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Synergistic growth in bacteria depends on substrate complexity

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Abstract

Both positive and negative interactions among bacteria take place in the environment. We hypothesize that the complexity of the substrate affects the way bacteria interact with greater cooperation in the presence of recalcitrant substrate. We isolated lignocellulolytic bacteria from salt marsh detritus and compared the growth, metabolic activity and enzyme production of pure cultures to those of three-species mixed cultures in lignocellulose and glucose media. Synergistic growth was common in lignocellulose medium containing carboxyl methyl cellulose, xylan and lignin but absent in glucose medium. Bacterial synergism promoted metabolic activity in synergistic mixed cultures but not the maximal growth rate (μ). Bacterial synergism also promoted the production of β -1,4-glucosidase but not the production of cellobiohydrolase or β -1,4-xylosidase. Our results suggest that the chemical complexity of the substrate affects the way bacteria interact. While a complex substrate such as lignocellulose promotes positive interactions and synergistic growth, a labile substrate such as glucose promotes negative interactions and competition. Synergistic interactions among indigenous bacteria are suggested to be important in promoting lignocellulose degradation in the environment.

Keywords

bacterial synergism; lignocellulose degradation; bacterial activity; enzyme production; microbial interaction

Introduction

Bacteria in nature do not live in isolation but interact dynamically with other bacteria in the community. Interactions can be either positive or negative, resulting in different community function outcomes (Newman and Banfield, 2002; Hansen *et al.*, 2007). Factors that mediate the way bacteria interact remain poorly studied.

Competition for resources has been suggested as the prevalent type of interaction among bacteria (Hibbing *et al.*, 2010; Foster and Bell, 2012). As labile nutrients are often limited in the environment, one bacteria might compete with other bacteria by producing antibiotics that inhibit the growth of others (D'Costa *et al.*, 2007; Hibbing *et al.*, 2010). For example, the majority of marine bacterial isolates exhibited antagonistic activity against others from

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the same environment through production of potential antimicrobial agents (Long and Azam, 2001; Grossart *et al.*, 2004; Rypien *et al.*, 2010).

Although competitive interactions among bacteria are ubiquitous in nature, there are multiple studies showing that using complex microbial communities enhances the rate of bioconversion of lignocellulose to more valuable products (Haruta *et al.*, 2002; Wongwilaiwalin *et al.*, 2010; Jiménez *et al.*, 2014). It is unclear however whether bacterial synergism is directly related to the structural complexity of the substrate and whether bacteria in the consortia switch from synergism to antagonism if labile substrates become available.

Lignocellulose consists of mainly cellulose, hemicellulose and lignin that are strongly cross-linked in plant tissue and thus is recalcitrant to degradation (Lynd *et al.*, 2002). To achieve better degradation, bacteria might form consortia and synergistically degrade lignocellulose (Lynd *et al.*, 2002; Perez *et al.*, 2002). It thus seems likely that the way bacteria interact may depend on substrate complexity with greater cooperation in the presence of recalcitrant substrate.

In this study, we test the hypothesis that the complexity of substrate mediates bacterial interactions. When lignocellulose is the only carbon source, bacteria form consortia and synergistically degrade the complex substrate. However, when labile substrate is available, bacteria compete for the limiting nutrient and antagonistic interaction prevails. To test this hypothesis, we isolated lignocellulolytic bacteria from salt marsh detritus. Bacteria were grown in single and three-species mixed cultures in both lignocellulose medium and glucose medium. We compared the growth, enzyme production and metabolic activity of three-species mixed cultures to those of pure cultures. The specific aims of this study are to determine: 1) the frequency of synergistic degradation among bacteria when grown in lignocellulose medium; 2) whether the occurrence of synergistic growth depends on the complexity of the carbon source; and 3) whether bacterial synergy affects the maximal specific growth rate, enzyme production and metabolic activity of bacteria in mixed cultures.

Materials and Methods

Culture media preparation

Three bacterial isolation media were used. They were composed of Bushnell Haas basal salt medium (Lo *et al.*, 2009) amended with one of the three main components of lignocellulose: carboxymethyl cellulose (CMC) (0.5%), xylan (0.5%) or lignin (0.3%). The basal salt medium was adjusted to pH 7.5 and 1% NaCl, and pre-filtered through G/C filter (1.0 μm) before adding any organic nutrients. Zobell marine medium (HiMedia, Cat# M385) (with 1.5% agar for plates) was used to grow purified bacterial isolates.

Two media, containing either glucose or lignocellulosic compounds, were used to study bacterial interactions. The glucose medium contained basal salt medium with 0.3% glucose and 0.05% yeast extract. The lignocellulose medium, used to simulate recalcitrant carbon substrates found in nature, contained basal salt medium with 0.3% CMC, 0.2% xylan, 0.1% lignin, and 0.05% yeast extract. The relative proportion of CMC, xylan, and lignin, 3:2:1,

mimics the typical lignocellulosic composition in grass material (Sun and Cheng, 2002). All components in the lignocellulose medium are water soluble, producing a clear solution that permitted measurement of bacterial growth directly by optical density (OD) in 96-well plates.

Bacterial isolation and classification

Natural detritus was collected from a salt marsh in Ocean Springs, MS, USA (30°23'32" N 88°47'56"W). Potential lignocellulolytic bacteria were isolated from the detritus using the three isolation media mentioned above. After incubation for 10–14 days at 25°C, representative single colonies were streaked on new agar plates of the same isolation media to obtain well-isolated colonies. Pure single colonies were then transferred and grown on Zobell marine agar. Based on the carbon source used during the initial isolation, the isolates were classified into three groups: cellulose-degrading (C), xylan-degrading (X), or lignin degrading bacteria (L).

To study bacterial interactions, nine bacteria with different colony morphology were randomly selected, three from each group. The nine bacteria were identified by 16S rRNA gene sequencing using universal primers 27F and 1492R (Weisburg *et al.*, 1991; Ciric *et al.*, 2010). The nine sequences have been deposited in GenBank database (Accession Number KJ158195-KJ158203). Putative taxonomic identities of the nine isolates were assigned to genus level using ribosomal database project (RDP) Bayesian classifier (Wang *et al.*, 2007) with minimum bootstrap confidence of 80%. Two of the nine isolates with bootstrap confidence less than 80% at the genus level were assigned to the family level. A phylogenetic analysis of the nine bacteria was conducted by MEGA (Tamura *et al.*, 2011) using neighbor-joining method (Saitou and Nei, 1987).

Culture preparation and growth measurements

The nine isolates were used to determine bacterial interactions in both lignocellulose and glucose media. To prepare the isolates for growth experiments, they were first grown in 3 ml Zobell marine broth in 100 mm × 16 mm polypropylene tubes. After 18 h at 25°C with shaking (250 rpm), 0.2 ml of each culture was used to inoculate 3 ml of either lignocellulose or glucose medium and grown at 25°C with shaking. Those in lignocellulose medium were grown for 30 h and those in glucose medium were grown for 24 h. To conduct growth experiments, seed cultures were prepared by diluting each culture to an optical density (OD₅₉₅) of 0.01 (ca. 10⁶ CFU/ml) with either lignocellulose or glucose medium.

Growth experiments using nine pure cultures and 27 mixed cultures were conducted to test whether the complexity of carbon source affects bacterial interactions. Each of the 27 mixed cultures were created by combining three pure cultures, one from each of the three groups (C, X, and L) described above. All growth experiments started with cultures at an OD₅₉₅ of 0.01 in clear flat-bottom 96-well plates. Growth experiments were conducted using 100 µl of glucose medium or lignocellulose medium. For three-species mixed cultures, each species contributed 1/3 of the starting volume. Each culture (pure or mixed) was replicated in four wells in each of three plates thus a total of 12 replicates were used for each pure culture and

bacterial combination. Each 96-well plate also included four wells filled with 100 μl sterile lignocellulose or glucose media as blank controls.

Bacterial cultures in growth experiments were grown at 25°C without shaking. Cultures in lignocellulose medium were incubated for 48 h and those in glucose medium for 30 h. Sterile growth media were optically clear at the start of each experiment and growth was determined by measuring the increase in OD₅₉₅ of each culture (minus the blank controls) using a Synergy 2 microplate reader (BioTek Instruments, Inc., Winooski, VT) over time. To determine maximal growth rates (μ, h^{-1}), optical densities of the cultures during the exponential growth phase were log-transformed and the slope for each culture used.

Definition of synergistic growth

Synergistic growth is defined as having occurred when a mixed culture grew more densely than any of the three corresponding pure cultures. The densest among the three pure cultures is referred to as the reference culture. Thus, a mixed culture is considered to exhibit synergism when it reached significantly higher density (OD₅₉₅) than its reference culture. No distinction was made whether the higher density resulted from the enhanced growth of all three bacteria or just one or two in the mixed culture. Of interest was the fact that enhanced growth of the mixed cultures indicated more bacterial biomass production and greater degradation of lignocellulose, the ecological process of interest.

Enzyme production assay

The production of lignocellulolytic enzymes was measured using fluorometric assays adopted from widely used methods (Sinsabaugh *et al.*, 1997, 2008; Marx *et al.*, 2001; Saiya-Cork *et al.*, 2002). The assays use enzyme-specific substrates labeled with 4-methylumbelliferone (MUB). The substrates are non-fluorescent, but fluorescence is observed when MUB is released upon substrate hydrolysis by enzymes in the growth medium (Hoppe, 1983; Marx *et al.*, 2001). Enzyme activity is quantified by measuring the amount of fluorescence. 4-MUB- β -D-glucoside, 4-MUB- β -D-cellobioside, and 4-MUB- β -D-xyloside, respectively, were used to test the activities of β -1,4-glucosidase (EC.3.2.1.21), cellobiohydrolase (EC.3.2.1.91), and β -1,4-xylosidase (EC.3.2.1.37). The first two degrade cellulose and the third degrades hemicellulose.

To perform assays, bacterial cultures growing in lignocellulose medium for 48 h were diluted five-fold with 50 mM MOPS buffer (pH 6.5) and 150 μl of each diluted culture were combined with 50 μl of one of the three 4-MUB-labeled enzyme-specific substrates (200 μM) in black 96-well plates. After incubation at 25°C in the dark for 1 h with shaking at 500 rpm, fluorescence in each well was measured using a Synergy 2 Bio-Tek microplate reader (365_{ex}, 450_{em}). The amount of lignocellulolytic enzyme in each culture was calculated based on the fluorescence of the sample relative to that of a standard. The standard contained 150 μl of dilute bacterial culture and 50 μl of 10 μM MUB. Blank controls contained 150 μl dilute culture and 50 μl buffer. Substrate controls contained 150 μl buffer and 50 μl substrate. The enzyme activity in each sample expressed in nmol/h/ml was calculated using the formula:

$$\frac{SF - BCF - SCF}{MF - BCF} \times \left(10 \frac{\text{nmol}}{\text{ml}} \times 0.05 \text{ ml} \right) \times \frac{1}{0.15 \text{ ml}}$$

where SF is the sample fluorescence, BCF the blank control fluorescence, SCF the substrate control fluorescence, and MF the MUB standard fluorescence. Results were multiplied by five to adjust for the initial five-fold sample dilution. Each sample, standard, blank control and substrate control was replicated four times. The experiment was repeated twice.

Microbial metabolic activity measurement

To test whether bacterial interactions enhance microbial activity, the metabolic activity of bacterial cultures was measured using the 2,3,5-triphenyltetrazolium chloride (TTC) assay (Gabrielson *et al.*, 2002; Burmølle *et al.*, 2006). The colorless TTC is enzymatically reduced by metabolically active bacteria to red 1,3,5-triphenylformazan (TPF) that can be quantified by measuring sample absorbance at 490 nm (Gabrielson *et al.*, 2002). After growth for 30 h in lignocellulose medium, 100 μl of each culture was combined with 25 μl of substrate solution (0.05% TTC and 1.5% glucose) and incubated at 25°C in the dark. Glucose was included to improve sensitivity (da Silva *et al.*, 2008). Sterile culture medium with the same amount of substrate solution served as negative control. Absorbance was measured at 0 and 3 h and the difference in absorbance (A_{490}) was used as a measure of metabolic activity. We also compared the growth-specific metabolic activity ($A_{490} \cdot \text{OD}_{595}^{-1}$) between mixed and pure cultures. The experiment was repeated twice using eight replicates each time.

Statistical analysis

One-way ANOVA and Tukey HSD test were performed to test whether mixed cultures grew better than their corresponding pure cultures. The effects of synergism on growth, maximal growth rate and metabolic activity were analyzed by one-way ANOVA, followed by Tukey HSD test. Due to the non-normal distribution of enzyme production data, the non-parametric Kruskal-Wallis test was used to test the effect of synergism on enzyme production. All statistical tests were performed in RStudio (www.rstudio.org).

Results

Bacterial isolates classification

The nine lignocellulolytic bacteria isolated from a natural salt marsh in Ocean Springs, MS, USA were taxonomically diverse (Fig. 1). Two isolates were classified as α -Proteobacteria including *Labrenzia* sp. (L1) and *Paracoccus* sp. (L3). Six isolates belonged to γ -Proteobacteria, including two *Vibrio* spp. (X3 and C3), one *Gallaecimonas* sp. (L2), one *Hahella* sp. (C1) and two presumptively new isolates (X1 and C2) that could not be assigned to the genus level. These two isolates were thus assigned to the family level and classified as a *Vibrionaceae* bacterium (X1) and a *Alteromonadaceae* bacterium (C2). The ninth isolate was *Jonesia* sp. (X2), belonging to *Actinobacteria*.

Synergistic growth in three-species mixed cultures

Synergistic growth among three-species mixed cultures was common in lignocellulose medium (Fig. 2). Synergistic growth is defined as having occurred when a mixed culture grew better than any of the three corresponding pure cultures. Fifteen among the 27 possible mixed cultures exhibited significantly greater growth when compared to their corresponding pure cultures (Fig. 2). In fact, all but one of these 15 mixed cultures reached higher densities ($OD_{595} > 0.209$) than all the pure cultures. The experiment was repeated twice more with similar results (data not shown). Hereafter, the 15 three-species combinations are designated as synergistic mixed cultures because those mixed cultures showed greater growth than their corresponding cultures that made up each of them. The remaining 12 mixed cultures are designated as non-synergistic mixed cultures.

Carbon source dependent synergistic interaction

To determine whether the synergistic growth was substrate dependent, we repeated the mixed-culture experiment using glucose as the sole carbon source. For comparing bacterial growth in glucose medium and lignocellulose medium, we plotted the growth of each mixed culture against the growth of its reference culture (the pure culture with the greatest growth among the three that made up the mixed culture) (Fig. 3). Although cultures grown in glucose medium reached higher densities than the same cultures in lignocellulose medium, none of the 27 mixed cultures in glucose medium reached higher density than their reference cultures. All had optical densities below the isometric line that indicates equal growth between mixed and reference cultures (Fig. 3). This result suggests the dominance of negative interaction or competition among three species in mixed cultures when grown in glucose medium. In contrast, most of the mixed cultures (21 in 27) reached densities above the isometric line when grown in lignocellulose medium (Fig. 3). Our results show that bacterial synergism occurred frequently in lignocellulose medium but never in glucose medium, suggesting that bacterial synergistic growth was dependent on the structural complexity of the carbon source.

Bacterial growth and activity during lignocellulose degradation

To explore the potential mechanism for bacterial synergism in lignocellulose medium, we compared the maximal growth rate and metabolic activity among the three culture groups: synergistic mixed cultures (the 15 mixed cultures that exhibited synergistic growth), non-synergistic mixed cultures and pure cultures. Synergistic mixed cultures reached the highest cell density among the three groups ($F_{2, 33} = 26.78$, $P < 0.001$) (Fig. 4A) but did not grow faster during the exponential phase (Fig. 4B). The mean growth density of synergistic mixed cultures ($OD_{595} = 0.280$) was more than twice that of pure cultures ($OD_{595} = 0.135$) and 1.6 times that of non-synergistic mixed cultures ($OD_{595} = 0.176$) (Fig. 4A). In terms of maximal growth rate, there was, however, no significant difference between synergistic mixed cultures (mean = 0.429 h^{-1}) and pure cultures (mean = 0.430 h^{-1}) (Fig. 4B). Interestingly, although they grew less than synergistic mixed cultures (Fig. 4A), non-synergistic mixed cultures had higher maximal growth rate (mean = 0.542 h^{-1}) than synergistic mixed cultures and pure cultures ($F_{2, 33} = 8.29$, $P = 0.001$) (Fig. 4B).

Bacteria in synergistic mixed cultures had higher metabolic activity (mean $A_{490} = 0.186$) than those in pure cultures (mean $A_{490} = 0.096$) ($F_{2, 33} = 3.79$, $P = 0.033$) but not significantly higher than non-synergistic mixed cultures (mean $A_{490} = 0.132$) (Fig. 4C). Although synergistic mixed cultures had higher metabolic activity than pure cultures, there was no significant difference in the growth-specific metabolic activity ($A_{490} \text{ OD}_{595}^{-1}$) among the three groups after normalizing for cell density (data not shown).

Bacterial production of lignocellulolytic enzymes

Bacterial synergism promoted the production of β -1,4-glucosidase (BG) but not the production of cellobiohydrolase (CBH) or β -1,4-xylosidase (BX) (Fig. 5). The mean BG activity among synergistic mixed cultures (133.5 nmol/h/ml) was over three times that of pure cultures (43.7 nmol/h/ml), and 2.6 times that of non-synergistic mixed cultures (52.3 nmol/h/ml) ($F_{2, 33} = 13.87$, $df = 2$, $P = 0.001$) (Fig. 6A). Activities of CBH and BX for synergistic group were slightly higher than two other groups but not significantly different from them (Fig. 5B and C). When adjusted for cell density, there was no significant difference in production of the three enzymes tested (nmol/h/ml/ OD_{595}) among synergistic mixed cultures, non-synergistic growth and pure culture groups (data not shown).

Discussion

We found that three-species mixed cultures composed of taxonomically diverse bacteria frequently exhibited greater growth during lignocellulose degradation. The results suggest that bacterial synergism may be important to detritus degradation in the salt marsh ecosystem where those bacteria were isolated. Whether bacterial synergism takes place appeared to depend on the chemical complexity of the carbon source. In lignocellulose medium most of the mixed cultures exhibited synergistic growth but none exhibited synergistic growth in glucose medium. Our results support the hypothesis that the complexity of carbon source plays a role in determining bacterial interactions in the environment.

Several mechanisms that promote synergistic growth have been proposed. For example, synergistic growth can result when multiple species produce complementary enzymes and take part in metabolite cross feeding (Wintermute and Silver, 2010; Kostylev and Wilson, 2012). Due to the complexity and recalcitrance of lignocellulosic substrate, the complete degradation of lignocellulose requires multiple enzymes (Kostylev and Wilson, 2012; Van Dyk and Pletschke, 2012). Furthermore, enzyme cocktails containing cellulases, xylanases and lignin peroxidases that are produced by multiple species can significantly enhance lignocellulose degradation rate (Lynd *et al.*, 2002; Guevara and Zambrano, 2006; Kostylev and Wilson, 2012; Van Dyk and Pletschke, 2012). In addition to making enzyme cocktails where enzymes act synergistically, mixed cultures can also promote production of enzymes that produce simple sugars promoting bacterial growth. In our study, β -1,4-glucosidase (BG) activity was more than three folds higher in synergistic mixed cultures than in pure cultures (Fig. 5A). The higher β -1,4-glucosidase activity likely produced more glucose that contributed to the enhanced growth of mixed cultures. Thus, it appears that mixed cultures in lignocellulose medium not only produced enzymes that were complementary but also

produced more active enzymes so that refractory substrates can be degraded more effectively compared to pure cultures.

Another mechanism that promotes bacterial synergism is metabolite cross feeding that allows bacteria to utilize complex substrate in a cooperative manner (Flint *et al.*, 2007; Wintermute and Silver, 2010). During late growth, some species in a mixed culture may produce metabolites that are toxic to themselves but are used by others. In this case, mixed cultures can alleviate problems of feedback regulation and metabolite repression present in pure cultures (Zuroff and Curtis, 2012). In the present study, synergistic mixed cultures reached higher densities than pure cultures but their maximal growth rate during exponential growth was not higher (Fig. 4A and B). One possible explanation is that after exponential growth when the primary nutrients are depleted, bacteria in mixed cultures can continue to grow by utilizing metabolites produced by others. As a result, mixed cultures reached higher growth density than pure cultures that had no partners to exchange metabolites with. This was supported by observations of higher metabolic activity in the synergistic mixed cultures after the exponential growth (Fig. 4C).

Although most mixed cultures (15 of 27) in lignocellulose medium exhibited synergistic growth and degraded more lignocellulose than pure cultures, some (12 of 27) did not under the same culture condition. This suggests that the specific combination of bacteria may be important. The physiological or metabolic response of each bacteria may depend on the presence of others in the mixed culture. Some combinations achieved enhanced growth while others exhibited competition perhaps due to resource overlap or competing metabolic capabilities of neighboring bacteria (Hibbing *et al.*, 2010; Freilich *et al.*, 2011; Elias and Banin, 2012).

It was striking that none of the 27 mixed cultures exhibited synergistic growth in glucose medium. The likely explanation is that cooperation serves no purpose in the presence of a labile carbon source such as glucose and thus competition is prevalent. The concept that substrate complexity regulates the type of bacterial interaction is supported by published studies. For example, Long and Azam (2001) and Grossart *et al.* (2004) reported that antagonism was common among bacteria found in marine ecosystems. A possible explanation lies in the marine (ZoBell) agar used in the laboratory studies. The limited amounts of peptone and yeast extract in the medium may have spurred competition among bacteria to result in the common antagonism observed. Similarly, both Nielsen *et al.* (2000) and Breugelmans *et al.* (2008) showed that when citrate was used as the carbon source, bacteria competed for the labile nutrient and usually formed separated biofilm colonies. In contrast, when complex benzyl compounds were the only carbon source in the medium, bacteria degraded the refractory substrate more efficiently than pure cultures and usually formed mixed-species biofilm. This suggests the possibility that bacteria grown together in glucose-limiting medium in the present study might compete for the labile substrate by producing antimicrobial chemicals to inhibit competitors, resulting in the predominance of antagonistic interactions.

In conclusion, our results show that certain combinations of indigenous bacteria from a salt marsh exhibit growth synergism that is dependent on the complexity of the substrate. When

lignocellulose is the only carbon source, bacteria tend to interact synergistically to degrade the complex substrate. When glucose is the only carbon source and in limited amounts, bacteria compete for the labile substrate resulting in the predominance of antagonistic interactions. To better understand the mechanism, additional studies on the relationship between the chemical complexity of the substrate and bacterial compositions in the consortia are needed. Currently, it is unclear how different species contribute in the consortia. Some may produce enzymes that degrade a particular component of lignocellulose more effectively while others may produce stimulatory exudates or quorum sensing molecules that coordinate interactions among bacteria.

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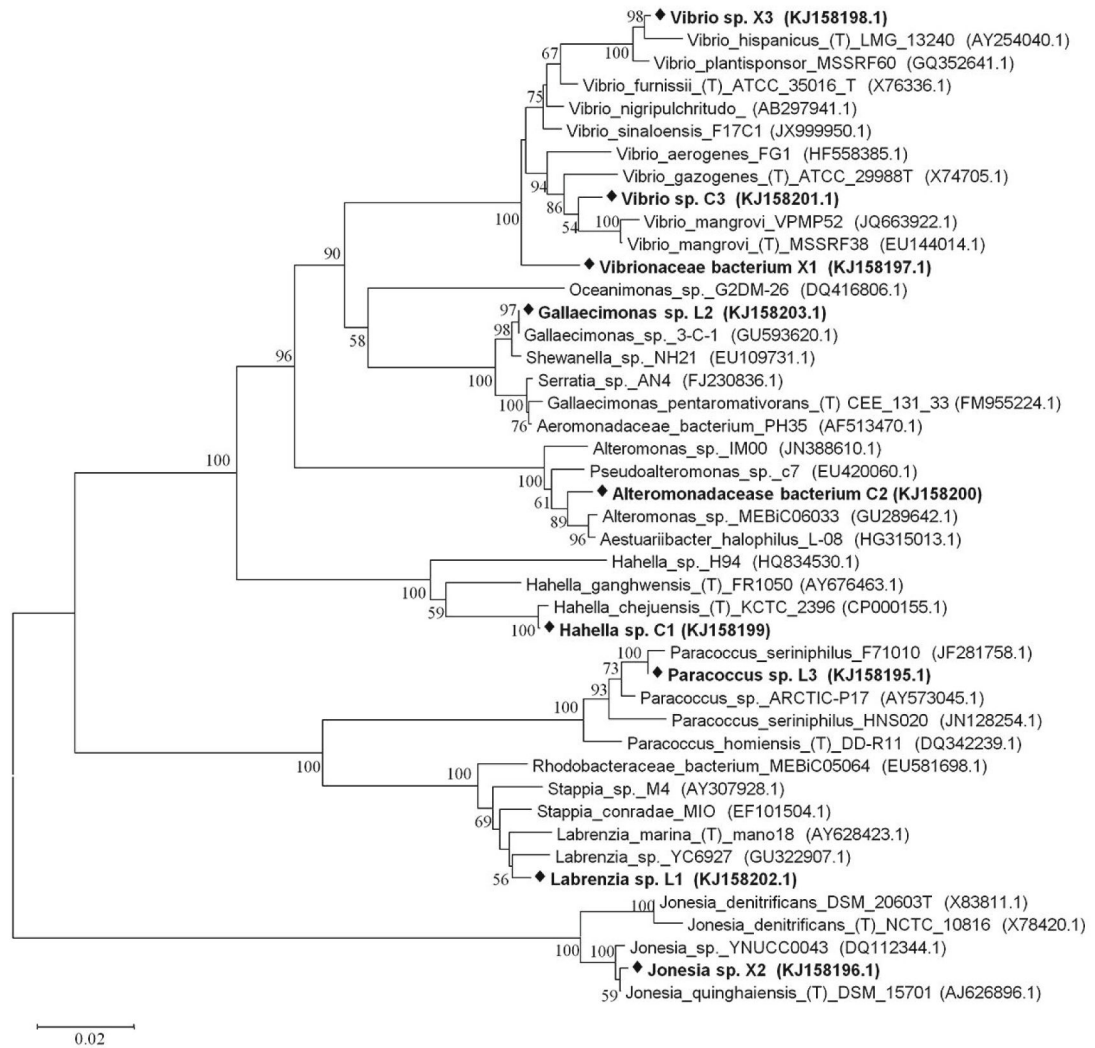


Fig. 1. Phylogenetic relationship of nine bacterial isolates used in study

Bold text with black diamond indicates the nine bacteria isolates used. The letter and number following the name of the bacterium denote the substrate used in isolating each bacterium, C for cellulose, L for lignin and X for xylan. Bootstrap values are shown as the percentage of 1,000 replicates when greater than 50%. The horizontal bar represents nucleotide substitutions per sequence position. GenBank accession numbers are in parentheses.

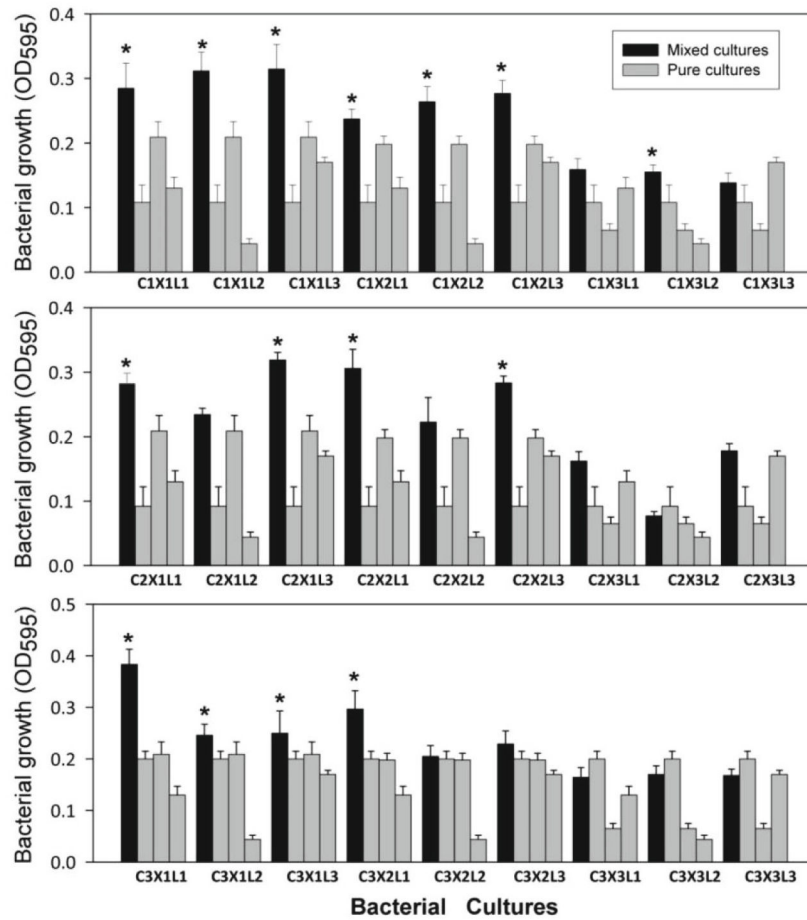


Fig. 2. Growth of mixed cultures compared to pure cultures in lignocellulose medium
 Growth of 27 mixed cultures (black bars) in relationship to the growth of their three corresponding pure cultures (gray bars). N = 12. Error bar is one standard deviation. Asterisks indicate significantly greater growth compared to pure cultures. One-way ANOVA ($F_{35, 396} = 151.77$, $P < 0.001$) followed by Tukey HSD test ($P < 0.05$).

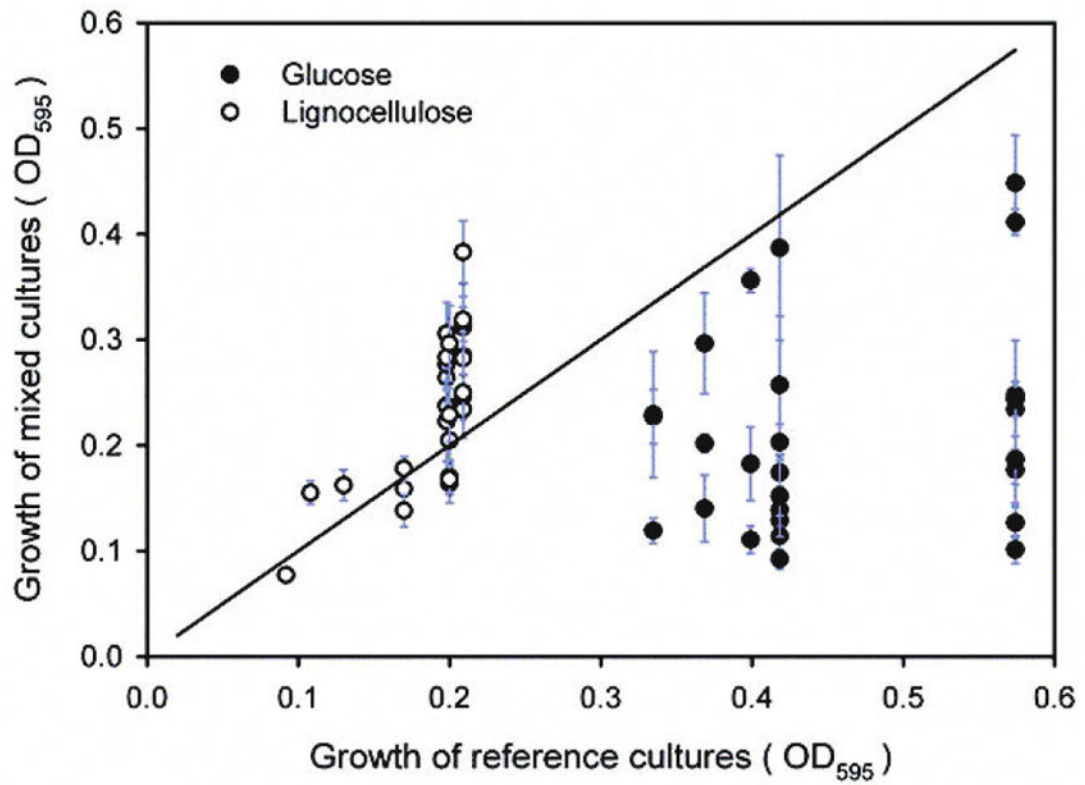


Fig. 3. Comparison of mixed culture growth in lignocellulose and glucose medium

Each data point is the OD₅₉₅ of one mixed culture (mean \pm SD, $n=12$) plotted against the OD₅₉₅ of its reference culture (the pure culture with the greatest growth among the three that made up the mixed culture). The isometric line represents equal growth between mixed cultures and their reference cultures.

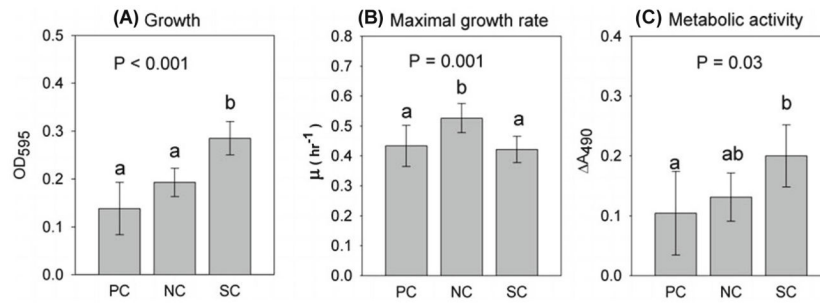


Fig. 4. Effect of synergy on bacterial growth, specific growth rate and metabolic activity
Gray bar is the mean value of each group. Error bars represent 95% confidence interval. Different letters indicate significant difference ($P < 0.05$). NC = non-synergistic mixed cultures, $n = 12$; PC = pure cultures, $n = 9$; SC = synergistic mixed cultures, $n = 15$.

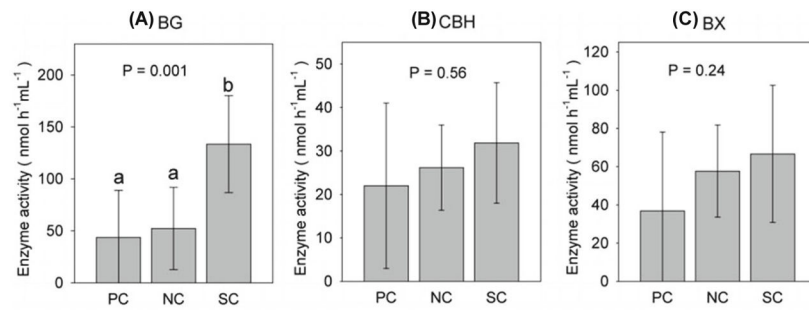


Fig. 5. Effect of synergy on bacterial production of lignocellulolytic enzymes
BG, β -1,4-glucosidase; CBH, cellobiohydrolase; BX, β -1,4-xylosidase. Gray bar is the mean enzyme activity of each group. Error bars represent 95% confidence interval. Different letters indicate significant difference ($P < 0.05$). NC = non-synergistic mixed cultures, $n = 12$; PC = pure cultures, $n = 9$; SC = synergistic mixed cultures, $n = 15$.