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Posted Date: 24 October 2023

doi: 10.20944/preprints202310.1502.v1

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Article

Arabinose Plays an Important Role in Regulating the Growth and Sporulation of *Bacillus subtilis* NCD-2

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Abstract: *Bacillus subtilis* NCD-2 showed a promising biocontrol effect against plant soil-borne diseases, and was developed as a commercial microbial fungicide against cotton verticillium wilt in China. Spores were main ingredient of the fungicide and played the crucial role in biological control of plant diseases. Therefore, the key to reducing the cost of fungicide was to find ways to increase the number of spores of strain NCD-2 during fermentation. In this study, 755 substances were evaluated by Phenotype Microarray technology, and 5 kinds of carbon sources and 1 kind of nitrogen source were found to promote the metabolism of strain NCD-2. Among carbon sources L-arabinose showed strongest ability to promote the bacteria growth and sporulation of strain NCD-2. When L-arabinose was used as a single carbon source, it could increase the bacteria concentration and the sporulation efficiency of strain NCD-2 by 2.04 times and 1.99 times, respectively, compared with D-glucose. Moreover, L-arabinose could significantly decrease the autolysis of strain NCD-2 revealed by microscopy observation. The mechanism for promoting the sporulation by L-arabinose was analysed by RNA-seq sequencing. Results showed that total of 790, 923 and 1270 genes were up-regulated and 639, 850 and 1001 genes were down-regulated under L-arabinose supplies when cultured at 8 h, 12 h and 16 h, respectively. Fourteen differentially expressed genes associated with arabinose transportation and sporulation were selected for qRT-PCR analysis, and the result showed basically consistent expression trend with transcriptome. Notably, genes associated with arabinose metabolism, sporulation, spore resistance to heat and spore coat formation were significantly up-regulated, and genes associated with sporulation-delaying protein were significantly down-regulated under L-arabinose supplies. *msmX* gene which was involved in arabinose transport in *Bacillus* genus was deleted, and the mutant decreased the growth and sporulation by 53.71% and 86.46%, respectively, when compared to strain NCD-2 wild type. Complementary of the mutant by importing intact *msmX* gene could restore the growth and sporulation of the mutant strain. In conclusion, arabinose played an important role in regulating the growth and sporulation of strain NCD-2.

Keywords: Phenotype Microarrays; *Bacillus subtilis*; L-arabinose; sporulation; transcriptome

1. Introduction

Plant soil-borne diseases, such as verticillium wilt and fusarium wilt caused serious loss in plant production, were very difficult to control. Microbial fungicide using living microorganisms as active compound were confirmed as effective and environmental friendly methods to suppress plant soil-borne diseases and reduce the amount of chemical fungicides [1,2]. The control capabilities of biocontrol agents were influenced by the concentration of biocontrol agent in plant rhizosphere. Therefore, it was important to increase the application dose of biocontrol agent.

 doi:10.20944/preprints202310.1502.v1

B. subtilis was an important resource for development of microbial fungicide due to its abilities to produce a variety of antibiotics and form highly resistance spores [3]. Wettable powders and dry powder seed-coating agents were the main formulations of microbial fungicides for suppressing plant soil-borne diseases. However, the processing of the two formulations involved of instantaneous high-temperature as high as 170°C to dry the bacteria, under such high temperatures only the spores could survive. Therefore, microbial fermentation should consider the bacteria concentration as well as the sporulation [4]. Ideal fermentation system was firstly to increase the fermentation level of the bacterium, and subsequently to make the maximum possible conversion of the bacteria into spores through nutrient regulation and other methods [5].

The carbon and nitrogen sources in the medium were the main factors affecting bacterial growth and sporulation [6–8]. Suitable and sufficient carbon and nitrogen sources could promote the growth of the bacterium, but sporulation generally occurred in unfavorable environments such as nutrient starvation [9]. During the stable phase of bacterial growth, residual carbon and nitrogen sources in the medium might inhibit the sporulation of *Bacillus.* Spp [10]. In microbes, carbohydrates were catabolized into pyruvate, which entered the tricarboxylic acid cycle, mainly through the glycolytic pathway (EMP) and pentose phosphate pathway (PPP). By comparing the effect of the sugars existed in pathway of EMP and PPP on sporulation of *B. subtilis*, it was found that the sugars existed in PPP pathway but not in EMP pathway could increase the sporulation of *Bacillus*. So it was concluded that PPP was an important carbohydrates catabolic pathway that affected sporulation [11]. In addition, metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} also affected the sporulation of *B. subtilis*, and addition of appropriate concentrations of metal ions in the medium could promote the sporulation [12–14]. Different strains had different nutrient requirements suitable for their growth and sporulation, therefore, specific nutrients needed to be explored for a specific strain.

Bacillus subtilis NCD-2 showed a promising biocontrol effect against plant soil-borne diseases, and was developed as a commercial microbial fungicide against cotton verticillium wilt in China. [15–17]. The objectives of this study were to screen the nutrients suitable for the growth and sporulation of strain NCD-2, and then explore the mechanism for regulating sporulation by the nutrients. Results of this study will provide important information for large-scale and efficient fermentation of strain NCD-2.

2. Results

2.1. Screening of nutrients that facilitate the metabolism of strain NCD-2

The metabolic activities of strain NCD-2 for carbon, nitrogen, phosphorus, sulfur and trace elements were determined by BIOLOG's Phenotype Microarrays (PMs) technology (Figure S1). For carbon source utilization, strain NCD-2 had higher metabolic activity in L-arabinose, D-arabinose, D-xylose, D-ribose and D-glucosamine supplies, respectively. For nitrogen source utilization, strain NCD-2 had higher metabolic activity in cysteine supply. For phosphorus and sulfur sources utilization, strain NCD-2 had low metabolic activities in 59 phosphorus sources and 35 sulfur sources.

2.2. Effects of L-arabinose, D-ribose, and D-xylose on growth and sporulation efficiency of strain NCD-2

The effects of different carbohydrates on the growth of strain NCD-2 were evaluated (Figure 1a). When D-glucose was used as carbon source, the bacteria concentration of strain NCD-2 reached the maximum of 2.55×10^7 CFU/mL, while that of L-arabinose was 5.20×10^7 CFU/mL, which was 2.04 times higher than that of D-glucose. When D-ribose and D-xylose were used as the sole carbon source, the bacteria concentration of strain NCD-2 reached the maximum of 3.90×10^7 CFU/mL and 3.10×10^7 CFU/mL, respectively. It was revealed that L-arabinose was the most suitable carbon source to promote the growth of strain NCD-2.

The effects of different carbohydrates on the sporulation of strain NCD-2 were evaluated (Figure 1b). Using D-glucose, L-arabinose, D-ribose and D-xylose as the sole carbon source, the sporulation efficiencies of strain NCD-2 were 2.33%, 27.12%, 11.40%, and 15.39% after 24 h of inoculation, respectively. The sporulation efficiencies of strain NCD-2 were 41.35%, 82.18%, 20.26% and 20.45%

 doi:10.20944/preprints202310.1502.v1

after 48 h inoculation, when using D-glucose, L-arabinose, D-ribose and D-xylose as carbon sources, respectively. The sporulation efficiencies of strain NCD-2 increased from 24 h to 48 h inoculation. In addition, the increase of sporulation by L-arabinose was also confirmed by microscopic observation (Figure S2). The results indicated that the L-arabinose could increase the sporulation of strain NCD-2 during the early and later growth stages, but the D-ribose and D-xylose only increased the sporulation of strain NCD-2 at early growth stage, when compared with D-glucose.

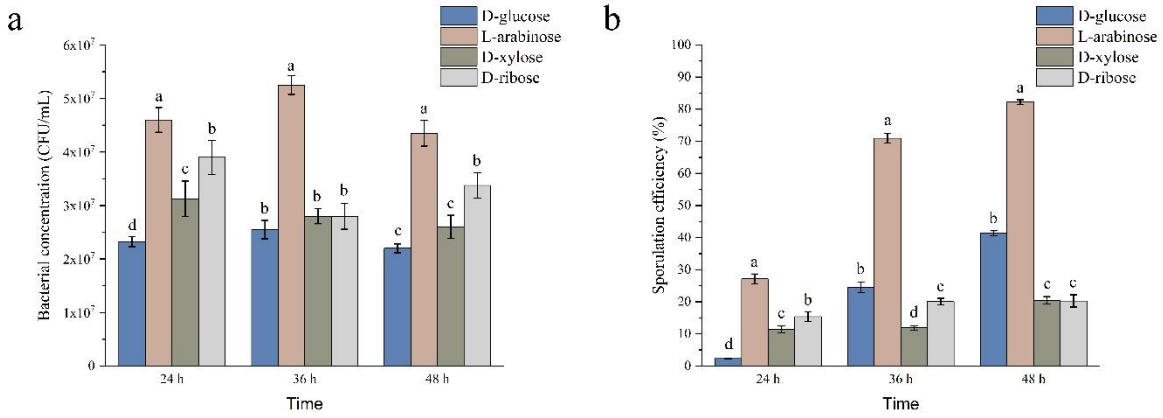

Figure 1. Effects of different carbon sources on the bacteria concentration (a) and sporulation efficiency (b) of strain NCD-2. Columns represent the average of four replicates, error bars show standard deviations, different letters indicate significant ($P<0.05$) difference according to ANOVA with Tukey's post hoc test.

2.3. Different proportions of L-arabinose and D-glucose on sporulation of strain NCD-2

The effects of different proportions of L-arabinose and D-glucose on the sporulation of strain NCD-2 were evaluated (Figure 2). Forty-eight hours after inoculation, the sporulation efficiencies of strain NCD-2 were 41.35% and 82.43% when using D-glucose and L-arabinose as sole carbon source, respectively. The sporulation efficiencies of strain NCD-2 gradually decreased with the decrease of L-arabinose proportions in the mixture of L-arabinose and D-glucose. The sporulation efficiencies of strain NCD-2 were 68.82%, 56.62%, and 32.79% when L-arabinose and D-glucose at proportions of 2:1, 1:1, and 1:2, respectively.

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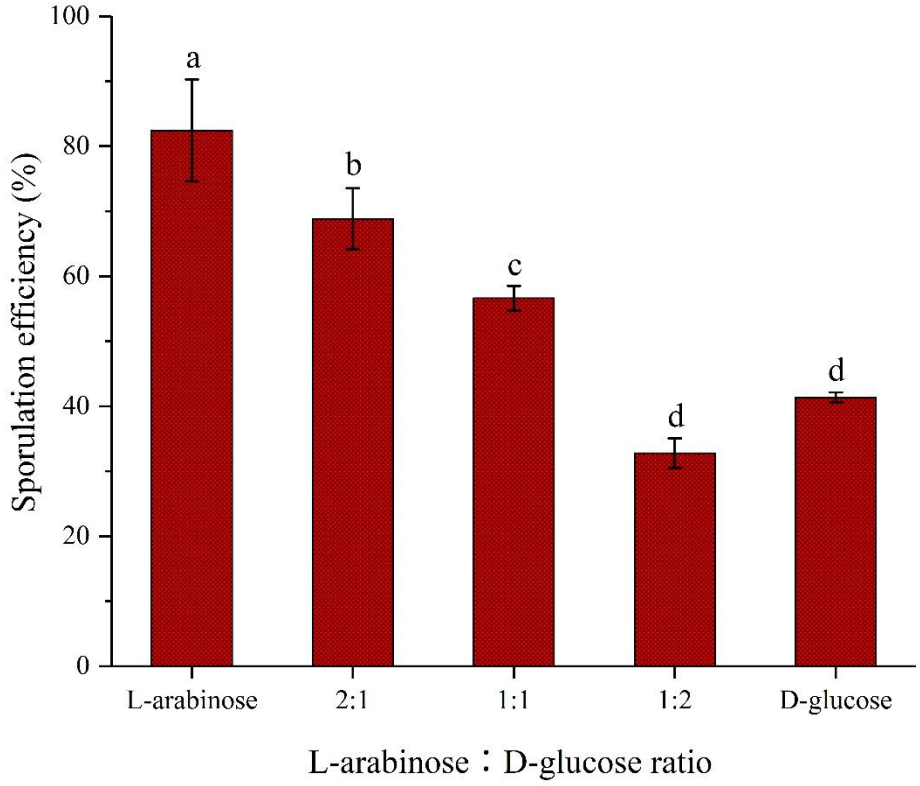

Figure 2. Different proportions of L-arabinose and D-glucose on the sporulation of strain NCD-2. Strain NCD-2 was cultured in M9 medium containing 4 g·L-1 carbohydrates, and the sporulation efficiency was determined 48 h after inoculation . Columns represent the average of four replicates, error bars show standard deviations, different letters indicate significant ($P<0.05$) difference according to ANOVA with Tukey's post hoc test.

2.4. Transcriptome analysis

The effects of L-arabinose and D-glucose on the gene expression of strain NCD-2 were compared by transcriptome sequencing. Compared with the D-glucose total of 1483 differential expression genes (DEGs) including 790 up-regulated genes and 693 down-regulated genes, were identified from L-arabinose treatment 8 h (Ara-8 h) after inoculation (Figure 3a). Total 1773 DEGs including 923 up-regulated genes and 850 down-regulated genes, were identified from L-arabinose treatment 12 h (Ara-12 h) after inoculation (Figure 3b). Total 2271 DEGs including 1270 up-regulated genes and 1001 down-regulated genes, were identified from L-arabinose treatment 12 h (Ara-16 h) after inoculation (Figure 3c). GO annotations revealed that the DEGs associated with sporulation (GO:0043934), spore wall (GO:0031160), endospore-forming forespore (GO:0042601) and asexual sporulation (GO:0030436) were significantly up-regulated at 8 h and 12 h after inoculation. However, only the DEGs associated with spore germination (GO:0009847) were enriched at 16 h after inoculation (Figure 4). KEGG enrichment analysis found that ABC transporters (map02010), polyketide sugar unit biosynthesis (map00523) and ribosome (map03010) were significantly enriched at 8 h and 12 h (Figure 5).

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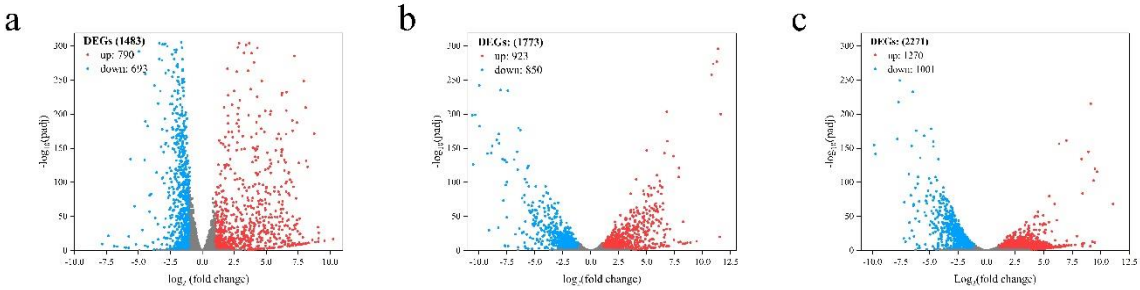

Figure 3. Volcano plots of transcriptomics comparison of strain NCD-2. X-axis indicates the average of log2 fold change from the replicates. Negative values indicate down-regulation and positive values indicate up-regulation. Y-axis is -log10 padj. Dots in blue or red indicate differentially expressed genes. Dots in black indicate proteins that are not significantly changed in genes expression. (a) 8 hours post inoculation. (b) 12 hours post inoculation. (c) 16 hours post inoculation.

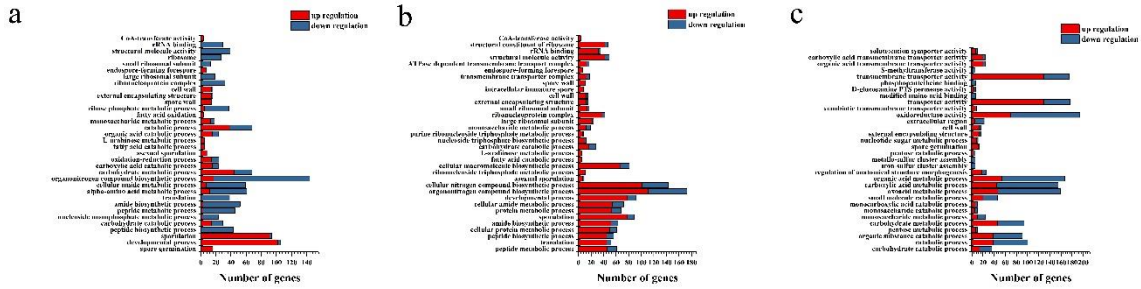

Figure 4. Go enrichment analysis based on the differential expression genes between L-arabinose and D-glucose-cultured strain NCD-2. The X-axis indicates the number of genes classified into regulatory or functional categories, as depicted on the Y-axis. Columns in blue indicate down-regulation and in red indicate up-regulation. (a) 8 hours post inoculation. (b) 12 hours post inoculation. (c) 16 hours post inoculation.

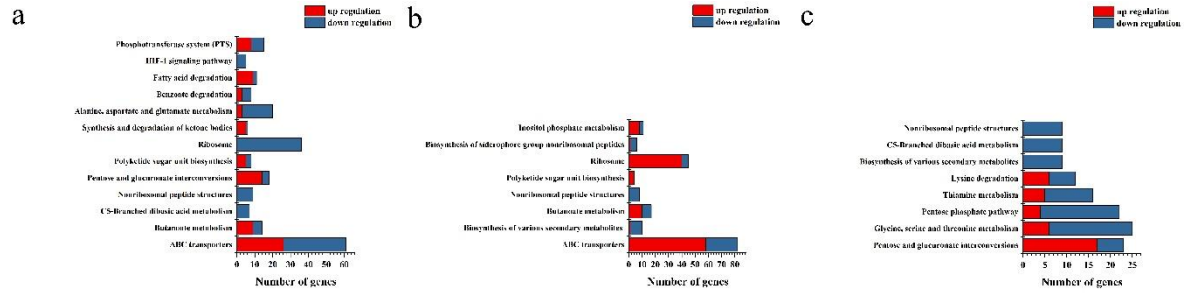

Figure 5. KEGG enrichment analysis based on the differential expression genes between L-arabinose and D-glucose cultured strain NCD-2. The X-axis indicates the number of genes classified into regulatory or functional categories, as depicted on the Y-axis. Columns in blue indicate down-regulation and in red indicate up-regulation. (a) 8 hours post inoculation. (b) 12 hours post inoculation. (c) 16 hours post inoculation.

2.5. Confirmation of transcriptional results by qRT-PCR

To verify the results of transcriptome, 14 DEGs associated with arabinose transportation, sporulation, spore resistance to heat, etc. were selected to analyze the expression in L-arabinose treatment by qRT-PCR. Results revealed that all the 14 genes showed basically consistent expression trend with transcriptome, It was indicated that the transcriptome results were reliable and could be used for further experimental analysis (Table S2).

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2.6. Analysis of genes associated with sporulation in strain NCD-2

The Venn plot showed that there were 717 genes significantly differentially expressed at all three time points (Figure S3), and most of the up-regulated genes involved in sporulation (Table 1). Among them *sigK* was a transcriptional regulator encoding spore formation. *cotE*, *cotF*, *cotG*, *cotS*, *cotT*, *cotV*, *cotW*, *cotX*, and *yheD* were responsible for spore coat proteins encoding. *dpaA* and *dpaB* were responsible for synthetases encoding pyridine dicarboxylic acid (DPA), a substance within the spore core. *gerBA*, *gerE*, *gerQ*, and *gerT* were responsible for encoding spore germination proteins. *spoIIIAH* and *spoIIQ* encoded polymeric complexes that connected forespore and mother cell [18]; *spoIVB* and *spoIVFB* encoded the activating proteins of protease and Sig-K, which catalyzed the formation of the spore cortex, respectively. *sspA*, *sspB*, *sspD*, and *sspE* encoding small acid-soluble proteins (SASPs) associated with spore resistance to heat. *sdpC*, encoded a cannibalism factor that delayed the sporulation in *B. subtilis*, were significantly down-regulated at all three time points.

Table 1. Differentially expressed sporulation related genes in L-arabinose treated strain NCD-2.

Accession ID	Gene nane	Log_2(Ara/Glc)			Production
		8 h	12 h	16 h	
WP_003231833.1	*cotE*	7.65	5.81	2.56	outer spore coat protein CotE
WP_003243364.1	*cotF*	6.59	9.47	5.54	spore coat protein CotF
WP_080344234.1	*cotG*	3.55	5.59	3.82	spore coat protein CotG
ADV92699.1	*cotM*	5.01	1.53	4.21	spore coat protein (outer)
WP_047183078.1	*cotS*	3.98	6.01	4.53	spore coat protein CotS
PSM02245.1	*cotT*	5.88	5.95	8.39	spore coat protein
AGE63031.1	*cotV*	4.37	6.32	5.46	spore coat protein (insoluble fraction)
WP_069486390.1	*cotW*	4.53	6.81	5.73	spore coat protein
WP_014476454.1	*cotX*	4.28	6.83	6.13	spore coat protein
WP_003231888.1	*dpaA*	6.29	6.84	4.81	dipicolinic acid synthetase subunit A
WP_003231884.1	*dpaB*	5.76	6.12	4.21	dipicolinate synthase subunit B
WP_015383228.1	*yheD*	5.17	3.35	2.27	spore coat associated protein YheD
WP_063336053.1	*gerBA*	4.55	2.20	4.73	spore germination protein GerKA
WP_003184172.1	*gerE*	3.94	5.01	3.91	spore germination protein GerE
WP_014478336.1	*gerQ*	7.95	4.77	1.32	spore coat protein GerQ
WP_047182746.1	*gerT*	5.10	6.02	6.03	spore germination protein GerT
AKE24397.1	*sigK*	6.45	4.08	3.26	RNA polymerase sporulation-specific sigma factor
WP_047182864.1	*spoIIIAE*	3.84	2.72	1.41	stage III sporulation protein AE
WP_003221804.1	*spoIIID*	10.25	6.81	4.40	sporulation transcriptional regulator SpoIIID
WP_004398593.1	*spoIIM*	1.78	1.84	2.35	stage II sporulation protein M
WP_047183325.1	*spoIIQ*	5.69	1.78	-1.82	stage II sporulation protein SpoIIQ
WP_004398697.1	*spoIVB*	6.19	1.77	2.12	SpoIVB peptidase
WP_015483522.1	*spoIVFB*	1.25	1.39	1.84	stage IV sporulation protein SpoIVFB
WP_003230465.1	*spoVAD*	6.29	1.70	4.08	stage V sporulation protein AD
AGE63365.1	*spoVD*	2.67	3.20	2.32	penicillin-binding protein
WP_047182441.1	*yjcA*	6.69	5.32	5.67	sporulation protein YjcA
WP_015383520.1	*ykvU*	3.60	3.01	4.18	sporulation protein YkvU
WP_003223491.1	*sspA*	7.22	3.14	3.04	alpha/beta-type small acid-soluble spore protein
WP_003233287.1	*sspB*	7.39	3.43	2.66	alpha/beta-type small acid-soluble spore protein
WP_003218568.1	*sspD*	7.57	3.93	4.00	alpha/beta-type small acid-soluble spore protein
BAI84385.2	*sspE*	6.59	4.34	3.30	gamma-type small acid-soluble spore protein
WP_003244950.1	*sdpC*	-1.40	-5.57	-6.81	sporulation delaying protein family toxin
WP_003228357.1	*sdpI*	-4.53	-3.43	-4.90	immunity protein SdpI
WP_003243541.1	*sdpR*	-4.51	-3.34	-2.64	sporulation delaying system autorepressor SdpR

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2.7. Deletion of the msmX gene decreased the sporulation efficiency in strain NCD-2

To confirm that L-arabinose influence sporulation, the *msmX* gene, which encoded the ATPase, responsible for arabinose uptake, was deleted from strain NCD-2 (Δ*msmX*). Additionally, the *msmX* complementary strain was developed for the Δ*msmX* mutant (CP*msmX*). The growth and sporulation efficiencies of the WT, Δ*msmX* and CP*msmX* strains were compared in M9 medium with L-arabinose as carbon source (Figure 6a). Results showed that the bacteria concentrations of WT were 4.60×10^7 CFU/mL, 5.25×10^7 CFU/mL and 4.35×10^7 CFU/mL at 24, 36 and 48 h after inoculation, respectively. Comparatively, strain Δ*msmX* decreased the growth, and the bacteria concentrations were 1.18×10^7 CFU/mL, 2.25×10^7 CFU/mL and 2.43×10^7 CFU/mL after 24 h, 36 h and 48 h of inoculation, respectively. However, the complementary strain (CP*msmX*) restored the growth, and the bacteria concentrations were 3.53×10^7 CFU/mL, 6.20×10^7 CFU/mL and 5.45×10^7 CFU/mL after 24 h, 36 h and 48 h of inoculation, respectively.

The sporulation efficiencies of strain NCD-2 wild type (WT), *msmX*-null mutant (Δ*msmX*) and its complemented strain (CP*msmX*) were also compared in M9 medium with L-arabinose as carbon source (Fingure 6b). The Results showed that the sporulation efficiencies of strain WT were 27.12%, 70.95% and 82.18%, at 24, 36 and 48 h after inoculation, respectively. Comparatively, strain Δ*msmX* decreased the sporulation efficiencies by 7.17%, 11.67% and 11.13%, at 24, 36 and 48 h after inoculation, respectively. However, the complementary strain (CP*msmX*) restored the sporulation efficiencies by 44.63% and 65.44%, at 36 h and 48 h after inoculation, respectively. The regulation of growth and sporulation of strain NCD-2 by *msmX* were also confirmed by microscopic observation (Figure S4).

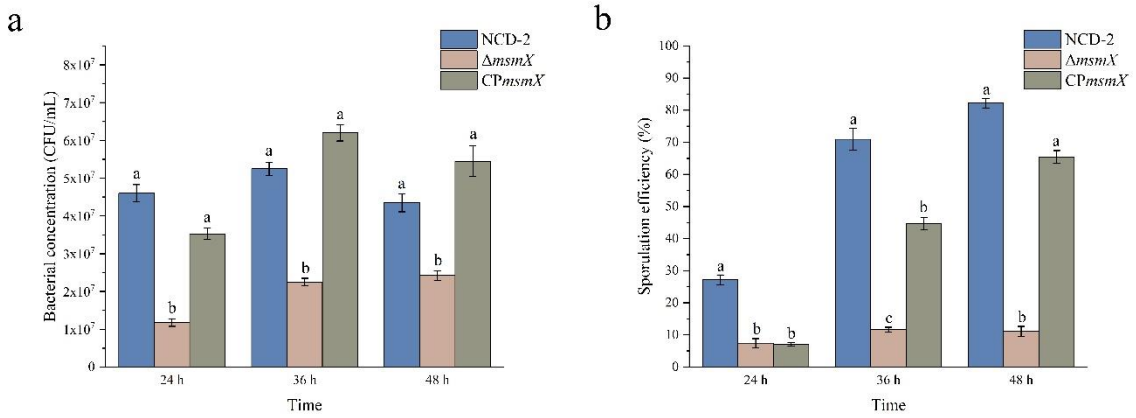

Figure 6. The bacteria concentration (a) and sporulation efficiency (b) of strain NCD-2 wild type (WT), msmX-null mutant (ΔmsmX) and its complemented strain (CPmsmX) in M9 medium with L-arabinose as carbon source, at 24 , 36 and 48 h after inoculation. Columns represent the average of three replicates, error bars show standard deviations, different letters indicate significant ($P<0.05$) difference according to ANOVA with Tukey's post hoc test.

3. Discussion

Spores of *Bacillus* species with strong resistant to stresses were the key ingredient in the formulation of microbial fungicide. Generally speaking, the control effect of plant soilborne diseases was positively correlated with the population of bacteria colonized in plant rhizosphere [19]. To obtain ideal biocontrol effect, it is expected to increase the amount of spore applied to the soil as much as possible. Therefore, promoting the yield of spores during fermentation process of *Bacillus* is definitely important for reducing the cost as well as ensuring the wide application of microfungicide. The key factor to reduce the cost of bio-fungicides was how to promote the growth of bacteria and yield of spores during fermentation process of *Bacillus*. It was known that the sporulation process and final spore yield depended on carbohydrate and amino acid [20]. The combined effects of yeast extract, peptone and glucose enhanced the spore yield of *B. megaterium* [21]. Likewise, the addition

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of glucose and ribose in the sporulation medium increased the spore yield of *B. subtilis* and *B. cereus* [6,11,22]. In this study, we focused on nutrients that promoted strain NCD-2 growth and spore formation, which required screening for a large number of nutrients due to different strains had different nutrient requirements. Phenotype Microarrays were commercially available microplate assays that could be used to test more than 1,000 phenotypic traits simultaneously by recording the microorganism's respiration over time on many distinct substrates [23,24]. Therefore, PMs could be used to screen nutrients suitable for the catabolism of a specific organism quickly and in high throughput, which had the advantages of large amount of information and time saving [25]. In this way, the catabolic capability of strain NCD-2 to approximately 200 carbon sources, 400 nitrogen sources, and 100 phosphorous and sulfur sources were determined by PMs. Results showed that strain NCD-2 had higher metabolic capacity to L-arabinose, D-xylose and D-ribose as sole carbon sources, among which L-arabinose could significantly increase the bacteria concentration and sporulation efficiency of strain NCD-2.

B. subtilis was able to grow on medium with L-arabinose as the sole carbon and energy source. In the absence of L-arabinose, AraR protein bounded to a site within the *araABDLMNPQ-abfA* operon promoter region preventing transcription. In the presence of L-arabinose a conformational change was induced in AraR such that recognition and binding to DNA was no longer possible, and the operon could be expressed [26]. After entering the cell, L-arabinose was sequentially converted to L-ribulose, L-ribulose 5-phosphate, and D-xylulose 5-phosphate by the action of L-arabinose isomerase (encoded by *araA*), L-ribulokinase (encoded by *araB*), and L-ribulose-5-phosphate 4-epimerase (encoded by *araD*), respectively. D-xylulose 5-phosphate was further catabolized through the pentose phosphate pathway [27,28]. Transcriptome analysis of this study showed that L-arabinose strongly up-regulated *araABDLMNPQ-abfA* operon expression in strain NCD-2 (Accession number: SUB12858722), and genes associated with sporulation were also strongly up-regulated (Table 1). Moreover, compared with L-arabinose as the sole carbon source, the sporulation efficiency of strain NCD-2 significantly decreased when both glucose and arabinose present in M9 medium (Figure 2), which might be attributed to the fact that the presence of glucose repressed the expression of both *araE*, a gene for L-arabinose transporter, and *ara* operon at the transcriptional level [29,30]. Thus, L-arabinose might be involved in regulating the expression of genes related to sporulation in strain NCD-2 by regulating *ara* operon.

Previous studies found that the AraNPQ-MsmX system was involved in the transport of arabinans, and knocking out *araNPQ* reduced the growth rate of *B. subtilis* [26,31]. Therefore, the deletion of the *msmX* gene, which encoded ATPase for providing energy to the AraNPQ transporter, inevitably led to a decrease in the growth rate of *B. subtilis*. In this study, *msmX* was deleted from strain NCD-2 wild type, and the mutant did reduce the bacteria growth and sporulation efficiency of strain NCD-2 with L-arabinose as the sole carbon source (Figure 6), which was consistent with the previously study, but not with D-glucose as the sole carbon source (Data not shown). It was clear that L-arabinose somehow entered the metabolic process of strain NCD-2 via the AraNPQ-MsmX system, then affected the growth and sporulation. The results would provide knowledge for effectively improving the growth and spore production during the fermentation of strain NCD-2.

The cell of *B. subtilis* might autolysed during fermentation, so resulting in a large number of cell deaths and reducing bacterial fermentation concentration [32]. There were many factors that led to autolysis of the bacterium [33–36]. Among them, a phenomenon of "cannibalism" was described [37,38], in which the master regulator of sporulation Spo0A was active, and released two toxins Skf and SdpC to kill Spo0A inactive sister cells. The nutrients released by the dead cells were used for the growth of cells that were not yet fully committed to sporulate. In this study, it was observed that cell autolysis produced a large amount of cell debris in the medium with D-glucose as sole carbon resource, but not in the medium with L-arabinose. In transcriptome, L-arabinose was significantly down-regulated the transcription of *sdpC*, compared with D-glucose at 8 h, 12 h and 16 h after inoculation. It was suggested that L-arabinose increasing the bacteria concentration of strain NCD-2 was due to L-arabinose inhibiting the process of "cannibalism" in strain NCD-2.

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4. Materials and Methods

4.1. Bacterial strains and growth conditions

The strains used in this study were listed in Table 2, *B. subtilis* strains were stored at -80℃ in LB medium containing 30% glycerol. Strains were cultured at 30℃ without special instructions and the appropriate concentration of antibiotics was added to the medium as needed.

Table 2. Strains used in this study.

Strain	Genotype	Source
WT	*Bacillus subtilis* NCD-2 wild type	Lab stock
Δ*msmX*	NCD-2 mutant, *msmX* deletion mutant	This study
CP*msmX*	Complementary of Δ*msmX* by intact *msmX*, Cm^R	This study

4.2. Phenotype microarrays analysis

The metabolic phenotype of *B. subtilis* strain NCD-2 on 755 nutrients was evaluated using the Biolog Phenotype MicroArrays system (Biolog, Hayward, CA, USA). In ninety-six-well PM1-8 microplates, including carbon source (PM1, PM2A), nitrogen source (PM3B, PM6-8), phosphorus source and sulfur source (PM5), were assayed , and the names of nutrients were described in the literature of Bochner et al [23]. The experiment was conducted according to the procedures developed by the manufacturer [25]. Briefly, strain NCD-2 was firstly scribed on BUG+B plates and cultured overnight at 33℃, single colony was selected and inoculated on BUG+B plates again, after cultured overnight at 33℃, Bacteria were dipped into the BUG+B medium with sterile cotton swabs and transferred to 20 mL of 1×IF-0a inoculum, stirred well to obtain a suspension of the bacteria, and the bacteria were continuously added until the turbidity of the suspension reached 81% T (T is the standard concentration unit of Biolog), and the suspension were added to the PM1-8 MicroPlates™ according to the instructions. The OmniLog software was set up and loaded, and the PM MicroPlates™ were incubated in the OmniLog incubator at 30℃ for 48 h. The plates were scanned every 15 min, and the results were analyzed and plotted using the OmniLog software at the end of the incubation.

4.3. Determination of cell concentration and sporulation efficiency

A preculture was prepared by incubating a colony of strain NCD-2 in 100 ml Luria-Bertani (LB) broth at 30℃ with agitation (180 rpm) for 12 h, centrifuged to collect the cells, and then resuspended with sterile water and adjusted to OD_{600}=1.0 with sterile water. The bacteria suspension was added to the M9 medium (12.8 g·L^{-1}, $Na_2HPO_4 \cdot 7H_2O$, 3 g·L^{-1} KH_2PO_4, 0.5 g·L^{-1} NaCl, 1 g·L^{-1} NH_4Cl, 0.24 g·L^{-1} $MgSO_4$, 0.011 g·L^{-1} $CaCl_2$, 4 g·L^{-1} D-glucose) at 1% inoculation volume. To evaluate the effect of different carbohydrates on sporulation of strain NCD-2, the D-glucose was replaced with same concentration of L-arabinose, D-xylose and D-ribose, respectively. Samples were harvested at 24, 36 and 48 h after inoculation, respectively, to calculate sporulation effeciency according to previous protocol [39].

4.4. RNA extraction and RNA sequencing

Strain NCD-2 was inoculated in M9 medium with D-glucose or L-arabinose as sole carbon source, respectively, strain NCD-2 was cultured at 30℃, 180 rpm, then the cells were collected at at 8 h, 12 h and 16 h after inoculation by centrifugation at 4℃ with 10,000 rpm for 5 min. The bacterium was rapidly frozen with liquid nitrogen and stored at -80℃, three replicates were set for each treatment. The total RNA of the collected bacteria was extracted according to the instructions of the RNAprep Pure Cell/Bactria Kit (TianGen Biotech, Beijing, China), and the quality and concentration of total RNA were measured with the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA library construction and RNA sequencing (RNA-seq) were performed by Illumina platform at Majorbio Co., Ltd. (Shanghai, China).

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4.5. Transcriptome data and differential gene expression analysis

The transcriptome raw data has been uploaded to NCBI-SRA database (Accession number: SUB12858722). Using fastp (https://github.com/OpenGene/fastp) to remove low-quality reads and adapters from the data. Then, these clean reads were mapped to reference genome (*Bacillus subtilis* NCD-2) using Bowtie (http://bowtie-bio.sourceforge.net/index.shtml). The screening criteria for differentially expressed genes (DEGs) were $|\log_2(\text{Fold Change})| > 1$ and adjusted *p*-value < 0.05. DEGs were used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis using Goatools (https://github.com/tanghaibao/goatools) and the R package ClusterProfile, respectively, and enrichment results were filtered with the parameters of *p*-value < 0.05.

4.6. Confirmation of transcriptome analysis results

To validate the transcriptome results, 14 genes (Supplementary Table S2) were selected for expression analysis by qRT-PCR. Primers were designed using primer premier 5.0 software (Applied Biosystems). *B. subtilis* strain NCD-2 were cultured in M9 medium with L-arabinose or D-glucose as the sole carbon source, and cultured at 30°C with shaking at 180 rpm, respectively. Strain NCD-2 was collected by centrifugation after 8 h, 12 h and 16 h of incubation, respectively. Total RNA was extracted as described above, and adjusted to 50 ng·μL^{-1}. The extracted total RNA was used as a template to synthesize the cDNA first strand using *TransScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China), and the cDNA concentration was then adjusted to 100 ng·μL^{-1} with double distilled water. The qRT-PCR reaction was conducted according to the instructions of *TransStart*® Top Green qPCR SuperMix (TransGen Biotech, Beijing, China). Three replicates were set up for each gene detection, and the *gyrB* gene was used as the internal reference gene, and the relative change in target gene expression was calculated by the formula $2^{-\Delta\Delta Ct}$ [40].

4.7. Function analysis of msmX gene

To delete the *msmX* gene from strain NCD-2, *msmX* upstream fragments were amplified using primers *msmX*-P1 (**CGAGCTC**TTTCAGCGGTTCGGGTG) and *msmX*-P2 (**GGGGTACC**GATCAAAAAAACCGGACATGGGG), *msmX* downstream fragments were amplified using primers *msmX*-P3 (**GGGGTACC**ACCCAGCCATCTAACATCCCCC) and *msmX*-P4 (**GCTCTAGA**TCCCGGTTCGATTGTGTCTG). The upstream and downstream amplification fragments were digested with *Kpn* I restriction enzyme respectively, and then the two fragments were ligated with T4 DNA ligase. Using the ligation product as a template, PCR amplified with *msmX*-P1 and *msmX*-P4 primers. The amplificon was digested with *Sac* I and *Xba* I restriction enzymes and then attached to the corresponding digestion site of the pKSV7 plasmid [41]. The recombinant plasmid was transformed into strain NCD-2 via electroporation, and the knockout of *msmX* was conducted by in-frame deletion, described as Arnaud et al [42]. The deletion of *msmX* (Δ*msmX*) was confirmed by PCR and sequencing with primers *msmX*-P1 and *msmX*-P4. To complementation of the Δ*msmX* mutant, intact *msmX* was amplified from strain NCD-2 with primers CP*msmX*-F (**GGGGTACC**TTATCGAATTCTCATTTCTG) and CP*msmX*-R (**GCAGGTCGAC**ATTGGAAATATGCACGAAAA), which included the *Kpn* I and *Sal* I restriction sites, respectively. The amplicon was digested with *Kpn* I and *Sal* I and inserted into pHY300PLK, which is a shuttle vector for *E. coli* and *B. subtilis* [43]. The recombinant plasmid was transformed into mutant strain Δ*msmX* by electroporation to obtain complementary strain (CP*msmX*). The wild type strain NCD-2 and mutants were cultured in M9 medium with L-arabinose as sole carbon source, after which the bacteria concentration and sporulation efficiencies were calculated by plate counting as described above.

 doi:10.20944/preprints202310.1502.v1

4.8. Statistical analyses

Statistically significant differences (P<0.05) in NCD-2 CFU, sporulation efficiency, as well as the CFU and sporulation efficiency between wild-type and mutant strains were evaluated by ANOVA using SPSS 18.0 software (SPSS, Chicago, IL, USA) followed by Tukey's post hoc test. Figures were prepared with Origin Pro 8.6 software (OriginLab Corporation, Hampton, MA, USA).

5. Conclusion

L-arabinose can significantly increase the bacteria concentration and sporulation efficiency of strain NCD-2. In this study, we used PMs technology to screen out several nutrients with high metabolic activity of strain NCD-2, among which L-arabinose can significantly increase the bacteria concentration and sporulation efficiency of strain NCD-2, and repress cells autolysis. Transcriptome results showed that L-arabinose up-regulated the expression of sporulation related genes and down-regulated the expression of cannibalism related genes. After knocking out *msmX*, which is responsible for transporting arabinose, significantly decreased the bacteria concentration and sporulation efficiency of strain NCD-2 in the medium with L-arabinose as the carbon source. Results will provide assistance in the study of directed fermentation and the mechanism of regulating sporulation of strain NCD-2.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Author Contributions: Conceptualization, Guo Qinggang and Ma Ping; Data curation, Y.F. and X.L. (Xiaomeng Liu); Formal analysis, Z.S.; Funding acquisition, Q.G. and P.M.; Investigation, Y.F.; Methodology, Y.F. and X.L. (Xiaomeng Liu); Software, Z.S. and Y.F.; Supervision, Q.G. and P.M.; Validation, Y.F. and X.L. (Xiaomeng Liu); Visualization, Y.F and Z.S.; Writing—original draft, Y.F.; Writing—review & editing, Z.S., P.W., Q.G. and P.M. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: This work was supported by the Chinese National Natural Science Foundation (32172487), the China Agriculture Research System of MOF and MARA (CARS-15-19), the Natural Science Foundation of Hebei Province (C2021301030), and the HAAFS Science and Technology Innovation Special Project (2022KJCXZX-ZBS-1).

Conflicts of Interest: The authors declare no conflict of interest.

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