 5.86 ± 0.43  | 6.48 ± 0.25  | 3.54 ± 0.35                                 | 4.52 ± 0.32  | 5.61 ± 0.32  | 3.46 ± 0.03                                 | 4.56 ± 0.04 | 5.48 ± 0.02 |
| 120                       | 5.41 ± 0.32                                | 6.53 ± 0.42  | 7.2 ± 0.35   | 4.35 ± 0.51                                 | 5.46 ± 0.12  | 6.52 ± 0.21  | 4.21 ± 0.03                                 | 5.14 ± 0.04 | 6.34 ± 0.03 |

**Table 2**

Central composite design for the production of cellulose using monoculture and co-culture along with the observed and predicted results.

| S. No. | Molasses (g/100 ml) | Cornmeal (g/100 ml) | Rice Bran (g/100 ml) | Cellulase activity (U/ml) |           |                 |           |
|--------|---------------------|---------------------|----------------------|---------------------------|-----------|-----------------|-----------|
|        |                     |                     |                      | Monoculture (T)           |           | Co-culture (TB) |           |
|        |                     |                     |                      | Observed                  | Predicted | Observed        | Predicted |
| 1      | 1                   | 1                   | 2                    | 32.76                     | 32.72     | 33.12           | 33.02     |
| 2      | 3                   | 1                   | 2                    | 33.69                     | 33.71     | 35.67           | 35.71     |
| 3      | 1                   | 3                   | 2                    | 33.69                     | 33.71     | 35.67           | 35.72     |
| 4      | 3                   | 3                   | 2                    | 33.46                     | 33.54     | 35.03           | 35.25     |
| 5      | 1                   | 1                   | 4                    | 33.93                     | 33.89     | 36.3            | 36.20     |
| 6      | 3                   | 1                   | 4                    | 33.22                     | 33.24     | 34.39           | 34.45     |
| 7      | 1                   | 3                   | 4                    | 33.69                     | 33.71     | 35.62           | 35.69     |
| 8      | 3                   | 3                   | 4                    | 31.82                     | 31.90     | 30.56           | 30.78     |
| 9      | 0                   | 2                   | 3                    | 29.01                     | 29.04     | 22.93           | 23.02     |
| 10     | 4                   | 2                   | 3                    | 28.31                     | 28.22     | 21.02           | 20.80     |
| 11     | 2                   | 0                   | 3                    | 32.76                     | 32.79     | 33.12           | 33.22     |
| 12     | 2                   | 4                   | 3                    | 32.52                     | 32.43     | 32.48           | 32.25     |
| 13     | 2                   | 2                   | 1                    | 32.29                     | 32.26     | 31.85           | 31.79     |
| 14     | 2                   | 2                   | 5                    | 31.82                     | 31.79     | 30.57           | 30.49     |
| 15     | 2                   | 2                   | 3                    | 39.7                      | 39.95     | 52.23           | 52.63     |
| 16     | 2                   | 2                   | 3                    | 40.01                     | 39.95     | 52.87           | 52.63     |
| 17     | 2                   | 2                   | 3                    | 40.01                     | 39.95     | 52.87           | 52.63     |
| 18     | 2                   | 2                   | 3                    | 39.78                     | 39.95     | 52.23           | 52.63     |
| 19     | 2                   | 2                   | 3                    | 39.78                     | 39.95     | 53.5            | 52.63     |
| 20     | 2                   | 2                   | 3                    | 40.48                     | 39.95     | 52.23           | 52.63     |

monoculture. For example, the FPase activity (3.25 U/ml) of TB2 at 120 h was higher than the monocultures (Table 1). Similarly, the pNPCase, pNPGase activities, and CMCase in co-cultures were also increased compared to that of monocultures at 120 h (Table 1). Furthermore, the xylanase I (6.24 U/ml) and xylanase II activities (6.34 U/ml) in TB2 were superior compared to the monocultures at 120 h (Table 1). Moreover, the extracellular protein content of TB1 and TB2 under repressive conditions also improved than that of monocultures at 120 h (Table 1). These results showed that co-culture could induce the production of secretory protein production under glucose.

### 3.2. The effect of co-culture on the transcript levels of cellulase-and xylanase related genes

To know the further mechanism of co-culture on the production of cellulase and xylanase the expression of cellulase-associated genes (Cellobiohydrolases 1 (*cbh1*), Cellobiohydrolases 2 (*cbh2*), Endoglucanases 1 (*egl1*), Endoglucanases 2 (*egl2*), and  $\beta$ -Glucosidase (*bgl1*)), xylanase (*xyn1* and *xyn2*), accessory protein (*swo1*), positive transcription regulators (xylanase regulator 1, cellulose-related TFs *ace2*, *ace3*, putative methyltransferase, Velvet A, beta-glucosidase regulator, *hap2*, *hap3*, and *hap5*) and repressors (cellulose-related TFs *ace1*, transcription repressor *rce1*, and carbon catabolite repressor 1) at 72 h were analyzed using RT-qPCR (Fig. 1). The co-culture showed improved expression of *cbh1*, *cbh2*, *egl1*, and *egl2* than the monoculture in the induction medium (Fig. 2a and b), which coincided with the enzyme activities of the co-culture. As shown in Fig. 1b, the relative quantification of the *cbh1*, *cbh2*, *egl1*, *egl2*, *bgl1*, *xyn1*, and *xyn2* genes in TB2 were upregulated 3.88, 3.46, 3.17, 2.71, 2.57, 2.51 and 2.31 fold, respectively in the presence of lactose compared to the monoculture. Furthermore, avicel increased the cellulase and xylanase gene expression compared to the lactose. Markedly, TB2 exhibited the highest expression compared to TB1. The expression of *cbh1*, *cbh2*, *egl1*, *egl2*, *bgl1*, *xyn1*, and *xyn2* in the TB2 was improved about 5.6, 5.4, 4.2, 4.1, 3.6, 4.1 and 3.4 fold, respectively in the avicel containing media compared to the monoculture (Fig. 1a). Further, the expression of the *swo1* gene in TB2 and TB1 was increased than that of monocultures (Fig. 1a). Moreover, the expression of inducer genes such as *xyr1*, *ace2*, *ace3*, *hap2/3/5* complex, *lae1*, *vel1*, and *bgl1r* also exhibited significant changes in the co-culture, however the expression of repressor genes was downregulated in the co-cultures compare to the monocultures (Fig. 1d and e). These results

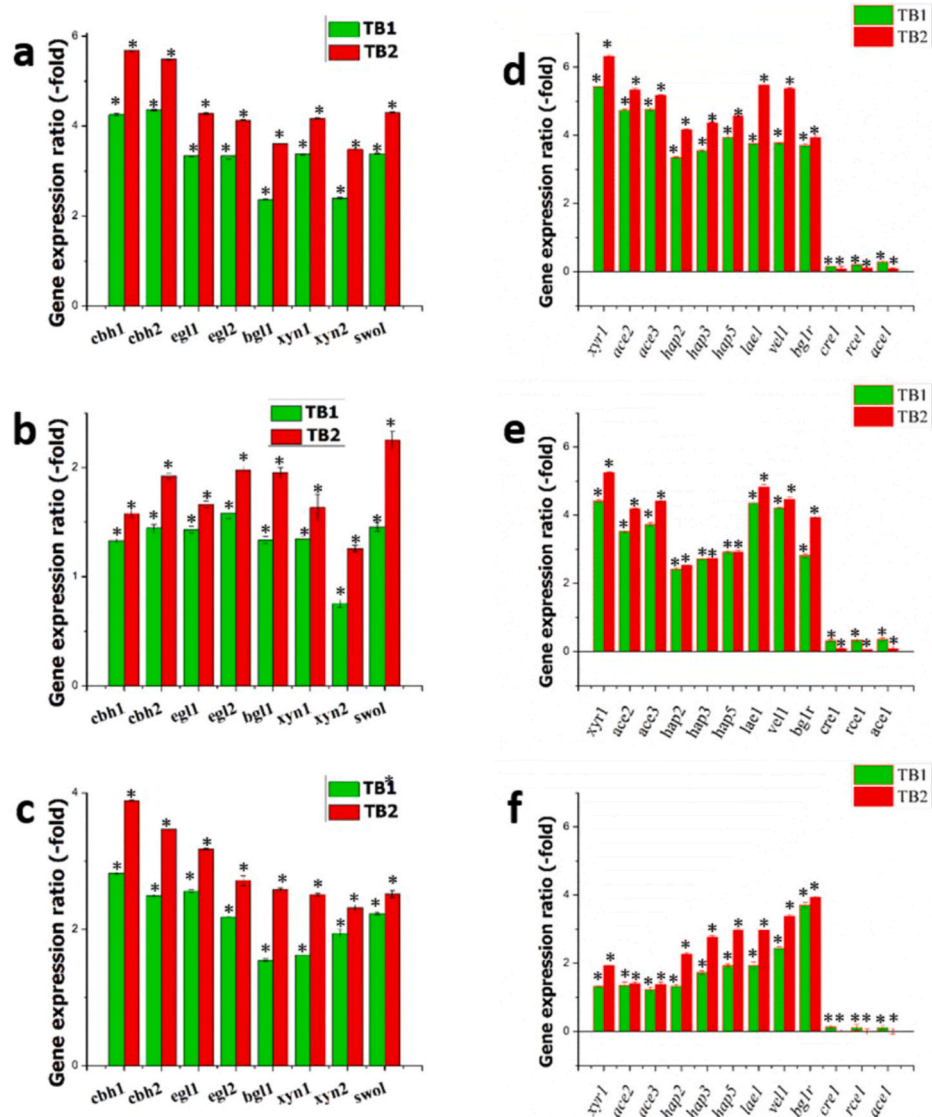
revealed that the co-culture induces the expression of the cellulase and xylanase genes by the regulation of the transcriptional regulatory genes.

Consistently, the expression of *cbh1*, *cbh2*, *egl1*, *egl2*, *bgl1*, *xyn1*, and *xyn2* in the TB2 were increased 1.5, 1.9, 1.6, 1.9, 1.9, 1.6, and 1.2 fold, respectively at 72 h than the monoculture under repressive conditions (Fig. 1c). The expression of *xyr1*, *ace2*, *ace3*, *hap2/3/5* complex, *lae1*, *vel1*, and *bgl1* were also increased in the TB1 and TB2 (Fig. 1f) under repression conditions. In the co-culture, the transcript levels of *xyr1*, *ace2*, *ace3*, *hap2/3/5* complex, *lae1*, *vel1*, and *bgl1r* were greater than the monoculture at 72 h. However, the fold of repressor genes was decreased in the co-cultures compared to the monocultures. The enhanced transcription of *xyr1*, *ace2*, *ace3*, *hap2/3/5* complex *lae1*, *vel1*, and *bgl1* genes by co-cultivation could induce the expression of *cbh1*, *cbh2*, *egl1*, *egl2*, and *bgl1* under repression condition (de Paula et al., 2018). Moreover, the expression of *swo1* was also increased in the co-culture of TB2 (Fig. 1c).

### 3.3. Optimization of cellulase production using the response surface methodology

In this study, the CCD method was selected to optimize the medium composition for the maximum production of enzymes to hydrolyze the lignocellulosic biomass. Table 2 shows the CMCase production by the monoculture and co-culture with a different range of substrates at 72 h. The observed and predicted values of CMCase activity of the monoculture and co-culture were shown in Table 2. The observed and predicted CMCase activity of all experiments was statistically analyzed using ANOVA (Table S3 and S4). The significance of each variable was determined using the P-value with 95% confidence. The P-value was less than 0.05 was considered as the most significant. The P-value 0.00 of T and TB demonstrate that the model is significant for both monoculture and co-culture. Similarly, linear, square, and 2-way interactions were also significant. The linear term of cornmeal for T was not significant, which reveals that it could not be utilized by the monoculture. But, the cornmeal is significant for TB, which reveals that it is required for the growth of *Trichoderma* and *Bacillus* during co-culture.

Moreover, the P-value of lack of fit showed that it was not significant, which designates that the design was suitable for the cellulolytic enzyme production by T and TB respectively. The adjusted R square (0.9970 and 0.9985) and predicted R square (0.9969 and 0.9980) of T and TB explained the closest variation between the predicted and observed



**Fig. 1.** Assessment of cellulase, xylanase (a–c), and transcription factors (d–f) related genes in monoculture (T) and the co-culture (TB1 and TB2). Gene expression fold changes in co-culture (TB1 and TB2) shown were relative to monoculture (T) on 2% avicel (a, d), lactose (b, e), and glucose (c, f) at 72 h. Values are the average of biological triplicates. Error bars represent the standard error. Asterisks refer to significant differences from monoculture (\* $p < 0.05$ , Student's t-test).

CMCase activity. The predicted R square of T and TB were also closer to the R square, which reveals that the model was fitted. The quadratic polynomial equation of T and TB in response to CMCase activity was shown below.

$$T = -0.961 + 12.922A + 8.706B + 13.170C - 2.8292AA - 1.8342BB - 1.9805CC - 0.2900AB - 0.4100AC - 0.2925BC \quad (1)$$

$$TB = -58.41 + 35.082A + 23.647B + 35.731C - 7.6805AA - 4.9742BB - 5.3717CC - 0.793AB - 1.110AC - 0.803BC \quad (2)$$

Where T and TB are the measured response of the CMCase activity of monoculture and co-culture, respectively. A, B, and C are the variables of molasses, corn meal and rice bran, respectively.

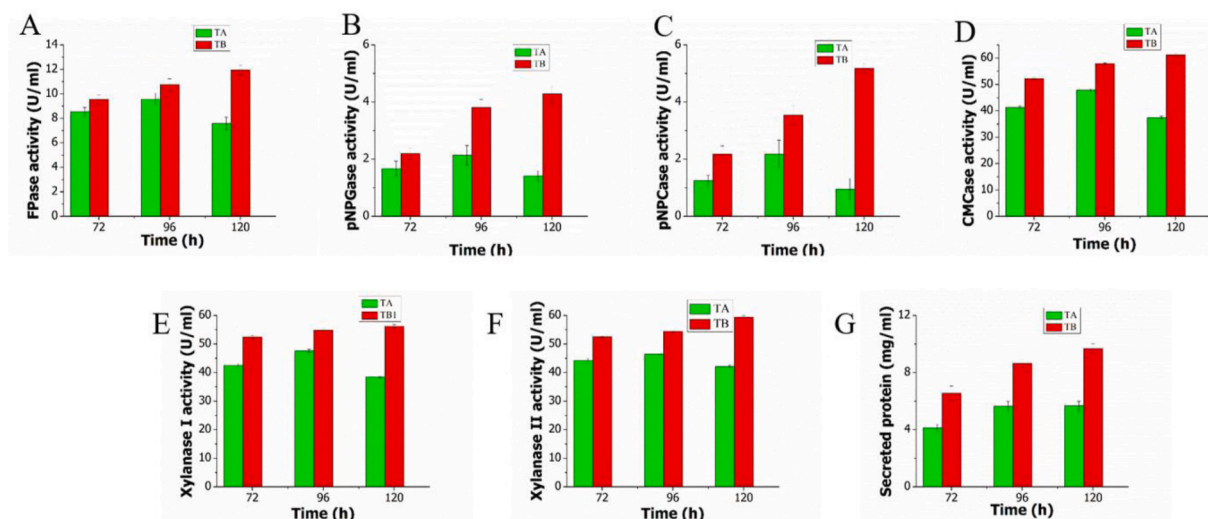
The response surface plot for each combination was plotted by keeping the remaining variables at the central level (Fig. S1). The surface plot showed that the maximum activity was recorded at 1.97 g of molasses and cornmeal and 2.97 g of rice bran, while the CMCase activity was reduced at the higher and lower level of all variables. The medium was optimized based on the response optimization results of response surface methodology and it was validated in the laboratory.

The 42.75 and 54.64 IU/ml of CMCase activity was obtained from T and TB respectively, which was higher than the predicted results of response optimization (Fig. S2).

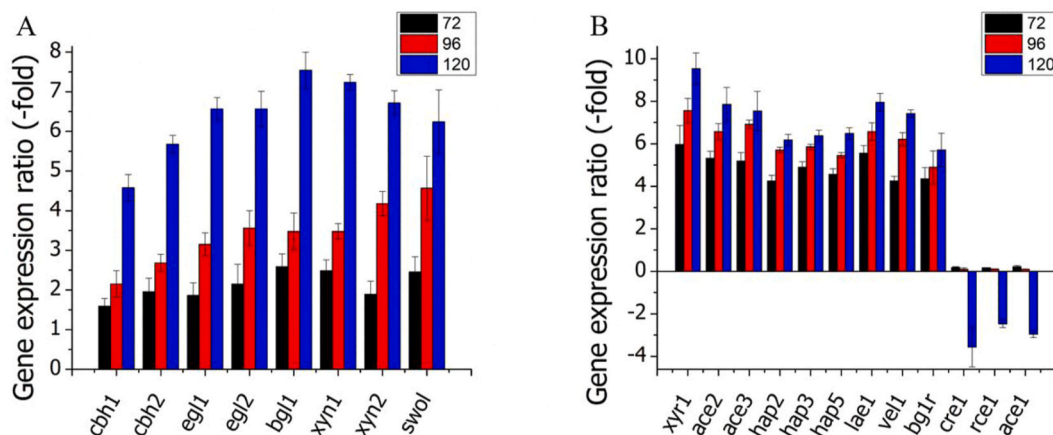
The optimized medium was consequently tested for the production of the cellulolytic enzymes and their gene expression. Noticeably, the activity of FPase, pNPCase, pNPCase, chemicals, xylanase 1 and 2 of the mono-culture and co-culture in the optimized medium was higher than that of the minimal medium containing avicel and lactose (Fig. 2). The results of the gene expression of cells-associated genes (*cbh1*, *cbh2*, *egl1*, *egl2*, and *bgl1*), xylanase (*xyn1* and *xyn2*), and accessory protein (*swol*) of co-culture were unregulated compared to the mono-culture (Fig. 3). Consistently, the expression of *xyn1*, *ace2*, *ace3*, *hap2/3/5* complex *lae1*, *vel1*, and *bgl1r* were increased in the co-culture. The repressor genes such as *ace1*, *rce1*, and *cre1* were highly downregulated in TB2 compared to the monoculture (Fig. 3).

#### 3.4. Hydrolysis of cellulosic and hemicellulosic biomass by co-culture

Scarification by lignocellulosic degrading enzymes of the mono-culture and co-cultures were performed using the pretreated corn



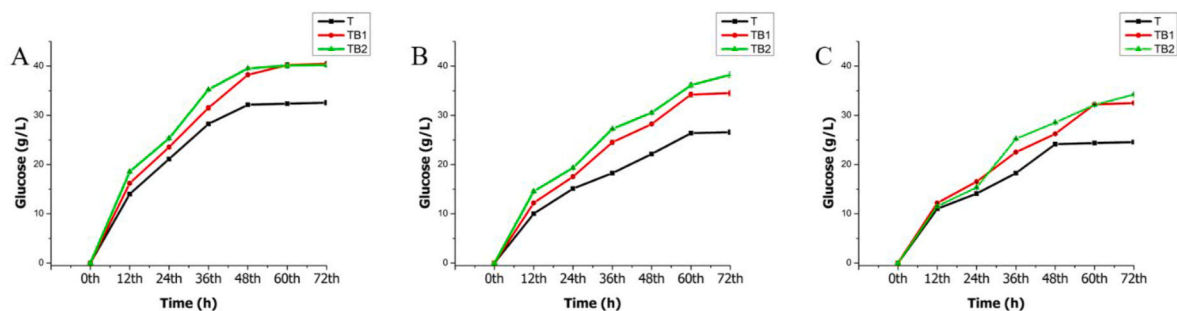
**Fig. 2.** Comparison of cellulase production by monoculture (T) and the co-culture (TB) at different time intervals in the newly designed medium. Fpase (A), pNPGase (B), pNPCase (C), cellulase (D) xylanase I (E), xylanase II (F), and secreted protein (G) were performed in 72, 96, and 120 h. Values are the average of biological triplicates. Error bars represent the standard error.



**Fig. 3.** Assessment of cellulase, xylanase (a-c), and transcription factors (d-f) related genes in monoculture (T) and the co-culture (TB). Gene expression fold changes in co-culture (TB) shown were relative to monoculture (T) on the newly designed medium at a different time interval. Values are the average of biological triplicates. Error bars represent the standard error.

Stover, wheat straw, and rice straw. The crude enzyme mixtures from the culture supernatant of monoculture and co-culture (TB1 and TB2) grown under an optimized medium were selected to evaluate the potential of biomass hydrolysis. After 48 h, crude enzyme mixture from the monoculture produced the hydrolysis yield of 32.15, 22.15, and 24.15 g/l of glucose from corn Stover, rice straw, and wheat straw,

respectively. After 48 h of saccharification, 31.59, 38.25, and 34.25 g/l glucose were obtained from corn Stover, rice straw, and wheat straw, respectively, from the crude enzyme mixture of TB2, which was higher compared to the monoculture (Fig. 4a-c). These outcomes recommend that cellulolytic enzymes synthesized by TB2 were more effective than monoculture for the hydrolysis of the agriculture biomass.



**Fig. 4.** Hydrolysis of pretreated corn Stover, rice straw and wheat straw by the crude enzyme of monoculture (T), and co-culture (TB1 and TB2). Corn Stover (a), rice straw (b), and wheat straw (c). Values are the average of biological triplicates. Error bars represent the standard error.

## 4. Discussion

### 4.1. Enhancement of lignocellulolytic enzyme production by co-cultivation

Various tactics have been used to improve lignocellulolytic enzyme production. Among them, supplementation of carbon sources in the fermentation medium could be used as an active inducer for cellulase and xylanase production. For example, Wang et al. (2012) supplemented the cellulose into the fermentation medium to stimulate the cellulase production from *A. fumigatus*. Dhillon et al. (2012) used lactose to induce the production of cellulase and hemicellulase. In the present investigation, avicel, lactose, and glucose were supplemented into the medium as a stimulator and repressor of the cellulase, and xylanase production. Our results revealed that avicel and lactose improved the production of cellulase and xylanase. It also demonstrates that avicel and lactose are the favorable substrates for cellulase and xylanase production. Besides, the presence of glucose at a higher level negatively regulates the production of cellulase.

Furthermore, the utilization of economic substrate and co-culture can offer a compelling methodology for the highest production of cellulase. The hydrolysis of cellulosic biomass by *T. reesei* hindered the hydrolysis rate (Malgas et al., 2020). In this specific situation, various investigations have been performed. Co-cultivation of *T. reesei* and *A. niger* in the solid-state fermentation of rice straw and wheat grain in the proportion of 3:1 produced higher cellulase than monocultures (Rabello et al., 2014). Ijoma et al. (2019) declared that co-cultivation improved enzyme productions compared to monocultures. The co-cultivation of *T. reesei* and *Aspergillus* augmented the hydrolysis of cellulose compared to the mono-culture (Kolasa et al., 2014). In our previous study, we observed that the enzymes involved in the mycoparasitism were upregulated by the *B. amyloliquefaciens* (Karuppiyah et al., 2019a and b). Karuppiyah et al. (2019a and b) discovered that co-inoculation of *T. asperellum* and *B. amyloliquefaciens* inhibit the growth of *T. asperellum* due to the faster growth of *B. amyloliquefaciens*, while the sequential inoculation of *B. amyloliquefaciens* into the 48 h grown *T. asperellum* allowed the growth of both organisms. The space and nutrient competition between the *T. asperellum* and *B. amyloliquefaciens* regulated the expression of *Bacillus* deficitin and macrolactin gene expression and thereby induced the *Trichoderma* signaling pathways to improve the production of mycoparasitism related enzymes and secondary metabolites. Further, the amino acids produced by *B. amyloliquefaciens* were consumed by *T. asperellum* for the maximum enzyme production (Karuppiyah et al., 2019a and b). The co-cultivation of genetically engineered *T. asperellum* and *B. amyloliquefaciens* was effectively used to hydrolyze the corn stover (Karuppiyah et al., 2021). In the present investigation, *B. amyloliquefaciens* significantly improved cellulase and xylanase production of *T. asperellum* based on the sequential inoculation method. Though the *T. asperellum* was grown in the presence of either stimulator or a repressor, the cellulase and xylanase production were significantly improved by the *B. amyloliquefaciens* compared with the monoculture of *T. asperellum*. Our results revealed that *B. amyloliquefaciens* could be a better inducer of cellulase and xylanase production. The present study revealed the *B. amyloliquefaciens* could induce the cellulase and xylanase production even under repressive conditions. To the best of our knowledge, the mechanism behind the induction of cellulase and xylanase production by the *B. amyloliquefaciens* is unknown.

### 4.2. Molecular mechanism behind the induction of lignocellulolytic enzyme production

Understanding the molecular mechanism behind the induction of cellulase and xylanase production of the *B. amyloliquefaciens* during co-culture with *T. asperellum* may perhaps reveal the induction mechanism of cellulase production. The expression of cellulase and xylanase were

coordinately regulated by the genes encoding endoglucanases (*egl1* and *egl2*), cellobiohydrolases (*cbh1* and *cbh2*), and xylanases (*xyn1* and *xyn2*) (Sun et al., 2008). Hence, in the present investigation, the expression of these genes under both inducing and repressing conditions was investigated and compared between the mono and co-culture conditions. An earlier study revealed that the expression of *cbh1* and *egl1* of *T. reesei* was reduced under the repressors compared to the inducers (Carle-Urioste et al., 1997). Inversely, *T. pseudokoningii* *cbh1* was expressed under the repressive (Wang and Gao, 1999). This transformation could be due to changes in the fermentation circumstances. Previous studies showed that *cbh1* and *cbh2* gene were involved in the basal expression of cellulase (Henrique-Silva et al., 1996; Seiboth et al., 1997). On the other hand, the *egl2* was also induced the production of cellulase by an inducer (Seiboth et al., 1997). Our results revealed that the expression of *cbh1*, *cbh2*, *egl1*, *egl2*, and *bgl1* were upregulated in the TB1 and TB2 than the axenic culture in the presence of both inducers and repressors.

The expression of these cellulase and xylanase encoding genes was regulated by the group of transcription regulators. *ace2*, *ace3*, *xyr1*, and *hap2/3/5* complex genes were positively regulating the expression of cellulase and xylanase encoding genes, while *ace1*, *rce1*, and *cre1* are the negative regulators (Portnoy et al., 2011). *xyr1* is a key gene to stimulate the transcription of cellulase and xylanase, while the *cre1* is an important regulator of carbon catabolite repression (CCR) to inhibit the transcription activation. *rce1* has the motif to bind with the promoter region of the *cbh1* and competes with the binding of *xyr1* and *hap2/3/5* complex to inhibit the enzyme activity. Similarly, *ace1* also competes with the *xyr1* to inhibit the production of enzymes. The *rce1* was also downregulated by the overexpression of *xyr1*, *hap2/3/5*, and *ace3* during the induction conditions. The results of the present study support the regulatory mechanism of transcription factors required for cellulase and xylanase production. Upon the stimulation by the avicel and lactose the *ace1*, *rce1* and *cre1* were downregulated and there increased the expression of positive regulators by activating the *xyr1* gene. The increased content of glucose, stimulated the *cre1* and *rce1* to negatively inhibit the *xyr1*. Fungus habitually involves the proper genetic regulation upon both repressor and stimulators.

Furthermore, *vel1* and *lae1* is a global regulatory gene of *Trichoderma*, which also involved the regulation of cellulase and xylanase gene expression (Karimi Aghcheh et al., 2014; Seiboth et al., 2012). The key regulatory gene of cellulase production, *xyr1* is further required *vel1* and *lae1* to induce the production of cellulase and xylanase. The expression of *vel1* and *lae1* is also further regulated by the carbon source and *B. amyloliquefaciens* (Karuppiyah et al., 2020). Similarly, in this study, the expression of *vel1* and *lae1* genes were increased in the presence of avicel and lactose. Further, we confirmed that the expression pattern of *xyr1* coincided with the expression of *vel1* and *lae1*. The expression of *vel1* and *lae1* genes were also upregulated by co-cultivation with *B. amyloliquefaciens*. Fascinatingly, the *B. amyloliquefaciens* induced the expression of *vel1* and *lae1* gene under the repressive conditions and thereby it upregulated the expression of positive regulators and down-regulated the negative regulators (Karuppiyah et al., 2020).

### 4.3. Optimization of lignocellulolytic enzyme production

Although the induction of cellulase and xylanase production by the co-culture has been predicted using the inducers and repressors, the cost of enzyme production using the inducers (avicel) limits their application. Hence, the commercial and agro-industrial byproducts were used to optimize the production of cellulase enzymes using response surface methodology. Based on our previous work, molasses, and corn meal have been selected to support the growth of *Trichoderma* and *Bacillus* during co-cultivation. In addition, rice bran has been selected as a source of cellulase for enzyme production. The RSM results showed the optimal level of each component for the maximum cellulase production. To further validate the optimum medium, the production of FPase,

pNPCase, pNPGase, CMCase, xylanase 1 and 2 at different times were estimated. The results demonstrated that the enzyme production of monoculture has been reduced at 120 h, it could be due to the feedback inhibition of the end product, while the co-culture produced more enzymes at 120 h, and which could be due to the induction of enzyme production by the *Bacillus*. These results have been further supported by the expression of regulating genes. Taken together, the co-cultivation of the *Bacillus* with *Trichoderma* positively regulates the genetic network involved in the production of cellulase and xylanase in the optimized medium for the lignocelluloses biomass degradation.

#### 4.4. Hydrolysis of lignocellulosic biomass

The degradation of lignocellulosic biomass requires a group of hydrolyzing enzymes. The cellulases produced by the *T. asperellum* contain a cocktail of enzymes such as endoglucanases, exoglucanases, glucosidase, and xylanase to hydrolyze cellulose and hemicellulose (Karuppiyah et al., 2021). In the present study, we identified FPase, pNPCase, pNPGase, CMCase, xylanase 1 and 2 from the *T. asperellum*. These enzymes have a broad range of specificity to hydrolyze the agriculture biomass residue such as corn Stover, rice straw, and wheat straw (Linder and Teeri, 1996). The cellulose and hemicelluloses are composed of pentoses, hexoses, hexuronic acids, and deoxyhexoses. For example, the distinctive composition of corn stover is cellulose (38.7%), hemicellulose (34.2%), lignin (21.3%), and proteins (Han and Chen, 2011). In the present study, we identified a group of enzymes produced during co-cultivation that completely hydrolyzed the cellulose and hemicellulose present in the corn Stover, rice straw, and wheat straw into the glucose for further application.

#### 4.5. Practical applications and future research prospects

Co-cultivation of *T. asperellum* and *B. amyloliquefaciens* provides the chance for simultaneous production of glucose and xylose from the lignocellulose, which could increase the production of bio-fuel. However, it has some challenges at the industrial level. More studies are required to develop the conversion of biomass to biofuel. Different pretreatment of lignocellulose biomass should be required to avoid the loss of cellulose and hemicellulose. Technology on the large-scale cultivation of the co-culture should be developed for the higher yield of biofuel. Further, Optimization of the downstream process should also be required to obtain the uniform metabolites for biofuel production. In the future, appropriate bioreactors with the proper method will be established for the co-cultivation of *T. asperellum* and *B. amyloliquefaciens* to obtain a precursor for biofuels.

## 5. Conclusions

The bioconversion of waste biomass into renewable energy is an important aspect of waste management. Several researchers have been made to increase the productivity of lignocellulolytic degrading enzymes on biomass conversion. Hence, the utilization of lignocellulosic biomass is the best method to produce biofuels. In this connection, the co-cultivation of *T. asperellum* and *B. amyloliquefaciens* was used to produce the cellulase and xylanase under both inducing and repressing conditions. Further, the optimized fermentation medium containing agricultural byproducts is cost-effective and effectively induced the production of enzyme cocktail by the co-culture. It could be an effective strategy to enhance enzyme production with cost-effectiveness. Further, the highest glucose yield from biomass hydrolysis using the crude enzyme of co-culture showed that the co-cultivation of *T. asperellum* GDFS1009 and *B. amyloliquefaciens* (TB2) is useful for rapid and complete conversion of lignocellulosic biomass waste materials into glucose for the next generation fuels. The present study triumphs over more cost-effective methods for the production of a cocktail enzyme to generate a range of novel products in the future.

## Credit author statement

**Valliappan Karuppiyah:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Lu Zhixiang:** Conceptualization, Investigation. **Hongyi Liu:** Conceptualization, Investigation. **Vallikkannu Murugappan:** Writing – review & editing, Visualization. **Subramanian Kumaran:** Writing – review & editing, **Anthonyraj Matharasi Perianaika Anahas:** Writing – review & editing, **Jie Chen:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jenvman.2021.113833>.

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