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The unconventional adverse effects of fungal pretreatment on iturin A fermentation by *Bacillus amyloliquefaciens* CX-20

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Summary

Fungal pretreatment is the most common strategy for improving the conversion of rapeseed meal (RSM) into value-added microbial products. It was demonstrated that *Bacillus amyloliquefaciens* CX-20 could directly use RSM as the sole source of all nutrients except the carbon source for iturin A fermentation with high productivity. However, whether fungal pretreatment has an impact on iturin A production is still unknown. In this study, the effects of fungal pretreatment and direct bio-utilization of RSM for iturin A fermentation were comparatively analysed through screening suitable fungal species, and evaluating the relationships between iturin A production and the composition of solid fermented RSM and liquid hydrolysates. Three main unconventional adverse effects were identified. (1) Solid-state fermentation by fungi resulted in a decrease of the total nitrogen for *B. amyloliquefaciens* CX-20 growth and metabolism, which caused nitrogen waste from RSM. (2) The released free ammonium nitrogen in liquid hydrolysates by fungal pretreatment led to the reduction of iturin A. (3) The insoluble precipitates of hydrolysates, which were mostly ignored and wasted in previous studies, were found to have beneficial effects on producing iturin A. In conclusion, our study verifies the unconventional adverse effects of fungal pretreatment on iturin A production by *B. amyloliquefaciens* CX-20 compared with direct bio-utilization of RSM.

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Introduction

Due to the growing international demand for oils with excellent nutritive value as well as renewable biofuels, driven by human health and environmental concerns, the production of rapeseed oil is continuously rising (Li et al., 2019, 2020; Song *et al*., 2019). Concomitantly, the global production of rapeseed meal (RSM), as the second most widely produced and less expensive byproduct after soybean meal (SBM), is approximately 40 million tons in 2019 (Sutter *et al*., 2017; Tie *et al*., 2020). RSM is a good source of high-quality protein for commercial swine and poultry diets with an even more favourable composition of essential amino acids (AA) than SBM (Kaewtapee *et al*., 2018). However, the utilization of RSM as an animal feed is limited because it contains anti-nutritional constituents such as phytic acid, erucic acid, glucosinolate, phenolic and fibre (Wang *et al*., 2010; Lomascolo *et al*., 2012; Drazbo *et al*., 2018). Moreover, the proteins from RSM are less digestible than those from other protein-rich waste materials such as fish meal or SBM, which has rendered them less valuable (Bos *et al*., 2007; Kiran *et al*., 2012). Consequently, alternative uses for this abundant byproduct are the subject of ongoing research.

In recent years, RSM has been widely used as a low-cost raw material in fermentation processes for the industrial production of various value-added microbial metabolites (Kiran et al., 2012, 2013; Chatzifragkou *et al*., 2014; Almeida *et al*., 2015; Jaszek *et al*., 2016; Prendecka *et al*., 2016). RSM can not only be used as a nitrogen source (Kiran *et al*., 2012; García *et al*., 2013; Chatzifragkou *et al*., 2014; Jin *et al*., 2015), but also provide carbon for the growth and metabolism of microorganisms (Chen *et al*., 2011; Almeida *et al*., 2015). However, these nutrients cannot be easily and directly assimilated by the majority of bacteria and industrial yeasts (Wang *et al*., 2010). Therefore, different techniques, such as fine milling, extrusion or the addition of chemicals and enzymes, were used to pretreat the RSM and thereby made its proteins accessible (Pustjens *et al*., 2012). Pretreatment with fungi in solid-state fermentation (SSF) is a good candidate for such processes (Wang *et al*., 2010; Kiran et al., 2012, 2013; García *et al*., 2013; Chatzifragkou *et al*., 2014; Salakkam *et al*., 2017; Salakkam and Webb, 2018). The growth of fungi can be more efficient than that of bacteria and yeasts because their morphology and physiology enable them to colonize the solid RSM material more easily (Wang *et al*., 2010). *Aspergillus oryzae* is as an efficient producer of protease, phytase, cellulase, xylanase and amylolytic enzymes, and is the most commonly used fungus for the pretreatment of RSM. For example, it has been used to produce RSM hydrolysates for the production of omega-3 docosahexaenoic acid (DHA) (Gong *et al*., 2015), microbial oil (Wang *et al*., 2010; Kiran et al., 2012, 2013), 1,3-propanediol (PDO) (Chatzifragkou *et al*., 2014) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)) (García *et al*., 2013). In addition, SSF of *Penicillium oxalicum* and *Neurospora crassa* was also used to hydrolyse RSM (Gong *et al*., 2015). However, there are still no fixed criteria for selecting the best fungi for RSM pretreatment.

Although fungal pretreatment is a feasible strategy for converting RSM into high-value-added microbial products, to the best of our knowledge, RSM hydrolysates produced by fungal pretreament have not yet been used in large-scale commercial production. Iturin A is an important antifungal cyclic lipopeptide with wide application prospects. In previous studies, we verified the advantages of direct bio-utilization of untreated RSM for effective iturin A production by *Bacillus* in submerged fermentation, which compared favourably with commercial nitrogen sources (Jin et al., 2014, 2015; Chen *et al*., 2019a). Moreover, the potential industrial strain *B. amyloliquefaciens* CX-20 was able to achieve high iturin A productivity by directly using RSM for all nutrients except for the carbon source (Chen *et al*., 2019a). Even though it was not optimal, when glucose was used as the carbon source, the maximum production of iturin A by *B. amyloliquefaciens* CX-20 after 72 h of batch fermentation in shake flasks reached up to 1.25 g l^{-1}. This value corresponded to a yield of 13.89 g iturin A per kg RSM, which was very close to the highest production level reported to date. This previous record of 14 g iturin A per kg dry substrate was obtained using *B. subtilis* RB14-CS in a medium based on soybean curd residue (Mizumoto *et al*., 2007; Chen *et al*., 2019a). However, whether fungal pretreatment can further improve the synthesis of iturin A is still unknown.

In this study, the effects of fungal pretreatment and direct bio-utilization of RSM for iturin A production by *B. amyloliquefaciens* CX-20 were comparatively analysed. Based on the chemical composition of solid fermented RSM (FRSM), nine fungi, including four *A. oryzae*, four *A. niger* and one *Trametes* sp. strain, were used to screen suitable fungal species. Moreover, the relationships between iturin A production and the composition of solid fermented RSM and liquid hydrolysates were explored systematically. Most previous studies focused on liquid supernatants of hydrolysates, while the insoluble precipitates were mostly ignored and discarded, so that the nutrient content of RSM could not be fully utilized. Based on *B. amyloliquefaciens* CX-20 that could directly utilize RSM for submerged fermentation, we used supernatants, precipitates and mixtures of hydrolysates as the sole source of nutrients except for carbon to produce iturin A (Fig. 1). In order to evaluate the advantages and disadvantages of fungal pretreatment objectively, the consumption of nutrients from RSM during fungal pretreatment was also taken into consideration.

Results

Screening of fungi for RSM pretreatment through SSF

The analysis of the nutrient contents of the unfermented RSM and nine FRSM was presented in Tables 1 and 2. After drying, the nine FRSM contained more dry matter, crude protein (CP) and total AA than RSM. The relative contents of CP and total AA were also analysed on a dry matter (DM) basis (Fig. 2). The relative CP contents of all FRSM were higher than that of the RSM, and the highest relative CP contents were measured in the FRSM produced by *A. oryzae* 92011 and *A. niger* 93298, reaching values of 48.31% and 50.60%, respectively. The relative CP content obtained by *Trametes* sp. 48424 was 47.05%. At the same time, the relative total AA contents of all FRSM were increased compared with RSM. The relative total AA contents produced by *A. oryzae* 92011 (92011F) and *A. niger* 93298 (93298F) were highest, at 41.31% and 41.84%, respectively. The relative total AA content of *Trametes* sp. 48424 FRSM

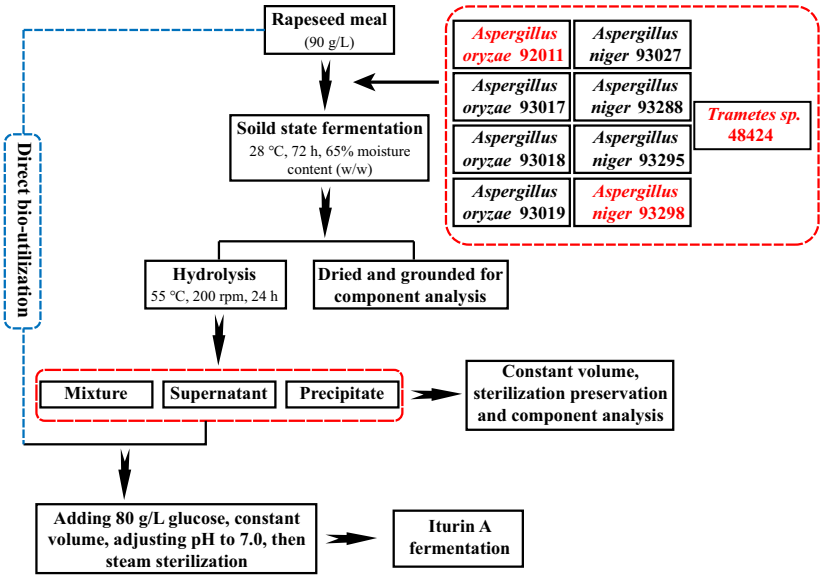

Fig. 1. A schematic of the overall experimental procedure. An initial RSM concentration of 90 g l^{-1} was used for the direct bio-utilization fermentation, while the same weight of RSM was pretreated by fungi in solid-state fermentation for the other experiments. Nine fungi were chosen and the chemical composition of the corresponding fermented RSM was analysed. Based on the contents of CP and total AA, the fungi *A. oryzae* 92011, *A. niger* 93298 and *Trametes* sp. 48424 were used for further hydrolysis and fermentation of iturin A. RSM pretreated by the three fungi corresponding to an initial concentration of 90 g l^{-1} was hydrolysed and then subjected to three treatments: mixture, supernatant and precipitate. After adding 80 g l^{-1} glucose, adjusting the pH to 7.0, and steam sterilization, all three treatment media were used for iturin A fermentation.

(48424F) was 38.38%. Moreover, as shown in Table 1, the content of crude fibre was decreased by every fungi, whereby *A. niger* had obvious advantages. After fermentation, most of the FRSM contained less neutral detergent fibre and acid detergent fibre, and had an increased content of ash. However, there was no clear change trend for crude fat. The crude fat contents of 92011F and 48424F were increased to 2.04% and 2.46% from 1.4%, while that of 93298F was decreased to 1.00%. In addition, the details of the AA contents of the FRSM were analysed as shown in Table 2. The AA profiles of samples fermented by different fungi showed different changes. However, the contents of the essential AA Met, His and Lys, as well as the non-essential AA Cys, Tyr and Pro were increased after fermentation by all fungi.

Effect of pretreatment with different fungal strains on the composition of RSM hydrolysates

Based on the analysis results of the chemical composition of RSM and the FRSM, *A. oryzae* 92011 and *A. niger* 93298 were selected for following hydrolysis and iturin A fermentation because the strains 92011F and 93298F had higher CP and total AA contents. Although the strain 48424F contained less CP and total AA contents than the strains 92011F and 93298F, it was also chosen as a candidate for hydrolysis and iturin A fermentation due to the high laccase activity, responsible for lignin degradation which was one of the most recalcitrant factors for efficient utilization of RSM. In a previous study, the optimal concentrations of initial RSM and glucose for iturin A fermentation by *B. amyloliquefaciens* CX-20 were confirmed to be 90 g l^{-1} and 80 g l^{-1}, respectively (Chen *et al*., 2019a). Therefore, 90 g l^{-1} of initial RSM was used for the direct bio-utilization and indirect utilization via fungal pretreatment as shown in Figure 1. After high-temperature steam sterilization, the chemical compositions of the RSM and FRSM hydrolysates (RSMH, 92011H, 93298H and 48424H) were analysed. Table 3 listed the concentrations of free ammonium nitrogen (FAN) and mineral elements in the three different hydrolysate preparations (mixture, supernatant and precipitate), as well as the corresponding iturin A production. The content order of FAN in the mixture of three hydrolysates was 48424H>93298H>92011H. However, there was no apparent change trend for the contents of FAN in the precipitates or supernatants of hydrolysates, which were respectively close to 250 mg l^{-1} or 1000 mg l^{-1}, in all cases. Interestingly, the content order of FAN in hydrolysate preparations was supernatant>mixture>precipitate. Similar change trends were also found for some mineral elements, including Ca, K, Mg, Mn and P. However, the content order of Cu, Fe, Si and Zn was mixture>precipitate>supernatant.

Table 1. Analysis of the nutrient composition of RSM and FRSM.

Strain	Dry matter	Crude protein	Crude fibre	Neutral detergent fibre	Acid detergent fibre	Ash	Crude fat
RSM	90.23 ± 0.75	39.48 ± 2.06	12.02 ± 0.04	30.38 ± 0.48	18.67 ± 0.29	7.68 ± 0.05	1.40 ± 0.01
92011F	94.21 ± 0.51	45.51 ± 0.89	10.14 ± 0.10	29.01 ± 0.69	16.93 ± 0.52	8.33 ± 0.14	2.04 ± 0.06
93017F	94.83 ± 0.17	42.81 ± 1.37	10.78 ± 0.13	27.31 ± 0.50	15.20 ± 0.35	8.01 ± 0.54	2.45 ± 0.08
93018F	94.65 ± 0.59	44.62 ± 0.79	11.27 ± 0.09	30.20 ± 0.22	17.27 ± 0.34	8.14 ± 0.17	1.74 ± 0.02
93019F	94.90 ± 0.25	43.84 ± 0.82	10.66 ± 0.09	28.74 ± 0.41	20.31 ± 0.26	8.14 ± 0.05	2.35 ± 0.01
93027F	94.18 ± 1.01	46.43 ± 1.54	9.68 ± 0.10	23.44 ± 0.30	14.72 ± 0.11	8.82 ± 0.03	1.25 ± 0.01
93288F	94.24 ± 0.17	42.45 ± 2.04	9.01 ± 0.07	27.92 ± 0.69	16.15 ± 0.29	8.59 ± 0.02	1.95 ± 0.03
93295F	94.21 ± 0.49	44.47 ± 1.65	9.52 ± 0.10	31.45 ± 0.41	20.74 ± 0.48	8.71 ± 0.13	1.25 ± 0.04
93298F	94.07 ± 1.11	47.60 ± 0.31	10.83 ± 0.02	28.67 ± 0.51	17.47 ± 0.46	8.92 ± 0.05	1.00 ± 0.01
48424F	94.66 ± 0.35	44.54 ± 0.46	10.14 ± 0.05	28.83 ± 0.22	16.38 ± 0.20	8.32 ± 0.23	2.46 ± 0.03

RSM indicates that no fungi were added. Fermented RSM by *A. oryzae* 92011, 93017, 93018 and 93019, *A. niger* 93027, 93288, 93295 and 93298, and *Trametes* sp. 48424 were designated as 92011F, 93017F, 93018F, 93019F, 93027F, 93288F, 93295F, 93298F and 48424F.

Table 2. Amino acid contents (%) of RSM and the FRSM treated by different fungal strains.

	RSM	92011F	93017F	93018F	93019F	93027F	93288F	93295F	93298F	48424F
Asp	2.77	3.40	2.21	3.36	2.40	3.13	2.74	3.19	3.03	3.34
Thr[a]	1.49	1.94	2.39	1.90	1.14	1.40	1.66	1.40	1.79	1.51
Ser	1.58	1.89	1.30	1.76	1.36	1.37	1.61	1.40	1.61	1.5
Glu	6.47	6.55	6.73	6.09	6.54	6.47	6.49	6.66	7.76	7.23
Gly	1.74	2.11	1.83	2.13	1.43	1.73	2.03	1.74	2.13	1.86
Ala	1.75	2.46	1.86	2.43	1.64	1.79	1.91	1.69	2.24	1.73
Cys	0.84	0.93	0.87	0.94	1.01	0.92	0.89	0.85	0.93	0.85
Val[a]	1.82	2.03	1.6	2.01	1.33	1.70	1.74	1.67	1.96	1.7
Met[a]	0.68	1.18	0.97	1.43	0.76	1.33	0.89	1.27	0.96	0.73
Ile[a]	1.37	1.66	1.21	1.56	1.00	1.27	1.34	1.23	1.53	1.3
Leu[a]	2.64	3.10	2.39	2.97	2.4	2.53	2.64	2.49	2.99	2.73
Tyr	1.08	1.53	1.34	1.39	1.85	2.4	1.33	2.11	1.57	1.64
Phe[a]	1.52	1.61	1.74	1.41	2.54	1.97	1.43	1.79	1.64	1.66
His[a]	1.13	1.87	1.5	1.76	2.07	1.6	1.51	1.51	1.71	1.47
Lys[a]	1.94	2.06	2.07	2.06	2.83	2.3	1.94	2.01	2.51	2.17
Arg[a]	2.28	2.39	2.46	2.37	3.79	2.81	2.06	1.86	2.23	2.03
Pro	2.16	2.21	2.65	2.37	2.53	2.81	2.58	2.46	2.77	2.88
Total AA	33.26	38.92	35.12	37.94	36.62	37.53	34.79	35.33	39.36	36.33

RSM indicates that no fungi were added. Fermented RSM by *A. oryzae* 92011, 93017, 93018 and 93019, *A. niger* 93027, 93288, 93295 and 93298, and *Trametes* sp. 48424 were designated as 92011F, 93017F, 93018F, 93019F, 93027F, 93288F, 93295F, 93298F and 48424F.
a. Indispensable amino acids (AA).

Effect of different fungal pretreatments on iturin A production by B. amyloliquefaciens *CX-20*

As shown in Figure 3, the iturin A productions from the three mixtures of hydrolysates were decreased with the increase of FAN concentration. Conversely, iturin A productions from the three precipitates of hydrolysates were increased with the increase of FAN concentration. However, no iturin A was produced by using the supernatants of hydrolysates as nutrient source. The maximum production was obtained by using the mixture of 92011H as the sole nutrient source except for the carbon source, followed by the mixtures of 93298H and 48424H. The corresponding iturin A concentrations were 0.93, 0.60 and 0.27 g l^{-1}, respectively. However, compared with direct bio-utilization of RSM, in which the iturin A production reached 1.25 g l^{-1} (Chen *et al.*, 2019a), fungal pretreatment had a negative effect on iturin A production. The maximum iturin A production from precipitates was obtained by the precipitate of 93298H, followed by the precipitates of 92011H and 48424H. The corresponding iturin A concentrations were 0.51, 0.37 and 0.24 g l^{-1}, respectively. Moreover, compared with the precipitate of RSM hydrolysate without fungal pretreatment, which yielded 0.64 g l^{-1} iturin A, fungal pretreatment was able to effectively release and solubilize nutrients from RSM into the supernatant of hydrolysate, so that the precipitate contained less nutrients for iturin A production.

The initial reducing sugar concentrations of all media were closed to 80 g l^{-1}, and the content order was supernatant>mixture>precipitate. However, the final reducing sugar concentrations of the supernatants did not change obviously. This was in agreement with the

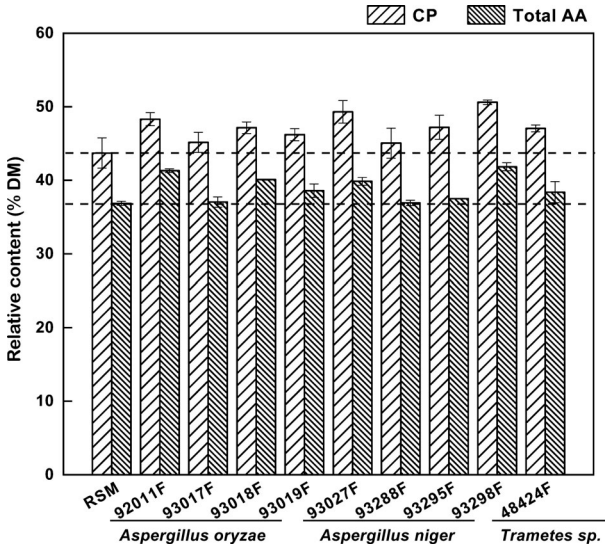

Fig. 2. The relative contents of CP and total AA of RSM and FRSM. RSM indicates that no fungi were added. Fermented RSM by *A. oryzae* 92011, 93017, 93018 and 93019, *A. niger* 93027, 93288, 93295 and 93298, and *Trametes* sp. 48424 were designated as 92011F, 93017F, 93018F, 93019F, 93027F, 93288F, 93295F, 93298F and 48424F.

fact that no iturin A was produced by using the supernatants as nutrient source. The content order of the final reducing sugar concentration among all the hydrolysates was supernatant > precipitate > mixture. Therefore, the change trends of sugar consumption ratios were also the same. The sugar consumption ratios of the mixtures were about 77%, while the sugar consumption ratios of the precipitates were about 71%.

Moreover, the changes of FAN before and after iturin A fermentation were also analysed. As shown in Figure 3B, the initial FAN (IFAN) concentrations of hydrolysates obtained following fungal pretreatment were generally higher than those of RSMH, either in the mixtures, precipitates or in the supernatants. The content order of corresponding IFAN was supernatant > mixture > precipitate. However, after fermentation by *B. amyloliquefaciens* CX-20, the final FAN (FFAN) concentrations changed, although there were no obvious rules as observed for IFAN. The IFAN of the mixtures of RSMH and 92011H was relatively low, at 72.74 and 448.46 mg l^{-1}, respectively, but their FFAN concentrations increased to 984.56 and 772.12 mg l^{-1}. Compared with IFAN, the FFAN concentrations of the mixtures of 93298H and 48424H were decreased, from 754.99 mg l^{-1} and 889.41 mg l^{-1} to 679.63 mg l^{-1} and 521.19 mg l^{-1}, respectively. However, the change trends of FFAN in the precipitates, which were higher than the IFAN, were similar. This was especially true for RSMH, which showed an increase from 44.44 mg l^{-1} to 499.18 mg l^{-1}. Except for the supernatant of RSMH, the FFAN concentrations of the supernatants of hydrolysates obtained following fungal pretreatment showed a slight decrease.

Influence of IFAN concentrations on iturin A fermentation

RSM and the FRSM contained a variety of nitrogen sources, including FAN, as well as water-soluble and water-insoluble proteins. Many reports indicated that the IFAN concentration had important effects on microbial production (Wang *et al*., 2010; Kiran et al., 2012, 2013; García *et al*., 2013; Chatzifragkou *et al*., 2014; Salakkam *et al*., 2017; Salakkam and Webb, 2018). Based on the presented results, we also analysed the relationship between the IFAN of the hydrolysates and iturin A yield (Fig. 4). There was an obvious linear relationship among the four media containing the mixture of hydrolysate, whereby the yields of iturin A were decreased gradually

Table 3. Mineral elements composition of mixture, precipitate and supernatant of different hydrolysates and their effects on IturinA yields.

	Ca (mg l^{-1})	Cu (mg l^{-1})	Fe (mg l^{-1})	K (mg l^{-1})	Mg (mg l^{-1})	Mn (mg l^{-1})	P (mg l^{-1})	Si (mg l^{-1})	Zn (mg l^{-1})	FAN (mg l^{-1})	Iturin A (g l^{-1})
Mixture of 92011H (Chen *et al*., 2019)	37.65	0.59	4.69	117.45	80.31	0.64	383.17	163.11	1.33	448.46	0.93
Precipitate of 92011H	18.71	0.48	3.88	69.84	50.96	0.48	231.04	114.33	0.98	250.95	0.37
Supernatant of 92011H	67.25	0.15	1.22	123.94	141.59	1.55	557.29	63.96	0.65	1005.56	nd
Mixture of 93298H (Chen *et al*., 2019)	31.25	0.79	13.26	126.47	90.62	2.26	588.34	187.79	5.90	754.99	0.60
Precipitate of 93298H	20.62	0.54	10.33	65.47	48.55	0.37	161.89	130.24	5.01	269.47	0.51
Supernatant of 93298H	52.58	0.34	1.71	133.04	153.02	2.22	858.89	62.20	1.58	957.20	nd
Mixture of 48424H (Chen *et al*., 2019)	62.95	0.59	6.90	117.51	139.02	1.66	633.78	183.36	3.49	889.41	0.27
Precipitate of 48424H	14.43	0.45	3.57	69.52	40.37	nd	92.23	160.81	2.18	205.68	0.24
Supernatant of 48424H	131.05	0.18	2.46	128.45	166.50	7.15	1065.42	43.69	2.27	1122.84	nd

FRSM hydrolysates by *A. oryzae* 92011, 93017, 93018 and 93019, *A. niger* 93027, 93288, 93295 and 93298, and *Trametes* sp. 48424 were designated as 92011H, 93017H, 93018H, 93019H, 93027H, 93288H, 93295H, 93298H and 48424H.
nd: not detected.

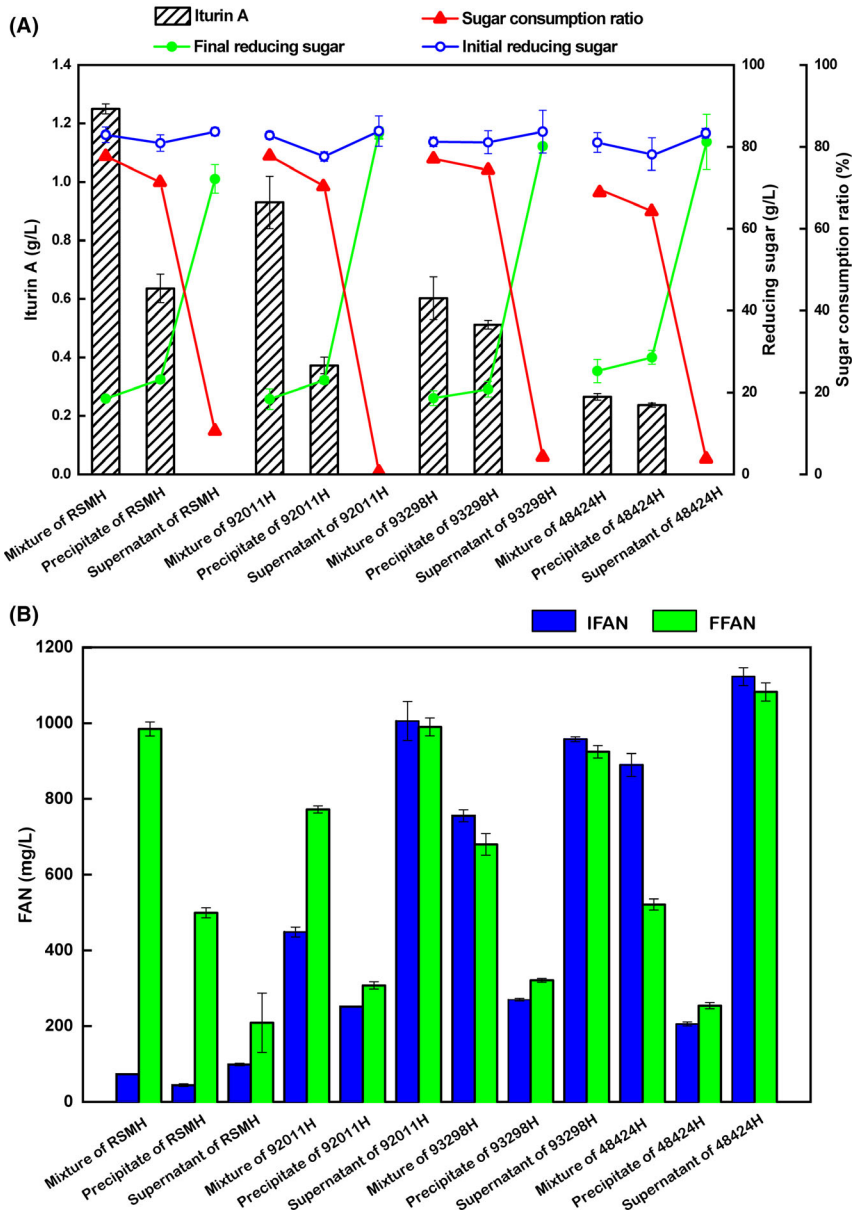

Fig. 3. Effect of hydrolysates pretreated by fungi on iturin A production by *B. amyloliquefaciens* CX-20. The hydrolysates included three treatments: mixture, supernatant and precipitate.
A. Effects of different hydrolysates on iturin A production, concentrations of initial and final reducing sugars, and the reducing sugar consumption ratio.
B. Effects of different hydrolysates on the concentrations of IFAN and FFAN.

with the increase of IFAN. However, the precipitates and the mixtures of hydrolysates obtained following fungal pretreatment showed opposite change trends.

Based on these results, we speculated that a high IFAN concentration might reduce the yield of iturin A by direct bio-utilization of RSM. Therefore, we analysed the influence of the IFAN concentration on iturin A fermentation by adding different concentrations of ammonium nitrate into medium containing only 90 g l^{-1} RSM and 80 g l^{-1} glucose (Fig. 5). As predicted, the concentration of IFAN was increased with the increase of the added ammonium nitrate. Conversely, the production of iturin A was decreased with the increase of IFAN concentration. When the concentration of ammonium nitrate was increased to 8 g l^{-1}, the IFAN concentration was increased from 72.74 mg l^{-1} to 433.95 mg l^{-1}, whereas the iturin A yield was decreased from 1.25 g l^{-1} to 1.07 g l^{-1}. Moreover, the FFAN concentrations were also increased with the increase of added ammonium nitrate, although the rate of increase was low. The initial

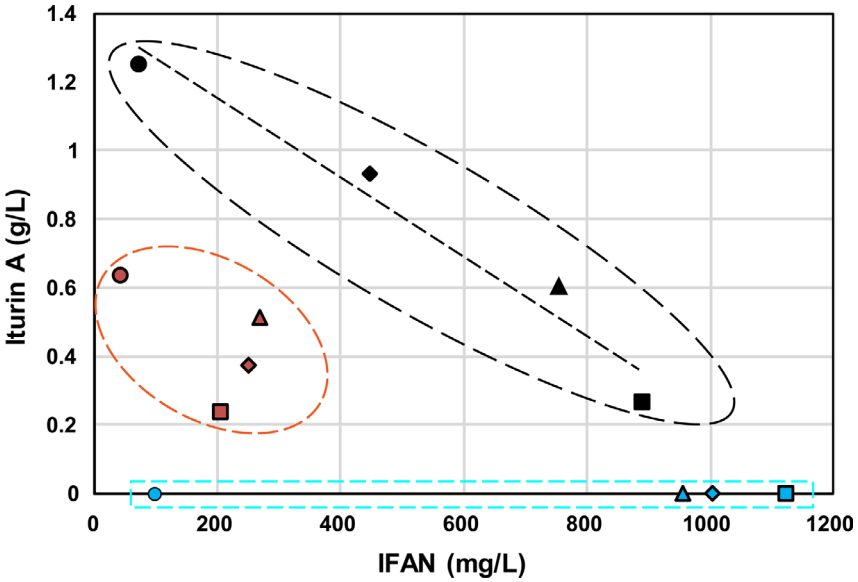

Fig. 4. The relationship between the IFAN and iturin A yield. Black circle, diamond, triangle and square represent the mixture of RSMH, 92011H, 93298H and 48424H, respectively. Yellow circle, diamond, triangle and square represent the precipitate of RSMH, 92011H, 93298H and 48424H, respectively. Blue circle, diamond, triangle and square represent the supernatant of RSMH, 92011H, 93298H and 48424H, respectively.

and final reducing sugar concentrations and sugar consumption ratios also did not exhibit significant changes.

Discussion

RSM is a good protein source for the microbial fermentation of high-value-added products (Wang *et al*., 2010; Kiran *et al*., 2013; Chatzifragkou *et al*., 2014; Jaszek *et al*., 2016; Prendecka *et al*., 2016; Boratyński *et al*., 2018), but its particular physical and chemical structure limited the nutrients to be directly assimilated by the majority of industrial microorganisms without pretreatment (Wang *et al*., 2010). Consequently, the application of RSM hydrolysate produced following fungal pretreatment as a feedstock for microbial bioconversions has been intensively investigated (Wang *et al*., 2010; Kiran et al., 2012, 2013; García *et al*., 2013; Chatzifragkou *et al*., 2014; Salakkam *et al*., 2017; Salakkam and Webb, 2018). However, RSM hydrolysate has not been used in industrial production on a large scale to date. The potential industrial strain *B. amyloliquefaciens* CX-20 demonstrated strong ability to directly use RSM as the sole source of all nutrients except for the carbon source for the fermentation of iturin A with high productivity (Chen *et al*., 2019a). Therefore, it was chosen as the object in this study, which aimed to analyse the reasons why fungal pretreatment appeared to be unsuitable for large-scale microbial fermentation of useful metabolites.

Although *A. oryzae* is the most commonly used fungus for the pretreatment of RSM, no criterion has been established to determine which fungi are more suitable for the hydrolysis of RSM. Chatzifragkou *et al*. (2014) studied the effects of temperature on the composition of RSM hydrolysate produced by *A. oryzae*, as well as the effects of the corresponding hydrolysates on biomass production and 1,3-propanediol (PDO) yield. Kiran et al. (2012, 2013) also used RSM hydrolysate produced by *A. oryzae* to produce microbial oil. The common reason for using *A. oryzae* is the fact that it is an excellent protease producer and the proteins present in the RSM can be broken down into peptides and AA (Wang *et al*., 2010; Kiran et al., 2012, 2013; García *et al*., 2013; Chatzifragkou *et al*., 2014; Salakkam *et al*., 2017; Salakkam and Webb, 2018). Similarly, *A. niger* has the capacity to synthesize proteases, amylases, fibre-degrading enzymes (cellulases, hemicellulases and pectinases), lipases and tannase, and has been widely used to improve the nutritional quality of RSM by SSF (Shi et al., 2015, 2016a,b). *Trametes* sp. 48424, a white-rot fungus with high laccase activity which is one of the important ligninolytic enzymes responsible for lignin degradation (Fan *et al*., 2011; Niu *et al*., 2015), has also been used in RSM pretreatment. Lignin, which is hydrophobic and crosslinks other structures, has been recognized one of the most natural recalcitrant factors and has played a negative role in efficient utilization of lignocellulosic substrates, such as RSM (Croat *et al*., 2017; Kainthola *et al*., 2019; You *et al*., 2019). Our results also demonstrated that *Trametes* sp. 48424 was more efficient than *A. oryzae* 92011 and *A. niger* 93298 in hydrolysing

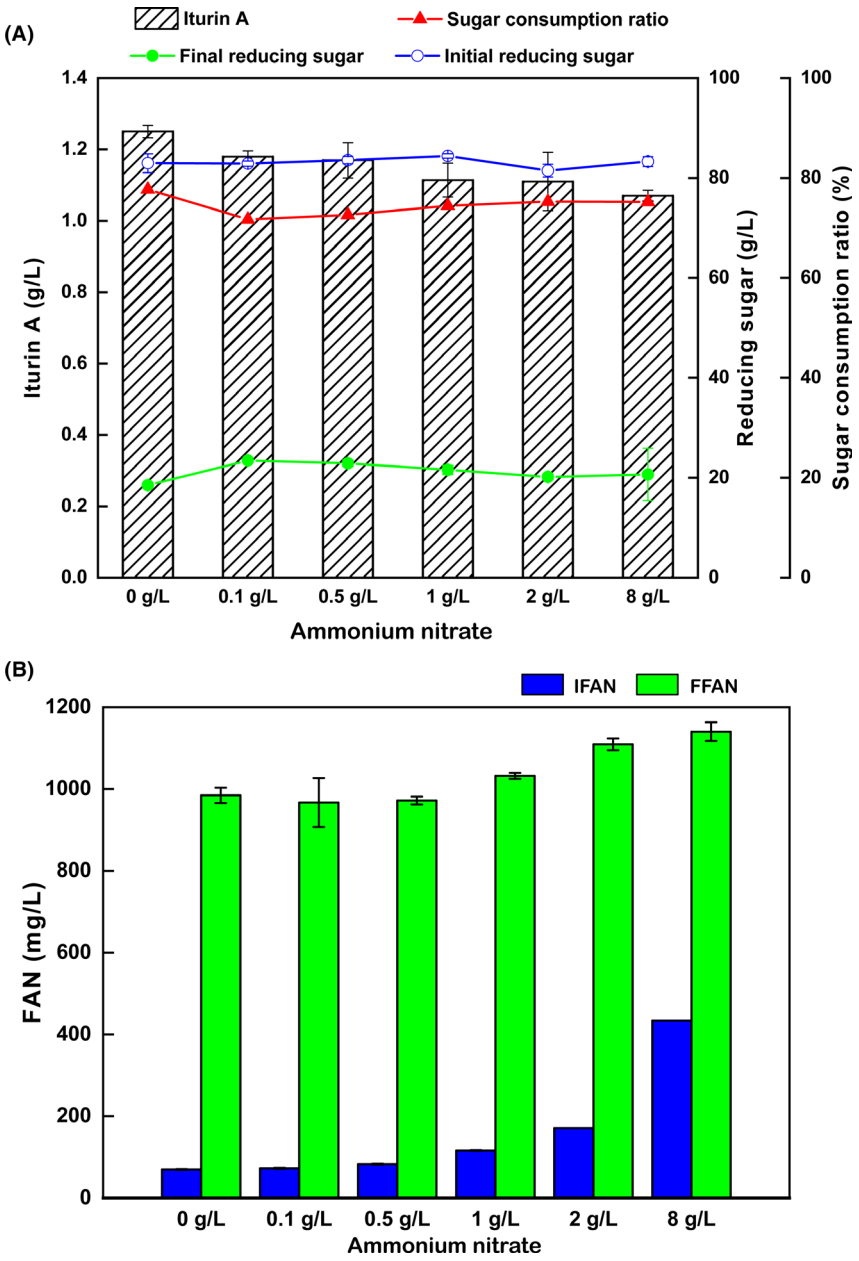

Fig. 5. Influence of IFAN concentrations on iturin A fermentation.
A. Effects of different IFAN concentrations on iturin A production, concentrations of initial and final reducing sugars, and the reducing sugar consumption ratio.
B. Effects of different concentrations of ammonium nitrate concentrations on the concentrations of IFAN and FFAN.

RSM, releasing FAN and most mineral elements (Table 3). Therefore, we chose *A. oryzae*, *A. niger* and *Trametes* sp. 48424 for further experiments.

In general, the chemical composition of RSM would be changed by fungal SSF (Shi *et al.*, 2015, 2016a,b). However, the chemical composition of solid FRSM has not been investigated in studies that used its corresponding hydrolysates as feedstocks for microbial fermentation. In this study, we compared the nutrient composition of solid RSM and FRSM. After fermentation of RSM by the fungi, the contents of CP, total AA and ash were increased significantly, while the contents of crude fibre, acid detergent fibre and neutral detergent fibre were decreased by most tested fungi. Although different fungi were used to pretreat RSM, FAN was an important evaluation parameter for liquid hydrolysates in all cases, since RSM was mainly used as nitrogen source. Therefore, FRSM with high CP and total AA content, such as 92011F and

93298F, were selected for the subsequent hydrolysis experiments. Although 48424F contained lower CP and total AA, it was also analysed for comparison because of the high laccase activity, responsible for lignin degradation which was one of the most recalcitrant factors for efficient utilization of RSM. The FAN concentrations in the liquid hydrolysates were not associated with the CP and total AA contents in the solid FRSM, either in the precipitates, supernatants or in the mixtures. Interestingly, the FAN concentrations in the supernatants were higher than those of the mixtures. The same was true for the mineral elements Ca, K, Mg, Mn and P (Table 3). We speculated that this might be related to the adsorption capacity of RSM, which might also be increased after fermentation by fungi due to the porous structure of FRSM, similar to activated carbon (Morosanu *et al*., 2019). After analysing the relationship between the FAN concentrations and iturin A yields, we found that the high concentrations of IFAN in the mixtures and supernatants reduced the yield of iturin A (Fig. 4). This was further corroborated by the observation that iturin A production using all hydrolysates was lower than that obtained using direct bio-utilization of RSM. To investigate the influence of IFAN on iturin A fermentation, ammonium nitrate was added to the untreated RSM medium. Although the results also showed a negative effect of IFAN on iturin A production, the impact was limited (Fig. 5). Moreover, the irregular correlation between iturin A yield and IFAN in the precipitates of hydrolysates indicated that FAN might be only one of multiple factors that influenced the production of iturin A (Fig. 4). For example, the proteins in the insoluble precipitates may also be important. This was consistent with the report by Jin *et al*. (2014) and Chen *et al*. (2019a), which demonstrated that almost no iturin A were produced when 8 g l^{-1} ammonium nitrate was used as the nitrogen source. However, when an equal amount of ammonium nitrate was added into RSM medium, the yield of iturin A reached 1.07 g l^{-1} in spite of the negative effects of ammonium nitrate (Fig. 5). Therefore, fungal pretreatment effectively released and solubilized nutrients from RSM so that a high FAN concentration was obtained in the mixture or supernatant of hydrolysate, which might have negative effect on iturin A fermentation.

Moreover, fungal pretreatment could also promote the release of pigments and phenolic compounds with strong antioxidative activity from RSM, which also showed inhibitory effects on the growth of *B. amyloliquefaciens* CX-20 in our laboratory (data not shown). Although different methods for the pretreatment of agricultural byproducts were reported to generate inhibitors (phenolic compounds, formic acid, hydroxymethyl furfural and furfural) that repressed microbial fermentation (Ravindran and Jaiswal, 2016; Munk *et al*., 2017), the negative effects of inhibitors released by fungal pretreatment on microbial metabolite production were not investigated in detail. The available literatures on the utilization of RSM hydrolysate produced by fungal pretreatment for microbial fermentation (Wang *et al*., 2010; Kiran et al., 2012, 2013; García *et al*., 2013; Chatzifragkou *et al*., 2014; Salakkam *et al*., 2017; Salakkam and Webb, 2018) indicated that diluting the hydrolysate to an optimal concentration was a feasible strategy to solve the problems caused by excess inhibitors, including FAN. Therefore, it stands to reason that other substances besides FAN in the RSM hydrolysate obtained by fungal pretreatment can also inhibit microbial fermentation. Appropriate dilution could partially solve these problems, which needs to be further studied.

AA has been used as stimulatory additives to improve the biosynthesis of antibiotics (Aharonowitz, 1980). Wu *et al*. (2018) reported that Ser was the most critical compound in the formation of iturin A. However, Peng *et al*. (2014) demonstrated that Asp addition was the most significant factor contributing to the iturin A yield, followed by Pro and Glu addition. After fungal fermentation, the contents of Ser, Asp, Pro and Glu were increased in all three FRSM, except for Ser in 48424F. However, the yields of iturin A produced from the three mixtures of hydrolysates were lower than that produced by the direct bio-utilization of RSM. As shown in Table S1, although SSF by fungi could improve the protein content of solid FRSM, it also resulted in decreases of total dry weight, total crude protein and total AA, which not only caused nitrogen waste by fungal consumption, but also produced pungent odour that would induce environmental pollution (Liu *et al*., 2016; Munk *et al*., 2017; Robledomahon *et al*., 2019). Similarly, while the relative contents of certain AA in solid FRSM might increase, the total quality decreased (Table S2). This is a clear disadvantage of RSM pretreatment by fungi in SSF.

Moreover, compared with the direct bio-utilization, fungal pretreatment increased the concentrations of IFAN in liquid hydrolysates, which had negative effects on iturin A production from RSM. This is the second disadvantage of fungal pretreatment. Judging by the results obtained using the precipitates of hydrolysates for iturin Aproduction (Fig. 3), the precipitates seemed to be the most valuable for the production of microbial metabolites. Notably, this was mostly ignored in previous studies and also caused nutrient waste in RSM (Wang *et al*., 2010; Kiran et al., 2012, 2013; García *et al*., 2013; Chatzifragkou *et al*., 2014; Salakkam *et al*., 2017; Salakkam and Webb, 2018). This is the third disadvantage of fungal pretreatment. In addition, compared with submerged fermentation, SSF by fungi has the drawbacks of low homogeneity of mass and heat transfer, difficult control

of pH, temperature and oxygen, less convenient handling and difficult monitoring (Mizumoto *et al*., 2007; Chen *et al*., 2011). All of these factors can lead to fluctuations in the composition of the hydrolysate from batch to batch, thus further affect the commercial feasibility of the overall process. And the process of preparing RSM hydrolysates obtained by fungal SSF needed an additional 4 days (three days for the fermentation and one day for the autolysis) in addition to the time of iturin A fermentation. This additional time would also increase the cost of production.

Above results are all specific to *B. amyloliquefaciens* CX-20 used in this study, which could directly use RSM as the sole source of all nutrients except for the carbon source for the fermentation of iturin A with high productivity. Whether these findings are likely applicable to many other microorganisms, remains to be further studied. In conclusion, our study verified the unconventional adverse effects of fungal pretreatment on iturin A production by *B. amyloliquefaciens* CX-20 compared with direct bio-utilization of RSM.

Experimental procedures

A schematic of the overall experimental procedure is shown in Figure 1. According to the previous study (Chen *et al*., 2019a), the initial optimal RSM and glucose concentrations for iturin A production by *B. amyloliquefaciens* CX-20 were 90 g l^{-1} and 80 g l^{-1}, respectively. Since RSM could not be dissolved in water, 1.8 g RSM was weighed and placed into a 250 ml flask in advance, after which an aqueous solution containing 80 g l^{-1} glucose was added to each flask, and the volume finally fixed at 20 ml for direct bio-utilization.

For the fungal pretreatment experiment, nine fungi, including four *A. oryzae*, four *A. niger* and one *Trametes* sp., were used to screen suitable fungal strains based on the chemical composition of solid FRSM. A sample comprising 1.8 g RSM was also weighed and placed into each 250 ml flask. After inoculation with different fungi for solid fermentation, the resulting FRSM in one part of parallel flasks were dried and ground for component analysis, and the FRSM in the other parallel flasks were hydrolysed by adding water to 20 ml for constant volume. After hydrolysis, this mixed liquid was designated as the mixture of hydrolysate. The mixture of hydrolysate in part of parallel flasks was divided into supernatant and precipitate fractions by filtration, and was then dissolved by adding water to 20 ml for constant volume, which was designated as the supernatant of hydrolysates and precipitate of hydrolysates, respectively. In addition to the component analysis, the mixture, supernatant and precipitate of hydrolysates were used for iturin A fermentation after 80 g l^{-1} glucose supplementation and sterilization. Each mixture, supernatant and precipitate of hydrolysates was produced independently in each 250 ml flask, instead of dispensing homogenous amounts of 20 ml volume into each 250 ml flask after hydrolysis process.

Microorganisms and media

The iturin A production strain *B. amyloliquefaciens* CX-20 (CCTCC No: M 2018794) was kindly provided by Professor Shouwen Chen (College of Life Sciences, Hubei University, Wuhan, China). Luria–Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 10 g NaCl and 1 l H_2O) was used for seed cultures of *Bacillus*. The fermentation medium was composed of 80 g glucose, 90 g RSM and 1 L H_2O. The initial pH of the medium was adjusted to pH 7.0, followed by sterilization at 121°C for 30 min. Flask experiments were performed in 250 ml flasks with a fermentation volume of 20 ml and an inoculation size of 5% (v/v). All fermentations were carried out at 28°C, under constant orbital shaking at 220 r.p.m.

A. oryzae 92011 (CCTCC No: AF 92011), 93017 (CCTCC No: AF 93017), 93018 (CCTCC No: AF 93018) and 93019 (CCTCC No: AF 93019), as well as *A. niger* 93027 (CCTCC No: AF 93027), 93288 (CCTCC No: AF 93288), 93295 (CCTCC No: AF 93295) and 93298 (CCTCC No: AF 93298), were kindly provided by the China Center for Type Culture Collection (Wuhan). *Trametes* sp. 48424 (Fan *et al*., 2011) was obtained from the School of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China. The nine fungal strains were employed in SSF to produce crude enzymes essential for RSM hydrolysis. The strains were maintained in the form of spores, in dry sand at 4°C. For the preparation of inocula for SSF, spores were purified and sporulated on slopes of medium containing 25 g l^{-1} RSM, 25 g l^{-1} wheat bran and 20 g l^{-1} agar.

SSF followed by fungal hydrolysis

SSF was conducted using nine fungal strains: *A. oryzae* 92011, 93017, 93018, 93019, *A. niger* 93027, 93288, 93295, 93298 and *Trametes* sp. 48424, respectively. Prior to each SSF, the fungi were sporulated on the surface of solid medium containing 25 g l^{-1} RSM, 25 g l^{-1} wheat bran and 20 g l^{-1} agar, in 20 ml test tubes incubated for 5 days at 28°C. Then, nine aqueous spore suspensions were formed by adding 10 ml of sterile distilled water with 0.01% Tween 80 (v/v) to every test tube. These spore suspensions served as inocula for SSF, which was performed in pre-sterilized (121°C for 30 min) 250 ml flasks containing 1.8 g of RSM as the sole source of nutrients. The moisture content was adjusted

to 65% (w/w) after inoculation with fungal spores. All flasks were seeded with approximately 10^6 spores/g RSM and incubated at 28°C for 72 h.

Hydrolysis of FRSM was subsequently conducted by mixing with distilled water to a final volume of 20 ml so that the initial RSM concentrations were approximately 90 g l^{-1}. The content was blended and incubated in flasks at 55°C for 24 h (García *et al*., 2013). RSM hydrolysate without fungal pretreatment was produced by weighing 1.8 g RSM and mixing with distilled water to a final volume of 20 ml for direct hydrolysis. The mixture of hydrolysate was either used directly, or divided into supernatant and precipitate fractions by filtration and mixed with distilled water to a final volume of 20 ml.

Analytical methods

Iturin A was extracted according to reported method (Chen *et al*., 2019b). Briefly, 0.3 ml of the mixed fermentation broth was pipetted into a 3 ml glass tube containing 1.2 ml of methanol, shaken well and then leached for 60 min. The mixture was centrifuged at 3,500 r.p.m. for 20 min, and the supernatant was filtered through a disposable filter with 0.22 μm pore size. The iturin A concentration was quantified using a Waters 2695 high-performance liquid chromatography (HPLC) system equipped with a reverse-phase column (ACQUITY UPLC BEA C18, 1.7 mm 2.16100 mm, Waters, USA). A mixture of acetonitrile and 10 mM ammonium acetate (35:65, v/v) was used as the eluent at a flow rate of 0.3 ml min^{-1}, and the elution was monitored at 210 nm. The concentration of iturin A was analysed and quantified using an authentic reference standard of iturin A (Sigma Chemicals, St. Louis, MO), and was measured in triplicate.

The FRSM and untreated RSM samples were analysed for dry matter (DM), crude protein (CP), ether extract, neutral detergent fibre (NDF), acid detergent fibre (ADF), ash, calcium (Ca) and phosphorus (P) content (Shi *et al*., 2016b). The amino acid (AA) profiles of the samples were analysed using a L8800 AA analyser (Hitachi, Tokyo, Japan). Before analysis, the samples were hydrolysed with 6 M HCl for 24 h at 110°C. Met and Cys were analysed as Met sulfone and cysteic acid after cold oxidation with performic acid overnight before hydrolysis.

The concentration of reducing sugars in the fermentation broth was determined using the DNS method (Miller, 1959) which was as follows: a defined volume of fermentation broth was added to a graduated 25 ml test tube, water was added to 2 ml, followed by 2 ml DNS, shaken well and then heated in boiling water for 5 min, cooled to room temperature and the volume was added to 25 ml. Finally, the absorbance was measured at a wavelength of 540 nm.

The concentration of free ammonium nitrogen (FAN) was determined using the ninhydrin method (Lie, 1973) which was as follows: a defined volume of fermentation broth was added to a graduated 10 ml test tube, water was added to 2 ml, and then, 1 ml of ninhydrin solution was added, shaken well and then heated in boiling water for 16 min, cooled in a cold water bath for 20 min and then 5 ml KIO_3 dilution solution was added, after which the absorbance at a wavelength of 570 nm was measured.

Ca, Cu, Fe, K, Mg, Mn, P, Si and Zn were measured using Agilent's 5110 Synchronous Vertical Dual View (SVDV) ICP-OES system (Agilent Technologies, Santa Clara, CA, USA).

All experiments were performed in triplicate. The data were processed using ORIGIN v8.6 software (Origin Lab Corp., Northampton, MA, USA).

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Conflicts of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Changes of the total nitrogen source from RSM before and after solid state fermentation treated by different fungal strains.

Table S2. Changes of the amino acid contents (mg/bottle) before and after solid state fermentation treated by different fungal strains.

