Artificial Consortia of *Bacillus Amyloliquefaciens* HM618 and *Bacillus Subtilis* for Utilizing Food Waste to Synthetize Iturin A

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Research Article

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Abstract

Food waste is a kind of biodegradable organic resource. It can be degraded into the small energy molecules for providing precursors to synthetize iturin A, which is a kind of lipopeptide with broad-spectrum antifungal activity for widely applying in biocontrol of the plant disease. However, the lower yield and higher cost during producing iturin A limit its application. In this study, the recombinants of *Bacillus subtilis* producing lipase and amylase were constructed to utilize food waste for producing iturin A under the consortium with *Bacillus amyloliquefaciens* HM618 produced iturin A and the engineering *B. subtilis*. The results showed that recombinant strain WB-A13 had the highest amylase activity of 23406.4 U/mL, and the lipase activity of WB-L01 was 57.5 U/mL. When HM618 was co-cultured with the engineering strain WB800N-AmyE or WB800N-Lip, the relatively highest yield of iturin A reached to 7.66 mg/L in co-cultured fermentation of consortium with HM618 and WB-A14, which was increased 32.9% compared to that of HM618 under pure culture. Under three-strains consortium containing HM618, WB-L02 and WB-A14, the yield of iturin A reached 8.13 mg/L, which was 38.8% higher than the control when the initial size OD_{600} of above three strains was 0.2, 0.15 and 0.15 respectively. Taken together, artificial consortium of *B. amyloliquefaciens* and the recombinant *B. subtilis* can better bioconvert food waste into iturin A, which provides a new strategy for the high-value utilization of food waste.

Introduction

Food waste (FW) is generally discharged from restaurants, family kitchens, public restaurants, etc (Zhang et al. 2014). In China, about 60 million tons of FW are generated annually according to China Statistical Yearbook 2011, and this value may reach 140 million in 2020 (Zhang et al. 2016; Ye et al. 2016). As such a large amount of FW has caused great troubles on our lives, there is an urgent need for reasonable and effective methods to dispose FW. The biological nature of FW is a mixture of organic substances, including starch, protein, lipid, and cellulose (Dinesh et al. 2018; Kim et al. 2011). From another perspective, FW can be considered as nutrients to maintain the growth of microorganisms, bio-transforming into value-added products (Banu et al. 2020). Currently, FW has been converted into pullulan (Rishi et al. 2020), Bt biopesticide (Zhang et al. 2015), bioflocculant (Liu et al. 2019), biolipid and protein (Zeng et al. 2017), biosurfactant (Chen et al. 2018), xanthan (Li et al. 2017), surfactin (Pan et al. 2021), ethanol (Wang et al. 2008), L-lactic acid (Tashiro et al. 2016) through microbial fermentation or methane (Park et al. 2019; Yirong et al. 2017), H_2 (Ortigueira et al. 2019), volatile fatty acids (Khatami et al. 2021) through anaerobic digestion. Compared with traditional disposal methods containing incineration and landfilling, biotransformation is more environmentally friendly and sustainable (Sindhu et al. 2019; Kiran et al. 2015).

Iturin A, which is composed of cyclic heptapeptide and β-amino fatty acid chain with 13-17 carbons, is a cyclic lipopeptide mainly produced by *Bacillus* (Zhao et al. 2019a; Kawagoe et al. 2015). Iturin A can be embedded in the fungal cell membrane to increase the permeability of K^+, conferring it broad-spectrum antifungal activity (Cochrane et al. 2016). Therefore, iturin A possess bright application prospects in the biological control of plant fungal pathogens (Wang et al. 2020; Calvo et al. 2019; Ambrico et al. 2017).

Despite this advantage, the high production cost coupled with low original yield limit the further industrial application of iturin A, thus, developing inexpensive materials is necessary to cheaper product iturin A (Dang et al. 2019; Shi et al. 2018). In current studies, various agro-industrial wastes or by-products such as rapeseed meal (RSM) (Chen et al. 2021), rapeseed cake (RSC) (Chen et al. 2019a), soybean curd residue (Mizumoto et al. 2006), soybean meal (Xu et al. 2020), sun□ower oil cake (SOC) (Kumar et al. 2017) have been targeted as substrates for iturin A production. These results have greatly stimulated the interest in exploring the degradation of FW to product iturin A.

It is well known that the degradation of complex substrates, such as food waste, is the rate-limiting step for microbial fermentation (Choi et al. 2018). Usually, it is preferred to add commercial enzymes to degrade FW into small energy molecules that can be directly used by microorganisms (Rishi et al. 2020; Al-Dhabi et al. 2020; Prasoulas et al. 2020). Although the effect is obvious, the cost is relatively high. Presently, the co-cultivation technology has been favored for the transformation of complex substrates into value-added products, by virtue of its advantages in the minimal operational cost and synergistic metabolism of two or more organisms (Mohapatra et al. 2020; Izmirlioglu and Demirci 2017; Hashem et al. 2021). Using FW as the substrate, two-strains consortia containing enzyme-producing fungus and *B. amyloliquefaciens* HM618 were constructed to produce surfactin (Pan et al. 2021). In another study, waste DGS were directly used to produce surfactin by co-cultures of *B. amyloliquefaciens* MT45 and *B. amyloliquefaciens* X82 and the maximum surfactin yield was reported as 3.4 g/L (Zhi et al. 2017). In addition, the progress of co-cultivating *Bacillus coagulans* and *Bacillus thermoamylovorans* to ferment L-lactic acid, co-cultivating *Saccharomyces cerevisiae* and *Pichia stipitis* fermenting to product ethanol, and mixed-cultivating *Bacillus* and *Enterococcus* to ferment poly-3-hydroxybutyrate has also been successively reported (Tashiro et al. 2016; Ntaikou et al. 2018; Sindhu et al. 2020). Above researches about co-culture fermentation provide ideas for valorization of FW for iturin A.

Bacillus subtilis WB800N, which has the advantages of relatively clear genetic manipulation background and strong protein secretion ability, is an excellent host for expressing heterologous proteins (Liu et al. 2018; Zhang et al. 2005). For the other hand, because of the defective ability to synthsize lipopeptides (Wu et al. 2018), WB800N is suitable for co-cultivation with *B. amyloliquefaciens* HM618 to produce iturin A without affecting product analysis. In this study, recombinant *B. subtilis* was engineered to heterologously express lipase and amylase for the purpose of better degradation of FW. Further, recombinant *B. subtilis* were mixed-cultured with HM618 to transform FW into iturin A. Moreover, the inoculation time of HM618 strain and initial size of engineering strains were explored. To our knowledge, this study firstly verified the suitability of valorizing FW for iturin A production through artificial consortia.

Materials And Methods

Strains, plasmids and reagents

The strains used in this study are listed in Table S1. *B. amyloliquefaciens* HM618 (CGMCC 7097) used to product iturin A was isolated from the soil. *B. subtilis* WB800N and the plasmid pHT43, used for

expressing the heterologous lipase and amylase, were purchased from Baosai Biological Co., Ltd. (Hangzhou, China). *Escherichia coli* DH5α was used for constructing plasmids which listed in Table S2. *Botrytis cinerea* and *Rhizoctonia solani* were stored in laboratory and used as indicators for the antifungal experiment.

Phanta DNA Polymerase was purchased from Novazan Biotechnology Co., Ltd. (Nanjing China). All restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beijing, China). The kits BM Seamless Cloning and Lethal Based Fast Cloning was purchased from Biomed (Beijing, China) and Tiangen (Beijing, China), respectively. Iturin A standard was purchased from Sigma (USA).

Cultivation Conditions

When constructing the plasmids, *E. coli* DH5α and the recombinant *B. subtilis* were cultured in Luria-Bertani (LB) medium (10 g/L NaCl, 10 g/L peptone, 10 g/L yeast extract). If necessary, 100 μg/mL ampicillin, or 6 μg/mL chloramphenicol was added. Before fermentation for the production of iturin A, all bacteria need to be activated twice in 250 mL flask containing 50 mL seed medium (60 g/L glucose, 10 g/L peptone, 10 g/L NaCl, 5 g/L yeast extract, 10 g/L beef extract) at 37 ℃, 220 rpm for 24 h. Followingly, the activated bacteria *B. amyloliquefaciens* HM618 and recombinant *B. subtilis* was inoculated with OD_{600} of 0.2 in 500 mL flask containing 200 mL FW medium which replaced glucose in Landy medium (60 g/L FW, 14 g/L sodium L-glutamate, 5 g/L yeast extract, 0.5 g/L KCl, 1 g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 0.15 mg/L $FeSO_4$, 5 mg/L $MnSO_4$, 0.15 g/L $CuSO_4$) with 10% (w/v) FW and cultured at 37 ℃, 180 rpm for 168 h. Two fungi strains of *B. cinerea* and *R. solani* were cultured in PDA medium (200 g/L potato extract, 20 g/L glucose, 18 g/L agar) 30 ℃.

Plasmids construction

The genes of lipase and amylase are listed in Table S3, among them *lip1a*, *lip2*, *lipA* and *amyMH* were synthetized directly into pHT43 by Tsingke Biotechnology Co., Ltd. (Beijing, China). For replacing the promoter P_{garc} or signal peptide SP_{amyQ} of plasmid pHT43 in situ, the fragments P43 and SP_{nprB} were PCR amplified from *B. subtilis* 168 with primers P1/P2 and P3/P4 (Table 1), respectively. Then, the fragment P43 were assembled into linearized pHT43 digested with *Sac* I and *Not* I using BM Seamless Cloning Kit, generating the promoter P43 replacement vector pHT-P43. Meanwhile, to obtain the signal peptide SP_{nprB} replacement vector pHT-SP_{nprB}, the fragment SP_{nprB} was ligated into linearized vector pHT43, which are digested with *Not* I and *Bam*H I simultaneously, by T4 DNA Ligase. Similarly, the promoter and signal peptide replacement vector pHT-P43SP_{nprB} was generated. Besides, *amyBa* was cloned from *B. amyloliquefaciens* HM618 with primers P6/P7, and four pHT43 derivatives carrying *amyBa* was generated through restriction coupled with ligation. Such as, the fragment amyEBa was ligated into linearized pHT-P43*lip1a*, which are digested with *Bam*H I and *Xba* I simultaneously, generating pHT-P43*amyEBa*.

Composition analysis of FW

The FW was collected from the Xueyi canteen of Tianjin university (Tianjin, China), and mainly included rice, vegetables, and little soup. After picking out the bones, toilet paper and other impurities, FW was homogenized using the homogenizer and stored at -40℃. Starch content was determined according to the method described by Ben (Ben et al. 2017). Based on Soxhlet extraction, lipid content was measured (Pan et al. 2021). Crude protein (CP) content was estimated through the Kjeldahl method described by Chen (Chen et al. 2017). Composition analysis of the FW is presented in Table 2.

Enzyme activity assay

All constructed plasmids were transformed into *B. subtilis* WB800N competent cells obtained through natural competence (Anagnostopoulos and Spizizen, 1961). Verified recombinant *B. subtilis* cultured in LB medium at 37 ℃, 180 rpm for 72 h. If necessary, 100 μg/mL ampicillin, 6 μg/mL chloramphenicol, or 0.5 mM IPTG was added. The biomass was measured by OD_{600} with a spectrophotometer (TU-1810, Beijing, China), followingly detecting the enzyme activity of fermentation supernatant centrifuged at 12000 rpm for 5 min.

Amylase assay

The amylase activity was determined through the DNS method. 1.5 mL reaction system consisted of 500 μL of phosphate buffer (50 mM, pH 6.0), 900 μL of soluble starch (1%, w/v) as substrate, and 100 μL supernatant. The mixture reacted at 37 °C for 10 min, followed by adding 1.5 mL of dinitrosalicylic acid (DNS) mixture to end the reaction. After boiling 10 min, the OD_{540} was measured with the TU-1810 spectrophotometer. The control contained corresponding volume of medium. One unit of amylase activity is defined as the amount of enzyme required to produce 1 μM reducing sugar per minute.

Lipase assay

For determination of lipase, the method as described by Liu (Liu et al. 2017a). Briefly, 1 mL reaction system consisted of 750 μL of Tris-HCl buffer (50 mM, pH 8.0), 50 μL of p-NPL (10 mM) as substrate, and 200 μL supernatant. The mixture reacted at 37 °C for 10 min, followed by boiling 3 min to end the reaction. Finally, the OD_{410} was measured with the TU-1810 spectrophotometer. The control contained corresponding volume of medium. One unit of lipase activity is defined as the amount of enzyme required to produce 1 μM pNP per minute.

Iturin A isolation and high performance liquid chromatography (HPLC) analyses

The 100 mL fermentation supernatant was collected by centrifugation at 11000 rpm and 4°C for 10min, subsequently pH was adjusted to 2.0 with 6 M HCl and stored overnight at 4°C. The lipopeptide precipitate was collected by centrifugation at 8000 rpm and 4°C for 10min, and then resuspended with 100 mL methyl alcohol (AR) for iturin A extraction overnight. After centrifuging at 8000 rpm for 10 min, and evaporating until almost completely dry, iturin A precipitate finally dissolved in 5 mL methyl alcohol

(HPLC). Crude iturin A filtered through a 0.22 μm nylon membrane for quantitative analysis (Zhao et al. 2019b; Shi et al. 2018).

Iturin A sample (10 μL) was determined by reversed-phase HPLC (LC-20A, Shimadzu, Japan) system equipped with a C18 column (Thermo Scientific, 250×4.6 mm, 5 μm) and a UV detector set to 220 nm. The mobile phase was mixture of water containing 0.1% formic acid and acetonitrile (55:45, v/v) at a flow rate of 1.0 mL/min (Dang et al. 2019). Based on the calibration curve made by iturin A standards, iturin A concentration was quantified.

Liquid chromatography-mass spectrometry (LC-MS) analyses

The iturin A produced by *B. amyloliquefaciens* HM618 was determined by LC-MS using Waters HPLC equipped with electrospray ionization mass spectrometry (Thermo Electron, USA). The ESI source was operated in positive ion mode, and the m/z scan end is 2000. The mobile phase and column were described above. The LC-MS analyses were performed on the micrOTOF-Q software (Bruker Daltonics, USA).

Glucose analysis

The content of glucose in the fermentation broth is an important indicator of the growth status of the bacteria. The fermentation broth centrifuged at 12000 rpm for 10 min was filtered with a 0.22 μm aqueous membrane. 10 μL of sample was determined by reversed-phase HPLC system equipped with Aminex HPX-87H Ion Exclusion particles column (7.8 mm×300 mm, BIORAD) and differential refractive index detector. The mobile phase was 5.0 mM sulfuric acid water at a flow rate of 0.6 mL/min and the column temperature was maintained at 65℃ (Liu et al. 2017b). The retention time of glucose is 9.3 min.

Statistical analysis

All experiments were handled in triplicate. Statistical analysis was performed using Microsoft Excel 2019 (Microsoft, USA). Differences with p-value <0.05 (*), p-value <0.01 (**), and p-value <0.001 (***) were labeled.

Results And Discussion

Identification of iturin A produced by B. amyloliquefaciens HM618

Iturin A is a mixture of several homologues (Chen et al. 2019b). As reported, the hydrogen adduct ions m/z of four isoforms of iturin A with a C14 to C17 chain length were 1043.55, 1057.55, 1071.56, and 1085.58, respectively (Chen et al. 2019b; Dang et al. 2019). Consistent with the reported results, LC-MS identification results showed that iturin A produced by *B. amyloliquefaciens* HM618 was composed of four homologues of C14, C15, C16, and C17 iturin A (Fig. S1). However, the tested C15 iturin A showed two liquid phase peaks, which may be of the same quality but different type. In addition, C14 iturin A

occupied the largest proportion in iturin A according to the large response, but C17 iturin A was very small.

Construction of engineering B. subtilis heterologously expressed lipase and amylase

Since food waste was composed of nearly half of starch and about a quarter of lipid, the artificial consortia using food waste as a substrate were constructed to biotransform food waste into iturin A. As presented in Fig. 1, three lipase genes and two amylase genes were introduced into *B. subtilis* WB800N, meanwhile the promoter coupled with signal peptide in plasmid pHT43 were also replaced. After screening, the recombinant strain WB800N-AmyE with higher amylase activity and the recombinant strain WB800N-Lip with higher lipase activity were mixed-cultured *B. amyloliquefaciens* HM618. In the mixed-culture fermentation, engineering *B. subtilis* are defined as the functional strains, secreting hydrolases to convert the FW into glucose and fatty acid chains that are precursors for the synthesis of iturin A.

Verified by sequencing, twenty pHT43 expression plasmids were designed and constructed in *B. subtilis* WB800N for lipase and amylase production (Fig. S2, S3, S4, S5, S6, S7, and S8). Three controls, *B. subtilis* WB800N, WB800N+IPTG that was added inducer during fermentation, and *B. amyloliquefaciens* HM618, were set. As shown in Fig. S9 and S10, the extracellular enzyme activities of the five genes consistently increased from 16 h, and reached the maximum at 48 h Combined with the biomass curve, the increase in enzyme activity was caused by the accumulation of biomass. Further analysis found that under the effect of the IPTG inducible promoter P_{garo}, the three lipase genes were better expressed. With the assistance of the signal peptide SPamyQ, the lipase activity of the recombinant strains was more excellent. In addition, the results showed that the signal peptide SPamyQ greatly promoted the secretion of amylase AmyEBa, and the amylase activity of the corresponding recombinant strain WB-A13 and WB-A14 was greatly higher than that of other strains. Regrettably, there was no repeated phenomenon that methyl parathion hydrolase activity was increased hundreds of times in recombinant WB800 under the control of the promoter P43 and signal peptide SP_{nprB} (Zhang et al. 2005).

Comparing the extracellular enzyme activity of each strain at 48 h, the recombinant strains with the higher amylase and lipase activity were selected. As shown in Fig. 2, the recombinant strain WB-A13 had the highest amylase activity, 23406.4 U/mL, which is 8.25 times that of the control group WB+IPTG and 5.26 times that of HM618 strain. In addition, the amylase activity obtaining from HM618 was 1.65 times higher than the WB800N. However, because of the difficultly genetic manipulation on *B. amyloliquefaciens* HM618, the *amyEBa* was selected to transfer into other host WB800N. The recombinant strain WB-L01 had the highest lipase activity, 57.5 U/mL, which is 1.96 times that of the control strain WB+IPTG. Undoubtedly, the recombinant strain WB-A13 and WB-L01 were selected to mixed-culture with HM618, converting starch or lipid into a small molecule to synthesis iturin A. In same principle, the recombinant strains WB-A14 with outstanding amylase activity, WB-L02 and WB-L09 with higher lipase activity also were selected to constitute artificial consortia with HM618 strain.

Production ituirn A by artificial consortia of HM618 and recombinant B. subtilis

Co-culture of HM618 with recombinant B. subtilis producing amylase

In view of the fact that nearly half of the organic components in FW are starch, improving the degradation efficiency of starch is conducive to obtaining reducing sugars from the substrate. Hence, artificial consortia of HM618 and recombinant bacteria expressing expertly amylase was constructed for the production of iturin A. *B. amyloliquefaciens* HM618 under pure culture served as a control. The iturin A production under co-culture of HM618 and WB800N-AmyE was observed in Fig. 3A. The yield of iturin A reached to 7.66 mg/L under the co-culture of HM618 and WB-A14, which was increased 32.9% (p-value <0.05) compared to that of HM618 under pure culture. Compared with co-culture of HM618 and WB-A14, the iturin A production was slightly less under co-culture of HM618 and A13. More iturin A yielded form the consortia than the pure culture, which might be attributed to the addition of recombinant *B. subtilis*. In the fermentation about degrading complex substrates, it seems feasible to improve the yield of target through adding functional strains based on the characteristics of the substrates. According to the related reports, compared to the co-cultivation of *B. amyloliquefaciens* and amylase-producing fungi, the surfactin was accumulated more when *B. amyloliquefaciens* was co-cultured with lipase-producing fungi. This result is attributed to the food waste which contain more lipid than starch (Pan et al. 2021). Furthermore, some studies have reported the use of cellulose in rice straw (Mohapatra et al. 2020) and corn stover (Minty et al. 2013) for biotransformation into biofuels.

The activity of amylase in the co-culture was detected every 24 hours. As observed in Fig. 3B, the changes of amylase activity in co-culture fermentation were almost the same as those of HM618 under pure culture, but they were obviously different from those of recombinant *B. subtilis* under pure culture. In specifical, the amylase activity in pure-culture fermentation of the recombinant strains reached more than 6000 U/mL at 24 h, and it remained until the late fermentation stage. However, the amylase activity in co-culture fermentation were lower, less than 3000 U/mL, which was almost equivalent to the amylase activity of HM618 under pure culture. Combined with the curves in Fig. S10, the phenomenon that the consumption rate of glucose by HM618 is faster than that of *B. subtilis* was obviously observed. Theoretically, HM618 could prioritize the use of energy materials to gain an advantage in quantity, causing the limited growth of recombinant *B. subtilis* in the co-culture fermentation. Thus, the amylase activity of co-culture was similar as the pure culture.

Co-culture of HM618 with recombinant B. subtilis producing lipase

Lipid is the second most abundant nutrient in FW, and the fatty acid chain, which is the degradation product of lipid, is also an important precursor for the synthesis of iturin A, making it promising to construct artificial consortia combining the recombinant *B. subtilis* producing lipase. However, the effect of co-culture of HM618 and WB800N-Lip was even less satisfactory for iturin A production (Fig. 3D). Although the yield of iturin A was 10.51 mg/L in co-cultivation of HM618 and WB-L02, which was the highest yield of iturin A in co-cultivation fermentation, it was still less than the yield of itruin A in the control group. An interesting phenomenon was that the yield of iturin A was different in the pure culture of HM618. This difference may be mainly due to the instability of the food waste components. In general,

the iturin A was produced relatively more under co-culture of HM618 and WB800N-AmyE, which may be related to the more starch content in food waste. Furthermore, the changes of lipase activity were same as the amylase changes (Fig. 3E). This phenomenon possibly reflects the relationship of the microorganisms in mixed-culture fermentation. The fact in fermentation was inferred that HM618 occupied a dominant position in mixed-culture fermentation by virtue of its stronger substrate utilization capacity. Due to the poor substrate utilization, recombinant *B. subtilis* strains could not accumulate biomass for the secretion hydrolase. This might be the other reason why artificial consortia of HM618 and WB800N-Lip produced less iturin A.

In order to make full use of food waste, the recombinant strain WB-A14 and WB-L02 were selected to mixed-culture with HM618 strain together based on above results. However, in mixed-culture fermentation, the interaction between microorganisms inevitably occurs, including competition for resources and sharing of metabolism (Mee and Wang, 2012). Therefore, a reasonable distribution of substrates and metabolic intermediates in the mixed culture is of great significance for increasing the yield of iturin A. According to reports, by optimizing the inoculation amount of *Bacillus polymyxa* which could synthesis acetoin and the inoculation time of *E coli* that produces vitamin B2, the artificial consortium can produce more acetoin (Liu et al. 2017b). Similarly, in the co-culture fermentation with distiller's grains, optimizing the inoculum of two *B. amyloliquefaciens* resulted in an increase in the yield of surfactant to 2.54 g/L (Zhi et al. 2017). Therefore, from the perspective of optimizing the inoculation methods, the energy distribution between *B. amyloliquefaciens* HM618 and recombinant *B. subtilis* strains should be balanced as much as possible.

Effect of inoculation method of mixed-culture fermentation of HM618, WB-L02, and WB-A14 on iturin A production

The recombinant strains that degrade the food waste were added into the FW medium earlier, to obtain more sufficient small molecules when the strain HM618 was inoculated. Taking into account the higher biomass of recombinant *B. subtilis* strains after 24 h, different experimental groups that increase the inoculate volume of HM618 were set up. As illustrated in Fig. 4A, when the strain HM618 was directly inoculated with other two recombinant strain, the yield of iturin A reached 8.12 mg/L, which was an increase of 38.8% compared with the control group (p-value <0.01). In contrast, in the group d which even was set to inoculate HM618 for maximum OD_{600} 0.6, the yield of iturin A only ranged between 2.05 to 2.52 mg/L under the delay of HM618 inoculation for 24 hours, greatly less than the control group with the yield of 5.86 mg/L (p-value <0.01). It is not achieved the expectation that the titer of iturin A was dramatically decreased when HM618 was postponed 24 h. The reason might be explained by analyzing other results in mixed-fermentation. As shown in Fig. 4B, the amylase activity in groups b-d at 24 h was higher than that in group a, and this advantage lasted until the later stage of fermentation. Unanimously, the glucose concentration in groups b-d at 24 h were almost 4 times higher than that in group a, and this difference gradually increased with the increase of fermentation time (Fig. 4D). The above results indicated that the amount of *B. subtilis* was fully accumulated when HM618 was inoculated at 24 h. Based on this speculation, the growth of HM618 was affected in a certain extent in the mixed-

fermentation, causing the decreased yield of iturin A in the mixed-fermentation. In addition, the changes of lipase activity shown in Fig. 4C were also embodied that HM618 strain no longer had the advantage of growth in the three-strains consortia when HM618 was inoculated after 24 hours. Although three-strains consortium containing HM618, WB-L02 and WB-A14 showed certain advantages in the production of iturin A compared with other fermentation methods, the energy distribution between the three strains required greater efforts to regulate.

Conclusions

Food waste is a suitable raw material for iturin A production through mixed-culture fermentation of *B. amyloliquefaciens* HM618 strain that produces iturin A and recombinant *B. subtilis* with excellent amylase and lipase activity that is constructed by synthetic biology method. Co-culture of HM618 and WB-A14 produced the relatively most iturin A under two-strains consortium containing HM618 and recombinant *B. subtilis*. Furthermore, under three-strains consortium containing HM618, WB-A14, and WB-L02, the yield of iturin A was 38.8% higher than that of HM618 pure culture when the initial size OD_{600} of above three strains was 0.2, 0.15 and 0.15 respectively. These results recommended the potential for biotransformation food waste into value-added chemicals by artificial consortia.

Declarations

Availability of data and materials The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions Chang-Hao Miao, proposed the research idea, analyzed, and wrote original draft; Xiao-Feng Wang, provided assistance in experimental design and results analysis; Bin Qiao, helped analyze the LC-MS results; Chun-Yang Cao, provided assistance in data processing; Qiu-Man Xu and Jing-Sheng Cheng, responsible for funding acquisition, resources and project administration.

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Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

References

1. Al-Dhabi NA, Esmail GA, Arasu MV (2020) Co-fermentation of food waste and municipal sludge from the Saudi Arabian environment to improve lactic acid production by *Lactobacillus rhamnosus* AW3 isolated from date processing waste. Sustainability 12: 6899. https://doi.org/10.3390/su12176899
2. Ambrico A, Trupo M (2017) Efficacy of cell free supernatant from *Bacillus subtilis* ET-1, an iturin A producer strain, on biocontrol of green and gray mold. *Postharvest* Biol Technol 134: 5-10. https://doi.org/10.1016/j.postharvbio.2017.08.001
3. Anagnostopoulos C, Spizizen J (1961) Requirements for transformation in *Bacillus Subtilis*. J Bacteriol 81:741-6. https://doi.org/10.1128/jb.81.5.741-746.1961
4. Banu JR, Merrylin J, Usman TMM, Kannah RY, Gunasekaran M, Kim SH, Kumar G (2020) Impact of pretreatment on food waste for biohydrogen production: A review. Int J Hydrogen Energy 45: 18211-18225. https://doi.org/10.1016/j.ijhydene.2019.09.176
5. Ben Taher I, Bennour H, Fickers P, Hassouna M (2017) Valorization of potato peels residues on cellulase production using a mixed culture of *Aspergillus niger* ATCC 16404 and *Trichoderma reesei* DSMZ 970. Waste Biomass Valorization 8: 183-192. https://doi.org/10.1007/s12649-016-9558-5
6. Calvo H, Mendiara I, Arias E, Blanco D, Venturini ME (2019) The role of iturin A from *B. amyloliquefaciens* BUZ-14 in the inhibition of the most common postharvest fruit rots. Food Microbiol 82: 62-69. https://doi.org/10.1016/j.fm.2019.01.010
7. Chen H, Shen H, Su HF, Chen HZ, Tan F, Lin JF (2017) High-efficiency bioconversion of kitchen garbage to biobutanol using an enzymatic cocktail procedure. Bioresour Technol 245: 1110-1121. https://doi.org/10.1016/j.biortech.2017.09.056
8. Chen CY, Sun N, Li DS, Long SH, Tang XY, Xiao GQ, Wang LY (2018) Optimization and characterization of biosurfactant production from kitchen waste oil using *Pseudomonas aeruginosa*. Environ Sci Pollut Res 25: 14934-14943. https://doi.org/10.1007/s11356-018-1691-1
9. Chen WH, Li X, Ma XL, Chen SW, Kang YP, Yang MM, Huang FH, Wan X (2019a) Simultaneous hydrolysis with lipase and fermentation of rapeseed cake for iturin A production by *Bacillus amyloliquefaciens* CX-20. BMC Biotechnol 19: 98. https://doi.org/10.1186/s12896-019-0591-x
10. Chen MC, Wang JP, Zhu YJ, Liu B, Yang WJ, Ruan CQ (2019b) Antibacterial activity against *Ralstonia solanacearum* of the lipopeptides secreted from the *Bacillus amyloliquefaciens* strain FJAT-2349. J Appl Microbiol 126: 1519-1529. https://doi.org/10.1111/jam.14213
11. Chen WC, Wang M, Gong YM, Deng QC, Zheng MM, Chen SW, Wan X, Yang C, Huang FH (2021) The unconventional adverse effects of fungal pretreatment on iturin A fermentation by *Bacillus amyloliquefaciens* CX-20. Microb Biotechnol 14: 587-599. https://doi.org/10.1111/1751-7915.13658
12. Choi JM, Han SK, Lee CY (2018) Enhancement of methane production in anaerobic digestion of sewage sludge by thermal hydrolysis pretreatment. Bioresour Technol 259: 207-213. https://doi.org/10.1016/j.biortech.2018.02.123
13. Cochrane SA, Vederas JC (2016) Lipopeptides from *Bacillus* and *Paenibacillus* spp.: A gold mine of antibiotic candidates. Med Res Rev 36: 4-31. https://doi.org/10.1002/med.21321

14. Dang YL, Zhao FJ, Liu XS, Fan X, Huang R, Gao WX, Wang SF, Yang C (2019) Enhanced production of antifungal lipopeptide iturin A by *Bacillus amyloliquefaciens* LL3 through metabolic engineering and culture conditions optimization. Microb Cell Fact 18: 68. https://doi.org/10.1186/s12934-019-1121-1
15. Dinesh GK, Chauhan R, Chakma S (2018) Influence and strategies for enhanced biohydrogen production from food waste. Renewable Sustainable Energy Rev 92: 807-822. https://doi.org/10.1016/j.rser.2018.05.009
16. Hashem M, Alamri SA, Asseri TAY, Mostafa YS, Lyberatos G, Ntaikou I (2021) On the optimization of fermentation conditions for enhanced bioethanol yields from starchy biowaste via yeast co-cultures. Sustainability 13: 1890. https://doi.org/10.3390/su13041890
17. Izmirlioglu G, Demirci A (2017) Simultaneous saccharification and fermentation of ethanol from potato waste by co-cultures of *Aspergillus niger* and *Saccharomyces cerevisiae* in biofilm reactors. Fuel 202: 260-270. https://doi.org/10.1016/j.fuel.2017.04.047
18. Kawagoe Y, Shiraishi S, Kondo H, Yamamoto S, Aoki Y, Suzuki S (2015) Cyclic lipopeptide iturin A structure-dependently induces defense response in *Arabidopsis* plants by activating SA and JA signaling pathways. Biochem Biophys Res Commun 460: 1015-1020. https://doi.org/10.1016/j.bbrc.2015.03.143
19. Khatami K, Atasoy M, Ludtke M, Baresel C, Eyice O, Cetecioglu Z (2021) Bioconversion of food waste to volatile fatty acids: Impact of microbial community, pH and retention time. Chemosphere 275: 129981. https://doi.org/10.1016/j.chemosphere.2021.129981
20. Kim DH, Kim SH, Kim HW, Kim MS, Shin HS (2011) Sewage sludge addition to food waste synergistically enhances hydrogen fermentation performance. Bioresour Technol 102: 8501-8506. https://doi.org/10.1016/j.biortech.2011.04.089
21. Kiran EU, Trzcinski AP, Liu Y (2015) Platform chemical production from food wastes using a biorefinery concept. J Chem Technol Biotechnol 90: 1364-1379. https://doi.org/10.1002/jctb.4551
22. Kumar PN, Swapna TH, Khan MY, Reddy G, Hameeda B (2017) Statistical optimization of antifungal iturin A production from *Bacillus amyloliquefaciens* RHNK22 using agro-industrial wastes. Saudi J Biol Sci 24: 1722-1740. https://doi.org/10.1016/j.sjbs.2015.09.014
23. Li PY, Zeng Y, Xie Y, Li X, Kang Y, Wang YB, Xie TH, Zhang YK (2017) Effect of pretreatment on the enzymatic hydrolysis of kitchen waste for production. Bioresour Technol 223: 84-90. https://doi.org/10.1016/j.biortech.2016.10.035
24. Liu W, Li MG, Yan YJ (2017a) Heterologous expression and characterization of a new lipase from *Pseudomonas fluorescens* Pf0-1 and used for biodiesel production. Sci Rep 7: 15711. https://doi.org/10.1038/s41598-017-16036-7
25. Liu L, Xu QM, Chen T, Cheng JS, Yuan YJ (2017b) Artificial consortium that produces riboflavin regulates distribution of acetoin and 2,3-butanediol by *Paenibacillus polymyxa* CJX518. Eng Life Sci 17: 1039-1049. https://doi.org/10.1002/elsc.201600239
26. Liu X, Wang H, Wang B, Pan L (2018) Efficient production of extracellular pullulanase in *Bacillus subtilis* ATCC6051 using the host strain construction and promoter optimization expression

system. Microb Cell Fact 17: 163. https://doi.org/10.1186/s12934-018-1011-y

27. Liu WJ, Dong Z, Sun D, Chen Y, Wang SW, Zhu JR, Liu C (2019) Bioconversion of kitchen wastes into bioflocculant and its pilot-scale application in treating iron mineral processing wastewater. Bioresour Technol 288: 121505. https://doi.org/10.1016/j.biortech.2019.121505
28. Mee MT, Wang HH (2012) Engineering ecosystems and synthetic ecologies. Mol BioSyst 8: 2470-2483. https://doi.org/10.1039/C2MB25133G
29. Minty JJ, Singer ME, Scholz SA, Bae CH, Ahn JH, Foster CE, Liao JC, Lin XN (2013) Design and characterization of synthetic fungal-bacterial consortia for direct production of isobutanol from cellulosic biomass. Proc Natl Acad Sci U S A 110: 14592-14597. https://doi.org/10.1073/pnas.1218447110
30. Mizumoto S, Hirai M, Shoda M (2006) Production of lipopeptide antibiotic iturin A using soybean curd residue cultivated with *Bacillus subtilis* in solid-state fermentation. Appl Microbiol Biotechnol 72: 869-875. https://doi.org/10.1007/s00253-006-0389-3
31. Mohapatra S, Mishra RR, Nayak B, Behera BC, Mohapatra PKD (2020) Development of co-culture yeast fermentation for efficient production of biobutanol from rice straw: A useful insight in valorization of agro industrial residues. Bioresour Technol 318: 124070. https://doi.org/10.1016/j.biortech.2020.124070
32. Ntaikou I, Menis N, Alexandropoulou M, Antonopoulou G, Lyberatos G (2018) Valorization of kitchen biowaste for ethanol production via simultaneous saccharification and fermentation using co-cultures of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*. Bioresour Technol 63: 75-83. https://doi.org/10.1016/j.biortech.2018.04.109
33. Ortigueira J, Martins L, Pacheco M, Silva C, Moura P (2019) Improving the non-sterile food waste bioconversion to hydrogen by microwave pretreatment and bioaugmentation with *Clostridium butyricum*. Waste Manage 88: 226-235. https://doi.org/10.1016/j.wasman.2019.03.021
34. Pan FD, Liu S, Chen XY, Cheng JS (2021) Bioconversion of kitchen waste to surfactin via simultaneous enzymolysis and fermentation using mixed-culture of enzyme-producing fungi and *Bacillus amyloliquefaciens* HM618. Biochem Eng J 172: 108036. https://doi.org/10.1016/j.bej.2021.108036
35. Park JG, Lee B, Kwon HJ, Park HR, Jun HB (2019) Effects of a novel auxiliary bio-electrochemical reactor on methane production from highly concentrated food waste in an anaerobic digestion reactor. Chemosphere 220: 403-411. https://doi.org/10.1016/j.chemosphere.2018.12.169
36. Prasoulas G, Gentikis A, Konti A, Kalantzi S, Kekos D, Mamma D (2020) Bioethanol production from food waste applying the multienzyme system produced on-site by *Fusarium oxysporum* F3 and mixed microbial cultures. Fermentation-Basel 6: 39. https://doi.org/10.3390/fermentation6020039
37. Rishi V, Sandhu AK, Kaur A, Kaur J, Sharma S, Soni SK (2020) Utilization of kitchen waste for production of pullulan to develop biodegradable plastic. Appl Microbiol Biotechnol 104: 1307-1317. https://doi.org/10.1007/s00253-019-10167-9

38. Shi JR, Zhu XY, Lu YJ, Zhao HZ, Lu FX, Lu ZX (2018) Improving iturin A production of *Bacillus amyloliquefaciens* by genome shuffling and its inhibition against *Saccharomyces cerevisiae* in orange juice. Front Microbiol 9: 2683. https://doi.org/10.3389/fmicb.2018.02683
39. Sindhu R, Manju A, Mohan P, Rajesh RO, Madhavan A, Arun KB, Hazeena SH, Mohandas A, Rajamani SP, Puthiyamadam A Binod P, Reshmy R (2020) Valorization of food and kitchen waste: An integrated strategy adopted for the production of poly-3-hydroxybutyrate, bioethanol, pectinase and 2, 3-butanediol. Bioresour Technol 310: 123515. https://doi.org/10.1016/j.biortech.2020.123515
40. Tashiro Y, Inokuchi S, Poudel P, Okugawa Y, Miyamoto H, Miayamoto H, Sakai K (2016) Novel pH control strategy for efficient production of optically active L-lactic acid from kitchen refuse using a mixed culture system. Bioresour Technol 216: 52-59. https://doi.org/10.1016/j.biortech.2016.05.031
41. Wang QH, Ma HZ, Xu WL, Gong LJ, Zhang WY, Zou DX (2008) Ethanol production from kitchen garbage using response surface methodology. Biochem Eng J 39: 604-610. https://doi.org/10.1016/j.bej.2007.12.018
42. Wang YY, Zhang CY, Liang J, Wu LF, Gao WB, Jiang JZ (2020) Iturin A extracted from *Bacillus subtilis* WL-2 affects phytophthora infestansvia cell structure disruption, oxidative stress, and energy supply dysfunction. Front Microbiol 11: 536083. https://doi.org/10.3389/fmicb.2020.536083
43. Wu Q, Zhi Y, Xu Y (2018) Systematically engineering the biosynthesis of a green biosurfactant surfactin by *Bacillus subtilis* 168. Metab Eng 52: 87-97. http://dio.org/10.1016/j.ymben.2018.11.004
44. Xu YX, Cai DB, Zhang H, Gao L, Yang Y, Gao JM, Li YY, Yang CL, Ji ZX, Yu J, Chen, SW (2020) Enhanced production of iturin A in *Bacillus amyloliquefaciens* by genetic engineering and medium optimization. Process Biochem 90: 50-57. https://doi.org/10.1016/j.procbio.2019.11.017
45. Ye M, Liu JY, Ma CN, Li YY, Zou LP, Qian GR, Xu ZP (2018) Improving the stability and efficiency of anaerobic digestion of food waste using additives: A critical review. J Cleaner Prod 192: 316-326. https://doi.org/10.1016/j.jclepro.2018.04.244
46. Yirong C, Zhang W, Heaven S, Banks CJ (2017) Influence of ammonia in the anaerobic digestion of food waste. J Environ Chem Eng 5: 5131-5142. https://doi.org/10.1016/j.jece.2017.09.043
47. Zeng Y, Bian DL, Xie Y, Jiang XL, Li X, Li PY, Zhang YK, Xie TH (2017) Utilization of food waste hydrolysate for microbial lipid and protein production by *Rhodosporidium toruloides* Y2. J. Chem. Technol Biotechnol 92: 666-673. https://doi.org/10.1002/jctb.5049
48. Zhang XZ, Cui ZL, Hong Q, Li SP (2005) High-level expression and secretion of methyl parathion hydrolase in *Bacillus subtilis* WB800. Appl Environ. Microbiol 71: 4101-4103. https://doi.org/10.1128/AEM.71.7.4101-4103.2005
49. Zhang CS, Su HJ, Baeyens J, Tan, TW (2014) Reviewing the anaerobic digestion of food waste for biogas production. Renewable Sustainable Energy Rev 38: 383-392. https://doi.org/10.1016/j.rser.2014.05.038
50. Zhang WY, Zou H, Jiang L, Yao JJ, Liang J, Wang QH (2015) Semi-solid state fermentation of food waste for production of *Bacillusthuringiensis* biopesticide. Biotechnol Bioprocess Eng 20: 1123-1132. https://doi.org/10.1007/s12257-015-0347-y

51. Zhang JY, Lv C, Tong J, Liu JW, Liu JB, Yu DW, Wang YW, Chen MX, Wei YS (2016) Optimization and microbial community analysis of anaerobic co-digestion of food waste and sewage sludge based on microwave pretreatment. Bioresour Technol 200: 253-261. https://doi.org/10.1016/j.biortech.2015.10.037
52. Zhao HB, Zhao XX, Lei SZ, Zhang YW, Shao DY, Jiang CM, Sun H, Shi JL (2019a) Effect of cell culture models on the evaluation of anticancer activity and mechanism analysis of the potential bioactive compound, iturin A, produced by *Bacillus subtilis*. Food Funct 10: 1478-1489. https://doi.org/10.1039/C8FO02433B
53. Zhao HB, Xu XG, Lei SZ, Shao DY, Jiang CM, Shi JL, Zhang YW, Liu L, Lei SZ, Sun H, Huang QS (2019b) Iturin A-like lipopeptides from *Bacillus subtilis* trigger apoptosis, paraptosis, and autophagy in Caco-2 cells. J Cell Physiol 234: 6414-6427. https://doi.org/10.1002/jcp.27377
54. Zhi Y, Wu Q, Xu Y (2017) Production of surfactin from waste distillers' grains by co-culture fermentation of two *Bacillus amyloliquefaciens* strains. Bioresour Technol 235: 96-103. https://doi.org/10.1016/j.biortech.2017.03.090

Tables

Table 1 Primer sequences

Primers	Sequences (5’-3’)
P1	ACGGCCAGTGAATTCGAGCTCagcttcgtgcatgcag
P2	TTCCTCCTTTAATTGGCGGCCGCgtgtacattcctctcttacctataatg
P3	*GCGGCCGC*atgcgcaacttgaccaag
P4	*GGATCC*agcagctgaggcatgtgttac
P5	GGTCAAGTTGCGCATgcggccgcgtgtacattcctctcttacctataatg
P6	*ggatcc*atgtttgaaaaacgattcaaaacctc
P7	*tctaga*ttaatgcggaagataaccgtttaaacc

Table 2 Components identification result of FW

Component	Content (%)
Total Solid (TS)	26.79±0.89
Moisture	73.21±0.11
Starch (based on TS)	47.77±5.33
Crude lipid (based on TS)	24.12±0.90
Crude protein (based on TS)	6.07±0.31

Figures

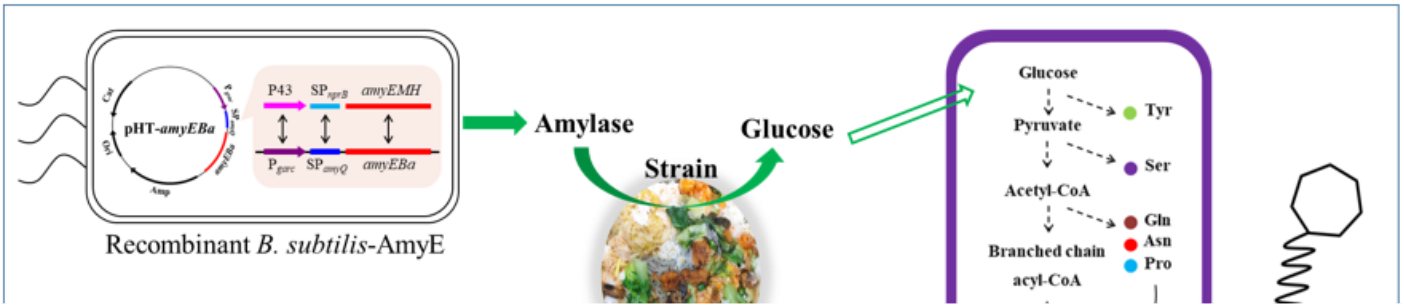

Figure 1

Schematic diagram of constructing artificial consortia.

Glucose is the energy molecule that supports the growth and metabolism of *B. amyloliquefaciens*. Fatty acid chains are the precursors for the synthesis of iturin A.

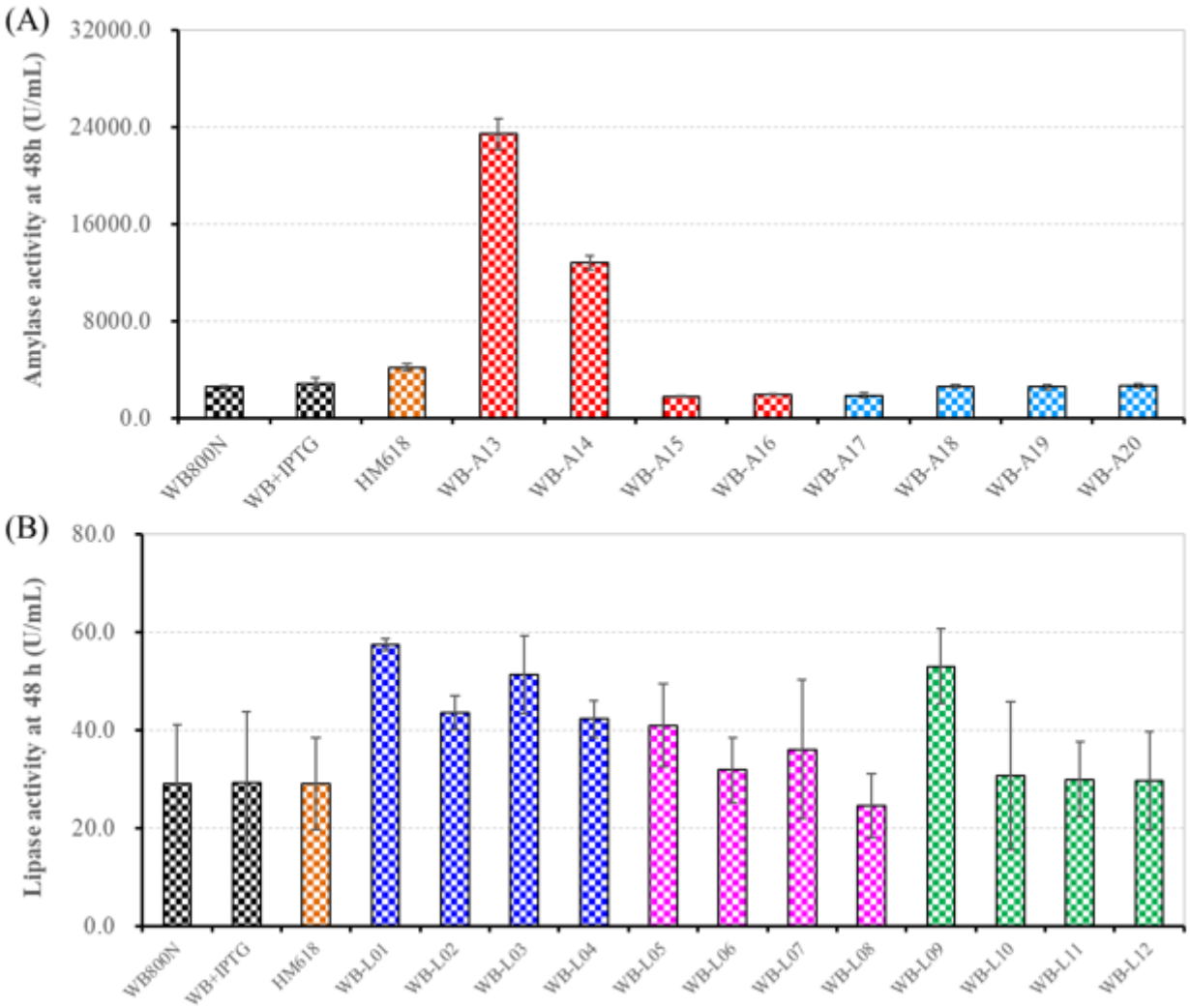

Figure 2

The amylase (A) and lipase (B) activity of recombinant *B. subtilis* at 48 h of fermentation.

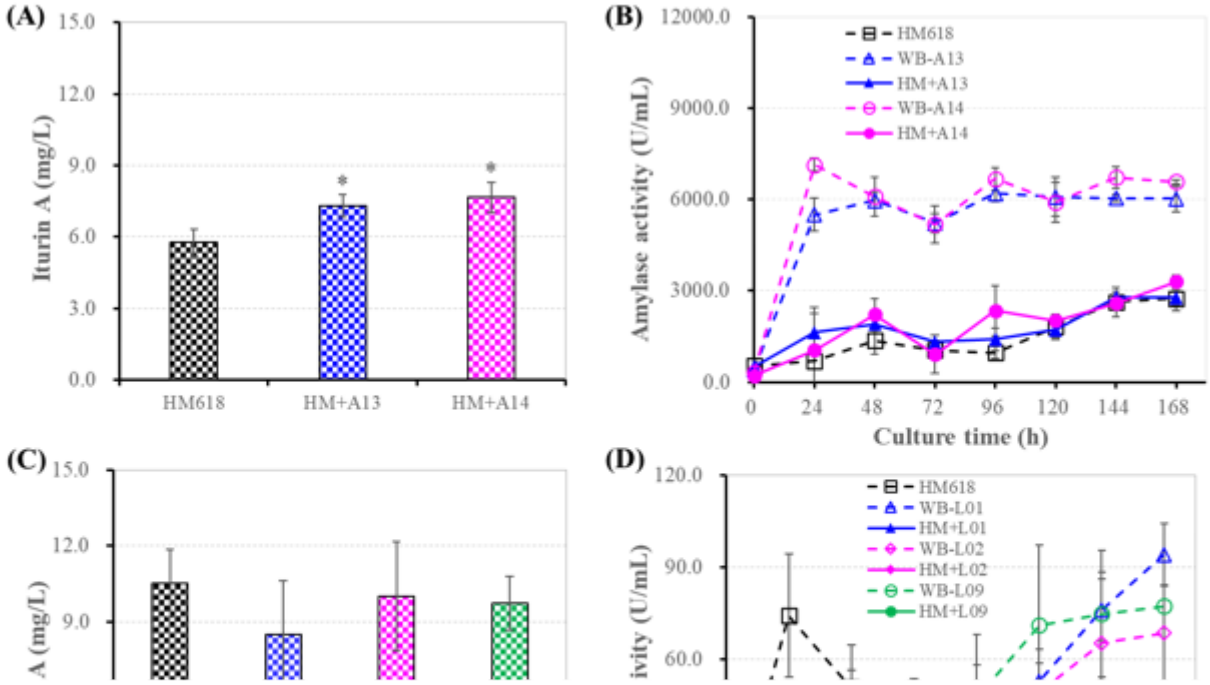

Figure 3

Co-culture fermentation of HM618 and recombinant *B. subtilis*.

Iturin A production (A) and amylase activity (B) under co-culture of HM618 and WB800N-AmyE. Iturin A production (C) and lipase activity (D) under co-culture of HM618 and WB800N-Lip.

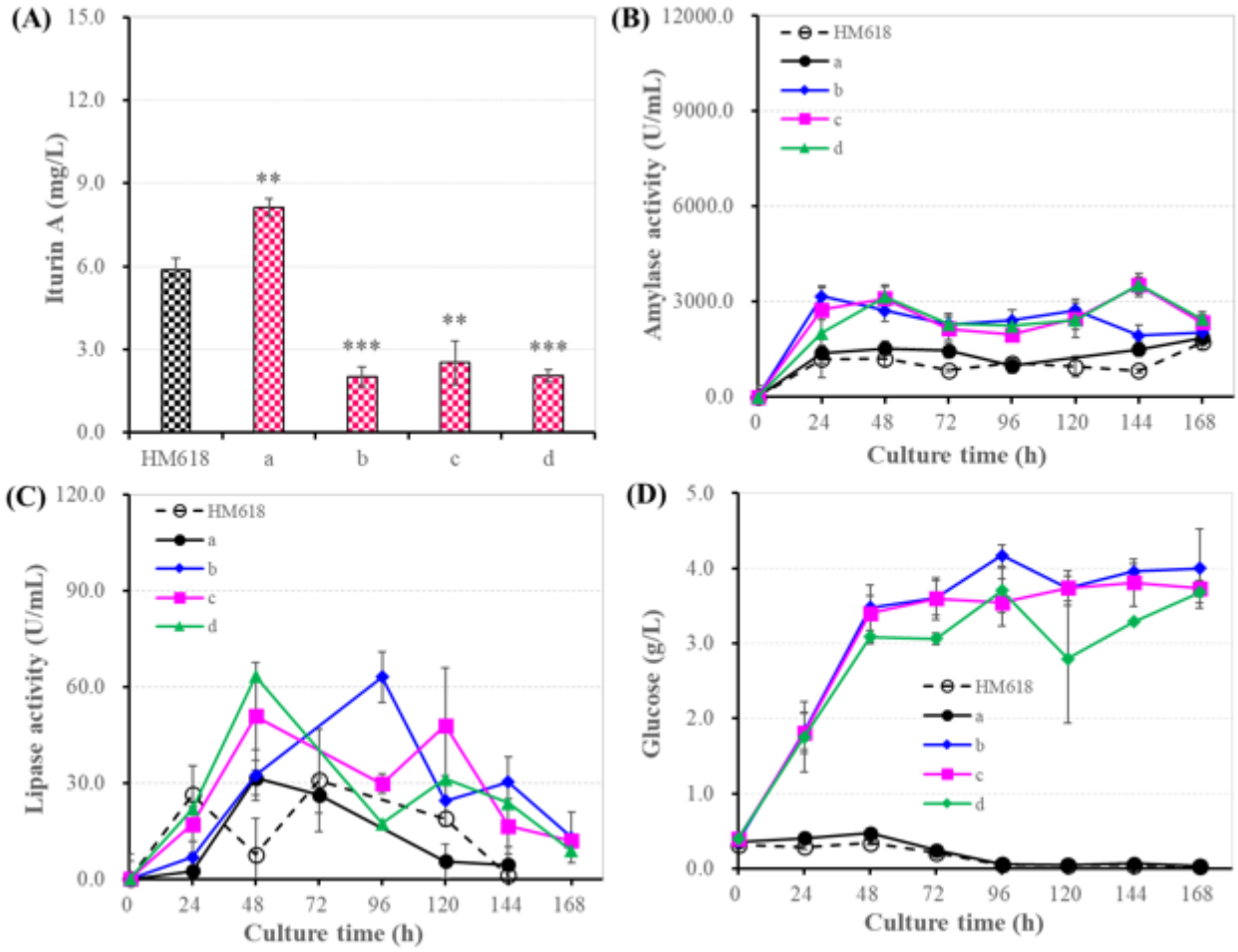

Figure 4

Effects of inoculation time in mixed-culture fermentation of three-strains consortium. Iturin A production (A), amylase activity (B), lipase activity (C), and glucose content (D) under mixed-culture of three-strains consortium. HM618 was solely cultured with initial OD_{600} of 0.2 as the control. Consortium a: $L02_{0.15}$+$A14_{0.15}$+$HM_{0\ h+0.2}$, b: $L02_{0.15}$+$A14_{0.15}$+$HM_{24\ h+0.2}$ (L02 and A14 was inoculated with OD_{600} 0.15 and 0.15 respectively, after 24 h HM618 was inoculated with OD_{600} of 0.2), c: $L02_{0.15}$+$A14_{0.15}$+$HM_{24\ h+0.4}$, d: $L02_{0.15}$+$A14_{0.15}$+$HM_{24\ h+0.6}$.

Supplementary Files

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