An auto-inducible phosphate-controlled expression system of *Bacillus licheniformis*

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**Abstract**  
**Background:** A promoter that drives high-level, long-term expression of the target gene under substrate limited growth conditions in the absence of an artificial inducer would facilitate the efficient production of heterologous proteins at low cost. A novel phosphate-regulated expression system was constructed using the promoter of the phytase encoding gene *phyL* from *Bacillus licheniformis* for the overexpression of proteins in this industrially relevant host.  

**Results:** It is shown that the *phyL* promoter enables a strong overexpression of the heterologous genes *amyE* and *xynA* in *B. licheniformis* when cells were subjected to phosphate limitation. Whether *B. licheniformis* can use phytate as an alternative phosphate source and how this substrate influences the *PphyL* controlled gene expression under growth conditions with limited inorganic phosphate concentrations were also investigated. It is shown that *B. licheniformis* cells are able to use sodium phytate as alternative phosphate source. The addition of small amounts of sodium phytate (≤ 5 mM) to the growth medium resulted in a strong induction and overexpression of both model genes in *B. licheniformis* cells under phosphate limited growth conditions.  

**Conclusions:** The *PphyL* controlled expression of the investigated heterologous genes in *B. licheniformis* is strongly auto-induced under phosphate limited conditions. The proposed *PphyL* expression system enables an overexpression of target genes in *B. licheniformis* under growth conditions, which can be easily performed in a fed-batch fermentation process.  

**Keywords:** *Bacillus licheniformis*, Heterologous gene expression, Phosphate starvation, Phytate

**Background**  
*Bacillus licheniformis* is a saprophytic bacterium that is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration [1]. The ability to produce and secrete high amounts of proteins into the extracellular medium (20-25 g/L) makes this bacterium to one of the most important industrial hosts for the large-scale production of industrial enzymes, such as amylases, proteases, phytases, and other specialty enzymes [2]. Another advantage of *B. licheniformis* is its ability to grow rapidly in simple media to high-cell-densities, which is favourable for an industrial-scale production.  

The expression systems used in *B. licheniformis* were mostly developed for *B. subtilis*. At present, three types of expression systems that contain (i) constitutive promoters, (ii) inducer-specific promoters and (iii) auto-inducible promoters have been used for high-level production of heterologous proteins in *Bacillus subtilis* (e.g., [3–5]). Among them, expression systems containing inducer-specific promoters (e.g., *Pspac* and *Pxyl*) are the most widely used type. However, the requirement for specific inducers, such as IPTG or xylose, increases the cost of their large-scale application [6, 7]. Constitutive expression systems allow for continuous transcription of their target gene, and thus, are not suitable for the production of potential toxic proteins. In contrast, auto-inducible expression systems that require no specific inducers are ideal for the industrial production of heterologous proteins at low cost. These expression systems are induced by a variety of environmental factors, which can be easily simulated in industrial fermentation processes [8]. For example, nutrient limitation, e.g.
glucose, is such a suitable signal for the induction of an auto-inducible promoter system [9]. Furthermore, an expression system using the phosphate starvation inducible pst promoter has been developed for B. subtilis [10]. However, a comparable auto-inducible promoter system has so far not been shown for B. licheniformis.

It has been recently demonstrated that phosphate starvation conditions induce a tightly regulated set of genes, which are involved in the mobilization of alternative phosphate sources by B. licheniformis cells. Among them, the phytase PhyL belongs to the most abundant extracellular protein under these conditions [11]. Phytase is an enzyme that catalyses the hydrolysis of phytate, the salt of phytic acid, to release a series of myo-inositol phosphate intermediates and inorganic phosphate (Pi) [12, 13]. Phytate is the major storage form of phosphate in plant seeds such as cereal and oilseeds (1 to 5% by weight) [14]. The strong expression of the phyL gene indicated that B. licheniformis cells might use phytate as an alternative phosphate source when concentration of Pi becomes limiting [11, 15]. It could be therefore concluded that the PphyL promoter would be a good candidate for the construction of a novel expression system that can be used for the production of heterologous proteins in B. licheniformis under phosphate-limited growth conditions.

In this study, the suitability of the phyL promoter as a novel auto-inducible phosphate-regulated expression system for B. licheniformis was investigated by means of translational reporter gene fusions with the heterologous genes amyE and xynA from B. subtilis both at the transcriptional and translational level. Furthermore, the role of phytate as an alternative, natural phosphate source for the growth of B. licheniformis cells and as an inducer for the expression of the phyL promoter were studied.

Methods

Strains and cultivation

All bacterial strains used in this study are listed in Table 1. B. licheniformis MW3 (B. licheniformis DSM13 (ΔhsdR1, ΔhsdR2)), which ensures high transformation efficiencies due to the deletion of two type I restriction modification systems hsdR1 and hsdR2, was used as the host strain [16]. Belitzky minimal medium (BMM) was used in all growth experiments [17]. The cells were cultured at 37°C with 200 rpm in 200 mL BMM (pH 7.0) containing 15 mM (NH₄)₂SO₄, 8 mM MgSO₄·7H₂O, 27 mM KCl, 7 mM sodium citrate dihydrate, and 50 mM Tris-HCl (pH 7.5) supplemented with 0.6 mM KH₂PO₄, 2 mM CaCl₂·2H₂O, 1 μM FeSO₄·7H₂O, 10 μM MnSO₄·4H₂O, and 11 mM glucose. For the phosphate starvation experiments, the concentration of phosphate was reduced to 0.15 mM KH₂PO₄.

| Table 1 Bacterial strains and plasmids used in this study |
|---------------------------------|-----------------|-----------------|
| Strains or plasmids             | Relevant genotype | Reference       |
| E. coli DH10B                   | F–, mcrA, Δmrr-hsdRMS-mrcBC, Ω80lacZ2, ΔM15, ΔlacX74, deoR, ecA1, endA1, araD139, Δ(amyE)7B97, galU, galK, h+, mpsL, nupG | Gibco BRL |
| B. licheniformis MW3            | ΔhsdR1, ΔhsdR2   | [16]            |
| B. licheniformis TH3            | ΔhsdR1, ΔhsdR2, pKUC3 | This study |
| B. licheniformis TH4            | ΔhsdR1, ΔhsdR2, pKUC4 | This study |
| Plasmids                        |                 |                 |
| pKUC                           | shuttle vector based on pUC18 and pKTH290 | [19] |
| pKUC3                          | pKUC containing the PphyL-amyE fusion | This study |
| pKUC4                          | pKUC containing the PphyL-xynA fusion | This study |

Construction of strains

The activity of the PphyL promoter was analyzed by means of translational reporter gene fusions. For this purpose, an approximately 300-bp fragment containing the PphyL promoter from B. licheniformis DSM13 (Additional file 1: Figure S2) was cloned in front of the α-amylase and xylanase reporter genes from B. subtilis 168 with the primer pairs 3/4 and 6/7, respectively (Additional file 1: Table S1). The amyE and xynA genes from B. subtilis 168 were amplified with the primer pairs 3/4 and 6/7, respectively (Additional file 1: Table S1). The PphyL-amyE and PphyL-xynA fusions were constructed by means of the precise gene fusion polymerase chain reaction strategy described by Yon and Fried [18] by using the primer pairs 1/4 and 1/7, respectively. The PCR-fusion fragments were then inserted into the XbaI and KpnI sites of the multi-copy plasmid pKUC (this shuttle vector is based on the pUC18 and pKTH290 plasmids [19]) resulting in vector pKUC3 and pKUC4, respectively. These vectors were used to transform the B. licheniformis strain MW3 by electroporation [20] resulting in the strains TH3 (pKUC3) and TH4 (pKUC4), respectively. These strains were then cultivated in phosphate-limited BMM as already described. Culture supernatants for enzyme assays were taken at different time points during the cultivation. The first sample was taken during the exponential growth phase (four hours after cultivation), the second sample during the transient phase and additional samples were taken 2, 4, 6, 8, 10, 12 and 14 h after onset of the stationary growth phase.

Enzyme assays

Activity of the amylase AmyE was determined with the Ceralpha kit (Megazyme International Ireland Ltd., Bray, Ireland). Amylase activity was calculated in “international units” (IU) by the equation: IU/mL = 4.6 χ
(ΔE₄₀₀ × 4.7 × Dilution) (ΔE₄₀₀ = Absorbance at 400 nm (reaction) – Absorbance at 400 nm (blank)). One international unit of activity is defined as the amount of enzyme required to release one micromole of glucose-reducing sugar equivalents per minute under defined conditions of temperature and pH (40 °C, pH 6.5) [21].

Activity of the xylanase XynA was measured using the modified dinitrosalicylic acid (DNSA) method [22] with some modifications as described in detail by Nguyen et al. [23]. One international unit (IU/mL) of xylanase activity was defined as the amount of enzyme that liberates one micromole of reducing sugar equivalent to xylose per minute under the assay conditions described.

Analysis of the extracellular proteins
The proteins in the supernatant samples were separated by one-dimensional (1D-) SDS-PAGE. In brief, 20 μL of supernatant samples were mixed with 5 μL SDS sample buffer (50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromphenol blue) and denatured at 90 °C for 10 min. The SDS-PAGE gel included a separating gel (10% “Acrylamide-Solution (30%)-Mix 37.5:1” (Bio-Rad, USA), 0.4 M Tris (pH 8.8), 0.1% SDS, 0.1% APS, 0.04% TEMED) and a stacking gel (4% “Acrylamide-Solution”, 0.125 M Tris (pH 6.8), 0.1% SDS, 0.05% APS, 0.1% TEMED). The protein separation according to their molecular weight was conducted at 150 V for one hour using a Protean II Cell system (BIO-RAD). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

Growth analysis
The growth of B. licheniformis cells on sodium phytate (Sigma-Aldrich Co, USA) as an alternative phosphate source was studied in a phosphate limited BMM by the translational fusion of P_{phyL'}−amyE and P_{phyL'}−xynA were cultivated in a phosphate limited BMM with sodium phytate, which was added to the growth medium at an OD (at 500 nm) of 1.0 with the final concentrations of either 0.5 mM or 5 mM. The cell samples were taken during the logarithmic growth phase at an OD of 1.0 and 1, 2, 3 and 4 h after onset of the stationary growth phase.

RNA isolation and northern blot analysis
Cell disruption was performed by using the RiboLyser Cell Disrupter (Thermo Electron Corporation, Germany) and total RNA was isolated and purified by using the KingFisher mL pipetting robot (Thermo Electron Corporation, Germany) by means of the MagNA Pure LC RNA isolation Kit I (Roche Diagnostics, Germany) as described in detail by Jürgen et al. [24]. The quality of the isolated total RNA was analyzed by means of the Bioanalyzer 2100 from Agilent (Germany).

The effect of phytate on the expression of the P_{phyL} controlled expression of the heterologous amyE gene was determined by Northern blot analyses as described by Wetzstein et al. [25]. The specific hybridization reaction for the amyE mRNA was performed with appropriate digoxigenin-labeled RNA probes. The probes were synthesized with the T7 RNA polymerase from the T7 promoter-containing internal PCR products of the amyE gene using the primer pairs 8/9 (Additional file 1: Table S1).

Results
Analysis of the phyL promoter sequence
A recent transcriptome analysis indicated a phosphate controlled expression of the phyL gene of B. licheniformis (Additional file 1: Figure S1) [11]. In addition, the study of Hoi et al. [11] also revealed that phytase is one of the most prominent proteins in the secretome of B. licheniformis cells under phosphate limited growth conditions. In order to elucidate the structure of the phyL promoter of B. licheniformis the predicted promoter region with the well-known promoter sequence of the phytase encoding gene phyC from Bacillus amyloliquefaciens FZB42, a close relative of B. licheniformis was aligned. It is shown that the phyL promoter region contains a σ^5-like promoter sequence, which well resembles the reported −10 and −35 sequences of the phyC promoter (Fig. 1). In addition, the phyL promoter region contained another −10-like consensus sequence, which is located between the putative −10 and −35 promoter sequence (Fig. 1). A pair of tandemly repeated PhoP TT(T/A/C)ACA binding boxes was found within and downstream of the −35 consensus promoter sequence. Another putative PhoP binding box was located downstream of the −10 consensus region of the phyL promoter. This suggests that the expression of the phyL gene in B. licheniformis is probably regulated by the PhoPR two component system as reported for the phyC gene in B. amyloliquefaciens FZB45 (Fig. 1).

PhyL controlled expression patterns of the amyE and xynA genes
The phosphate regulated promoter, P_{phyL}, was cloned upstream of the reporter genes amyE and xynA, resulting in...
the plasmids pKUC3 (strain TH3) and pKUC4 (strain TH4) respectively. The expression pattern of the B. licheniformis strains containing one of those plasmids revealed that both promoter fusions started to express the amyE and xynA reporter genes when cells entered the transient phase (6 h after inoculation) in a phosphate limited BMM (Fig. 2). In order to verify that the synthetic constructs of the phyL promoter and the reporter genes are indeed mainly phosphate-dependent regulated during the onset of the stationary phase, the strain B. licheniformis TH3, carrying the PphyL-amyE fusion, was exemplarily cultivated with different phosphate concentrations (0.15, 0.3 and 0.6 mM) (Additional file 1: Figure S3). The data demonstrate that α-amylase activity was significantly increased under phosphate-limited conditions (0.15 mM). In contrast, only low α-amylase activities were detectable in cultivations with high phosphate concentrations. Furthermore, no amylase or xylanase activity was detected during the exponential phase without phosphate limitation.

The amylase activity in the PphyL-amyE fusion strain TH3 increased gradually throughout the stationary phase and reached a final maximal activity of about 2.5 IU/mL (Fig. 2a). The PphyL-xynA fusion strain TH4 showed a similar final xylanase activity throughout the stationary phase of about 1.5 IU/mL (Fig. 2b).

The SDS-PAGE analysis of the extracellular protein fraction of the strain TH3 revealed that the heterologous AmyE protein started to secrete and accumulate in the extracellular medium at the transient phase (6 h after cultivation). The AmyE level increased gradually throughout the stationary phase (Fig. 2c). The production pattern of the heterologous XynA protein in strain TH4 was similar to the production pattern observed for the AmyE protein (Fig. 2d).

Analysis of phytate as alternative substrate and inducer
B. licheniformis was then tested for its ability to use phytate as alternative phosphate source when cells were
subjected to phosphate limitation. The growth of *B. licheniformis* cells was monitored in phosphate-limited BMM in the absence or presence of sodium phytate. In growth experiments without the addition of sodium phytate, *B. licheniformis* cells entered the transient phase after 6 h of cultivation at an OD$_{500}$ nm of about 0.8. The growth rate of the control and the phytate containing culture remained constant until an OD of around 1.0 was reached. However, in the culture with 0.5 mM sodium phytate the OD values increased gradually up to an OD of about 2. When higher amounts of sodium phytate (5 mM) were added to the growth medium, *B. licheniformis* cells grew more slowly to an OD value of 1.0 within 8 h, however, the biomass significantly increased up to an OD value of 2.7 at the end of the cultivation (Fig. 3).

In order to investigate whether phytate might serve as an inducer of the *phyL* promoter, the *B. licheniformis* strains TH3 and TH4 bearing the *PphyL′-amyE* and *PphyL′-xynA* fusions, were cultivated in phosphate limited BMM supplemented with different concentrations of sodium phytate. Under growth conditions without sodium phytate, the *PphyL′-amyE* fusion strain TH3 showed a maximal amylase activity of about 2.6 IU/mL, while the *PphyL′-xynA* fusion strain TH4 reached a maximal xylanase activity of about 1.5 IU/mL (Fig. 3a and b). When 0.5 mM sodium phytate was added to the growth medium, the *phyL′-amyE* fusion strain TH3 reached a higher maximal amylase activity of 4.5 IU/mL (Fig. 3a). This observation was supported by Northern blot analyses of the strain TH3, which revealed a significant higher *amyE* transcript level under growth conditions with 0.5 mM sodium phytate compared to the control without phytate addition (Fig. 4). The addition of 0.5 mM phytate to the growth medium of the *phyL′-xynA* fusion strain TH4 resulted in higher maximal xylanase activity of 9.2 IU/mL (Fig. 3b). Such an increase in the expression pattern could also be observed for the XynA protein level in strain TH4 under growth conditions with 0.5 mM phytate (Fig. 3d). The application of a higher phytate concentration (5 mM) did not result in higher amylase or xylanase activities (Fig. 3a and b). It is worth mentioning that the native phytase protein PhyL was overproduced and secreted when phytate was added to the phosphate limited BMM (Fig. 3d).

**Discussion**

A global transcriptome and proteome analysis revealed that more than 100 genes are significantly upregulated in *B. licheniformis* in response to phosphate limitation [11]. The *phyL* transcript belonged to the most strongly induced and abundant mRNAs during the transition phase from the exponential to stationary phase. A sequence analysis indicates that the *phyL* gene is similar to other phosphate-controlled genes regulated by the PhoPR two component system. Furthermore, the −10 and −35 regions of the *phyL* promoter reveal typical SigmaA-dependent sequences (Fig. 1). Putative PhoP
binding boxes were found within and downstream of the phyL promoter region. Such a specific PhoP control was recently shown for the highly similar phyC promoter of the closely related species B. amyloliquefaciens [26]. Data of this study reveal that the phyL promoter was only induced when cells are exposed to phosphate limitation. As suggested by Makarewicz et al. [26], both E σ^A RNAP holoenzyme and PhoP-P are necessary and sufficient to establish the transcriptional activation of the phyC promoter in B. amyloliquefaciens under such growth conditions. Thus, the expression of the phyL promoter in B. licheniformis might be similarly regulated as described for the phyC promoter in B. amyloliquefaciens.

Data of this study indicate an efficient expression of two heterologous model proteins by using the tightly regulated and strongly inducible PphyL promoter in B. licheniformis under phosphate-limitation conditions. Neither protein bands in a 1D-SDS-PAGE nor activities of the two model proteins, the amylase or the xylanase, could be detected during the exponential growth phase. Furthermore, the growth behavior of the recombinant strains also indicates that perturbing effects of the proposed expression system on the growth of B. licheniformis cells during the exponential growth phase, can be excluded as long as sufficient inorganic phosphate is available. In addition, it is shown that sodium phytate is a suitable alternative phosphate source for the growth of B. licheniformis when cells were subjected to phosphate limited growth conditions. Experiments in this study suggest that moderate concentrations of sodium phytate (≤ 5 mM) would be more favorable to induce the activity of the phyL promoter in B. licheniformis. The addition of higher concentrations of phytate (e.g. 1% w/v or 15 mM) to the growth medium could hamper the activity of the promoter of the phytase gene in B. licheniformis (data not shown). This could be due to a critical increase of inorganic phosphate levels by the phytase hydrolysis of the induced phytase enzyme, which would down-regulate the activity of the phosphate responsive PhoPR two-component system and thus result in a lower PphyL activity.

For B. subtilis a similar phosphate controlled expression system based on the pst promoter was suggested [10]. However, a crucial starvation of an essential substrate, such as phosphate, during the protein over-production phase could diminish the protein synthesis capacity of the host. Therefore, to reach optimal yields, either the promoter of an appropriate expression system has to be switched on before the complete exhaustion of the critical substrate or the limited nutrient needs to be replaced by another suitable substrate, which does not lead to a down-regulation of the promoter system. Thus, the perfect alternative substrate should be metabolized and in parallel be an inducer of the system [9]. Data of this study indicated that the expression system using the phyL promoter is not only strong and tightly regulated by the level of inorganic phosphate but also easily inducible by the alternative phosphate source phytate. The suggested promoter system is comparable to the pst promoter system of B. subtilis [10] but exhibits an additional feature due to its expression stimulation by the alternative phosphate source phytate.
Conclusions
The results of this study demonstrate that the phyL promoter is a suitable candidate for an auto-inducible expression system for Bacillus licheniformis. It is shown that phytate is not only an appropriate alternative phosphate source for this bacterium, but also an inducer of this expression system. The PphyL expression system might be used to overexpress target genes in Bacillus licheniformis under growth conditions, which can be easily performed in industrial batch-fermentation processes. However, further studies are required to investigate in detail the suitability of this auto-inducible system for large-scale heterologous protein production in Bacillus licheniformis fed-batch fermentation processes.

Additional files

Additional file 1: Figure S1. Heat map of expression levels of selected phosphate starvation-inducible genes in Bacillus licheniformis [11]. Low and high values are given in blue with different gradients. Figure S2. The 323 bp large DNA-sequence of the phytase promoter (PphyL), which was used to express amyA and xynA. Figure S3. The phyL promoter driven expression of the recombinant amylase (n = 3 independent cultivations) of Bacillus licheniformis TH3 in BMM supplemented with different concentrations of phosphate. Lines indicate cell growth, while bars indicate enzyme activity. Growth: triangles with 0.15 mM phosphate, circles with 0.3 mM phosphate, squares with 0.6 mM phosphate. Enzyme activity: grey bars with 0.6 mM phosphate, black bars with 0.3 mM phosphate, white bars with 0.15 mM phosphate. Table S1. Sequences of primers used in this study. (DOCX 50 kb)

Abbreviations
APS: Ammonium persulfate; BMM: Belitzky minimal medium; IU: International unit; OD: Optical density; PAGE: Polyacrylamide gel electrophoresis; PI: Inorganic phosphate; RNAP: RNA polymerase; SDS: Sodium dodecyl sulfate; TEMED: Tetramethylammoniumhydroxide

Acknowledgements
This work was supported by the National Foundation for Science and Technology Development of Vietnam (NAFOSTED) [106.16-2012.23]. We thank Claudia Borgmeier (Universität Münster) for providing the pKUC vector. We acknowledge support for the Article Processing Charge from the Publication Fund of the University of Greifswald.

Funding
This work was supported by the National Foundation for Science and Technology Development of Vietnam (NAFOSTED) [106.16-2012.23].

Availability of data and materials
All data generated or analysed during this study are included in this published article and in supplemental files (Additional file 1: Figures S1 and S2 and Additional file 1: Table S1).

Authors’ contributions
TTN designed and conducted the experiments, evaluated the results and drafted the manuscript. MHN helped in research design and cloning experiments. HTN helped in amylase and xylanase assays as well as the analysis of extracellular proteins. XCN helped in analyzing the substrate induction experiments. TS and BJ initiated this study, directed the project and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 8 January 2018 Accepted: 5 December 2018
Published online: 09 January 2019

References
1. Gupta R, Beg QK, Khan S, Chauhan B. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl Microbiol Biotechnol. 2002;60:381–95.
2. Schallmey M, Singh A, Ward OP. Developments in the use of Bacillus species for industrial production. Can J Microbiol. 2004;50:1–17.
3. Lee SJ, Pan JG, Park SH, Choi SK. Development of a stationary phase-specific autoinducible expression system in Bacillus subtilis. J Biotechnol. 2010;140:16–20.
4. Schumann W. Production of recombinant proteins in Bacillus subtilis. Adv Appl Microbiol. 2007;62:137–90.
5. Wenzel M, Müller A, Siemann-Herzberg M, Altenbuchner J. Self-inducible Bacillus subtilis expression system for reliable and inexpensive protein production by high-cell-density fermentation. Appl Environ Microbiol. 2011; 77:6419–25.
6. Bhavsar AP, Zhao X, Brown ED. Development and characterization of a xylose-dependent system for expression of cloned genes in Bacillus subtilis: conditional complementation of a teichoic acid mutant. Appl Environ Microbiol. 2001;67:403–10.
7. Phan TTP, Nguyen HD, Schumann W. Novel plasmid-based expression vectors for intra- and extracellular production of recombinant proteins in Bacillus subtilis. Protein Expr Purif. 2006;46:189–95.
8. Chan AY, Chan MM, Lo HM, Leung VC, Lim BL. A dual protein expression system in Bacillus subtilis. Protein Expr Purif. 2002;26:337–42.
9. Silbersack J, Jürgen B, Hecker M, Schneider R, Schweder T. An acetoin-regulated expression system for reliable expression system of Bacillus subtilis. Appl Microbiol Biotechnol. 2006;73:895–903.
10. Kerovuo J, Weyman MJ, Povelainen M, Auer S, Miasnikov A. A novel efficient expression system for Bacillus and its application to production of recombinant phyrase. Biotechnol Lett. 2000;22:1311–7.
11. Hoi LT, Voigt B, Jürgen B, Ehrenreich A, Gottschalk G, Evers SC, Friescher J, Maurer KH, Hecker M, Schweder T. The phosphate-starvation response of Bacillus licheniformis. Proteomics. 2006;6:3382–601.
12. Kerovuo J, Rovinen J, Hatzack F. Analysis of myo-inositol hexakisphosphate hydrolysis by Bacillus phytase: indication of a novel reaction mechanism. Biochem J. 2000;363:2–8.
13. Singh B, Kunze G, Satyanarayana T. Developments in biochemical aspects and biotechnological applications of microbial phytase. Biotechnol Mol Biol Rev. 2011;6:69–87.
14. Rao DE, Rao KV, Reddy TP, Reddy VD. Molecular characterization, physicochemical properties, known and potential applications of plant phytases: an overview. Crit Rev Biotechnol. 2009;29:182–98.
15. Voigt B, Schweder T, Bibald MJ, Albrecht D, Ehrenreich A, Bernhardt J, Friescher J, Maurer KH, Gottschalk G, van Dijl JM, Hecker M. The extracellular proteome of Bacillus licheniformis grown in different media and under different nutrient starvation conditions. Proteomics. 2006;6:268–81.
16. Waschkau B, Waldeck J, Wieland S, Eichstadt R, Meinhardt F. Generation of readily transformable Bacillus licheniformis mutants. Appl Microbiol Biotechnol. 2008;78:181–8.
17. Stülke J, Hillen W. Regulation of carbon catabolism in Bacillus species. Annu Rev Microbiol. 2000;54:949–80.
18. Yon J, Fried M. Precise gene fusion by PCR. Nucleic Acids Res. 1989;17:4895.
19. Truong VL. Characterization of the pectinolytic enzymes of the marine psychrophilic bacterium Pseudoalteromonas haloplanktis strain ANT/505. Ph. D thesis, University of Greifswald, Germany. Available at https://epub.ub.uni-greifswald.de/frontdoor/deliver/index/docId/254/file/Truong_thesis_20.9.06.pdf. 2006.
20. Sambrook J, Fritsch E, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1989.
21. McCleary BV, McNally M, Monaghan D, Mugford DC. Measurement of alpha-amylase activity in white wheat flour, milled malt, and microbial enzymes preparations, using the Ceralpha assay: collaborative study. J AOAC Int. 2002; 85:1096–102.
22. Bailey JM, Biely P, Poutanen K. Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol. 1992;23:257–70.
23. Nguyen TT, Nguyen MH, Nguyen HT, Nguyen HA, Le TH, Schweder T, Jürgen B. A phosphate starvation-inducible ribonuclease of Bacillus licheniformis. J Microbiol Biotechnol. 2016;26:1464–72.
24. Jürgen B, Barken KB, Tobisch S, Pioch D, Wümpelmann M, Hecker M, Schweder T. Application of an electric DNA-chip for the expression analysis of bioprocess-relevant marker genes of Bacillus subtilis. Biotechnol Bioeng. 2005;92:299–307.
25. Wetzstein M, Völker U, Dedio J, Lobau S, Zuber U, Schiesswohl M, Herget C, Hecker M, Schumann W. Cloning, sequencing, and molecular analysis of the dnaK locus from Bacillus subtilis. J Bacteriol. 1992;174:3300–10.
26. Makarewicz I, Dubrac S, Masedek T, Borns R. Dual role of the PhoP~P response regulator: Bacillus amyloliquefaciens FZB45 phytase gene transcription is directed by positive and negative interactions with the phyC promoter. J Bacteriol. 2006;188:6953–65.