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Property Improvement of α-Amylase from *Bacillus stearothermophilus* by Deletion of Amino Acid Residues Arginine 179 and Glycine 180

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SUMMARY

To improve the properties of α-amylase from *Bacillus stearothermophilus* (AmyS), a deletion mutant AmySΔR179-G180 was constructed by deleting arginine (Arg179) and glycine (Gly180) using site-directed mutagenesis. AmyS and AmySΔR179-G180 were expressed in *Bacillus subtilis* and purified by ammonium sulfate precipitation, after which the enzymatic properties were characterized and compared. By deleting amino acids Arg179 and Gly180, the thermostability of α-amylase AmySΔR179-G180 was enhanced and the half-life at 100 °C significantly increased from 24 to 33 min. In addition, AmySΔR179-G180 exhibited greater acid resistance and lower calcium requirements to maintain α-amylase activity. The secretory capacity of the recombinant strain was evaluated by fed-batch fermentation in a 7.5-litre fermentor in which high α-amylase activity was obtained. The highest activity reached 3300 U/mL with a high productivity of 45.8 U/(mL·h).

Key words: α-amylase, *Bacillus subtilis*, fermentation, site-directed mutagenesis, thermostability

INTRODUCTION

α-Amylases (1,4-α-D-glucan glucanohydrolases; EC 3.2.1.1) are classical calcium-binding enzymes, which randomly hydrolyze internal α-1,4-glucosidic linkages in starch to produce smaller molecular mass maltodextrins, maltooligosaccharides and glucose (*1*). α-Amylases have great commercial value, as they can be used in the initial stages of starch processing (liquefaction) in the production of fructose and glucose syrups, detergent matrices as cleaning agents, in the baking industry for improving flour and in the textile industry for the removal of starch from textiles (*2*).

In the industrial production of glucose and fructose, starch liquefaction by α-amylases and saccharification by β-amylases are carried out sequentially. During liquefaction, the pH must be adjusted to between 5.5 and 6.0 and calcium ions need to be added due to the poor properties of widely used α–amylases produced by wild type strains such as *Bacillus amyloliquefaciens* (BAA) (*3*), *Bacillus stearothermophilus* (BSA) (*4*) and *Bacillus licheniformis* (BLA) (*5*), which significantly increase the production cost of glucose and fructose. Hence, much work has been performed on protein engineering, including random mutagenesis, site-directed mutagenesis, and DNA shuffling, all of which have been widely applied to improve thermostability, reduce operating pH range and remove the calcium requirement of α-amylases to meet the needs of industrial production (*6*). While random mutagenesis and DNA shuffling require highly efficient screening methods, rational design, based on sequence alignment and structural analysis of site-directed mutagenesis, is an effective method to obtain beneficial mutants. In previous studies, the thermostability of BAA (*7*) and *Bacillus* KSM-1378 α-amylase (LAMY) (*8*) was greatly improved by deletion of an Arg-Gly peptide. Furthermore, a truncated *Bacillus* sp. strain TS-23 α-amylase (BACΔNC) (*9*) had the same effect by deleting Arg210-Ser211. The half-life of *B. stearothermophilus* AmyUS100 was also increased by the deletion of Ile214-G215 (*10*).

In this study, we deleted the amino acid residues Arg179 and Gly180 in AmyS by site-directed mutagenesis to optimize the properties of AmyS. The mutant AmyS was found to be

more acid-resistant at pH=4.5-6.0 than the wild type AmyS, the thermostability was significantly improved and the requirement for calcium to maintain activity was decreased. Furthermore, we used a 7.5-litre fermentor to evaluate the recombinant production of AmySΔR179-G180 in fed-batch cultivation.

MATERIALS AND METHODS

Materials

Peptone and yeast extract were obtained from Oxoid, Thermo Fisher Scientific (Basingstoke, UK). Coomassie Brilliant Blue R-250 staining solution was obtained from Bio-Rad (Hercules, CA, USA). The 3,5-dinitrosalicylic acid (DNS) was obtained from Sigma-Aldrich (St Louis, MO, USA). NaCl, $CaCl_2$, K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, sodium acetate, sodium citrate, sodium phosphate, starch, glucose, L-tryptophan, acid-hydrolyzed casein and antibiotics were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, PR China). NuPAGE 12 % Bis–Tris Gel and MOPS SDS Running Buffer were obtained from Invitrogen (Carlsbad, CA, USA). PrimerSTAR Max DNA Polymerase and *Dpn*I were purchased from Takara Biotechnology (Dalian) Co., Ltd. (Dalian, PR China).

Plasmids, strains, medium and culture conditions

Plasmids and bacterial strains used in this study are described in **Table 1**. The pMA5 is an *Escherichia coli/B. subtilis* shuttle plasmid, which was donated by the *Bacillus* Genetic Stock Center (Columbus, OH, USA). *E. coli* DH5α was chosen as a host for molecular cloning and plasmid construction. This strain was cultivated in Luria–Bertani (LB) medium at 37 °C, which contained 5 g/L of yeast extract, 10 g/L of peptone and 5 g/L of NaCl. *B. subtilis* 1A751 was used as the host strain for AmyS (α-amylase from *B. stearothermophilus*) expression, and this strain was grown in Super rich (SR) medium (2.5 % yeast extract, 1.5 % peptone and 0.3 % K_2HPO_4, pH=7.2) at 37 °C. Antibiotics were added as follows: kanamycin 25 μg/mL and ampicillin 100 μg/mL.

The components of the medium used for *Bacillus subtilis* transformation were as follows: Spizizen minimal medium consisting of (in g/L): $(NH_4)_2SO_4$ 2, KH_2PO_4 6, K_2HPO_4 18.3 and sodium citrate 1.2. GMI (growth medium I) was Spizizen minimal medium supplemented with 0.8 % glucose, 0.04 % acid-hydrolyzed casein, 0.1 % yeast extract, 0.02 % $MgSO_4 \cdot 7H_2O$ and 0.005 % L-tryptophan, and GMII (growth medium II) was Spizizen minimal medium supplemented with 0.8 % glucose, 0.02 % acid-hydrolyzed casein and 0.16 % $MgSO_4 \cdot 7H_2O$.

Site-directed mutagenesis

The Easy Mutagenesis System (TransGen Biotech Co. Ltd., Beijing, PR China) was used to construct the mutant plasmid in accordance with the supplier's instructions. First, a linear plasmid carrying the desired mutation was PCR-amplified with primers AmySΔR179-G180-F (5′-TTACATATGAAACAACAAAAACGGCTTTACG-3′) and AmySΔR179-G180-R (5′-CGCGGATCCTTAAGGCCATGCCACCAGTC-3′) with PrimerSTAR Max DNA Polymerase using the plasmid pMA5-AmyS as a template. The PCR products were then digested with *Dpn*I, which degraded the methylated DNA, removing the template. Finally, the nicked plasmid DNA carrying the desired mutation was transformed into *E. coli* DH5α, according to the methods described by Green and Sambrook (*11*). The newly constructed plasmid was designated pMA5-AmySΔR179-G180 and verified by DNA sequencing (*12*) (Genewiz, Suzhou, PR China).

B. subtilis transformation

First, GMI and GMII were prepared as mentioned previously (*13*). *B. subtilis* 1A751 and derivatives were cultivated in GMI overnight at 37 °C. A volume of 0.5 mL of overnight culture was transferred into 4.5 mL of GMI medium and grown at 37 °C for 4.5 h. A volume of 0.75 mL of culture was then mixed with 4.25 mL of GMII medium and the culture was incubated for 1.5 h at 37 °C. A mass of plasmid of 1 μg was mixed with 1 mL of competent cells and incubated for 1 h at 37 °C. The cells were then plated on LB agar plates containing kanamycin (25 μg/mL) and incubated for 12 h at 37 °C.

Table 1. Strains and plasmids used in this study

Strains and plasmids	Properties	Source
Strain		
E. coli DH5α	F⁻Δ*lac*U169(Ø80d *lac*ZΔM15) *sup*E44 *hsd*R17 *rec*A1 *gyr*A96 *end*A1 *thi*-1 *rel*A1	Invitrogen**
B. subtilis 1A751	eglSΔ102 bglT/bglSΔEVaprEnprE his	BGSC***
Plasmid		
pMA5	*E. coli/B. subtilis* shuttle vector, $P_{Hpa\,II}$; Ap^r, Km^r	Lab stock
pMA5-AmyS	pMA5 derivative, *amyS* from *B. stearothermophilus*	Lab stock
pMA5-AmySΔR179-G180	pMA5-AmyS* derivative, AmySΔR179-G180	This work

*AmyS GenBank: AF032864.1, **Invitrogen, Carlsbad, CA, USA, ***BGSC=Bacillus Genetic Stock Center, Columbus, OH, USA

Expression and purification of AmyS and AmyS mutant, and electrophoresis

The plasmids pMA5-AmyS and pMA5-AmySΔR179-G180 were individually transformed into *B. subtilis* 1A751 for protein expression. *B. subtilis* 1A751 cells harbouring pMA5-AmyS and pMA5-AmySΔR179-G180 were grown overnight and 1-mL culture was transferred into 100 mL of SR medium containing kanamycin (25 μg/mL) and the culture was then incubated at 37 °C for 36 h. The supernatant was collected by centrifugation (4 °C, 5000×*g*, 10 min) using centrifuge model 5810R (Eppendorf, Hamburg, Germany). The enzyme in the supernatant was precipitated by adding solid ammonium sulfate to 70 % (by mass per volume). This was performed at 4 °C with continuous stirring and then kept on ice for 30 min. The precipitated proteins were collected by centrifugation (4 °C, 12 000×*g*, 30 min; model 5810R; Eppendorf), dialyzed against 100 mM sodium acetate (pH=6), and then concentrated to minimum volume. The purified enzymes were analyzed by SDS-PAGE using the NuPAGE 12 % Bis–Tris Gel together with MOPS SDS Running Buffer. Protein bands were visualized by Coomassie Brilliant Blue R-250 staining.

α-Amylase activity assay

α-Amylase activity was measured according to the 3,5-dinitrosalicylic acid (DNS) method (*14*). One unit of α-amylase activity was defined as the amount of enzyme that liberates 1 μmoL of glucose per min under the detection conditions. In our experiment, 40 μL of diluted enzyme solution were mixed with 80 μL of 100 mM sodium acetate (pH=6) and 80 μL of 1 % (by mass per volume) soluble starch and then incubated for 20 min at 75 °C. The reaction was stopped by quenching on ice, after which 200 μL of 1 % DNS were added and the sample was boiled for 10 min. Absorbance was measured at 540 nm using Microplate Reader SpectraMax M5 (Molecular Devices LLC, San Jose, CA, USA) after cooling to room temperature. A reaction mixture without the enzyme was prepared as a negative control. All measurements were performed in triplicate.

Assay for optimal temperature and pH

The α-amylase activity at 50, 55, 60, 65, 70, 75, 80, 85 and 90 °C was determined, and the optimal temperature was obtained. α-Amylase activity was measured at pH=4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 and the optimal pH was determined. The buffers used in the experiment were as follows: 100 mM sodium acetate buffer (pH=4.0 to 6.0) and 100 mM sodium phosphate buffer (pH=6.0 to 7.5).

Thermostability evaluation

The purified enzyme was incubated with different concentrations of $CaCl_2$ (0, 0.25, 0.5 and 1 mmol/L) at 100 °C for 0-1 h. Subsequently, the residual enzyme activity was assayed to analyze thermostability.

Computer modelling methods

The homology models of native AmyS and mutant AmyS-ΔR179-G180 were created with Discovery Studio v. 4.1 (*15*), using the wild type BStA (1HVX PDB entry) as a template. The structure was viewed using the PyMol editor (*16*).

Fed-batch cultivation

B. subtilis 1A751 strain harbouring pMA5-AmyS-ΔR179-G180 was used for high density fermentation in a 7.5-litre BIO FLO 310 fermentor (New Brunswick Scientific Co Inc., Edison, NJ, USA), with a fed-batch strategy. The medium was supplemented with 2.5 % yeast extract, 1.5 % peptone, 0.3 % K_2HPO_4 and 50 μg/mL of kanamycin. The airflow rate was controlled at 6.0 L/min and, by automatic regulation of stirring speed, dissolved oxygen tension was kept between 20 and 40 % air saturation. Temperature and pH were controlled at 37 °C and 7.0, respectively. A silicone-based anti-foaming agent was used to eliminate foam. Fermentation was carried out with a working volume of 3.5 L at 37 °C for 72 h. When cell growth rate reached a constant level, fed-batch cultivation was initiated. Soluble starch (8.0 % by mass per volume) was added as the substrate at a constant flow rate until its final mass per volume ratio reached 4.0 %. Cell growth was monitored by measuring cell dry mass. α-Amylase activity was analyzed by measuring the cell-free supernatant.

Statistical analysis

The experiments (α-amylase activity assay and dry cell mass measurement) were performed in triplicates. Data are presented as mean relative value±standard deviation.

RESULTS AND DISCUSSION

Expression of AmyS and AmySΔR179-G180 in B. subtilis

In our previous study, we cloned the α-amylase gene (GenBank: AAB86961.1; *17*) from a wild type *B. stearothermophilus* strain and successfully expressed this α-amylase (AmyS) in *B. subtilis* (*18*). According to sequence alignment, we found that an Arg-Gly peptide present in AmyS was similar to that in BAA (*7*) and LAMY (*8*). The deletion of the Arg-Gly peptide greatly improved the thermostability of BAA and LAMY. Therefore, we attempted to optimize AmyS by deleting amino acid residues Arg179 and Gly180 simultaneously by site-directed mutagenesis. The mutation (ΔR179-G180) was introduced into AmyS using the Easy Mutagenesis System (TransGen Biotech) and the altered sequence was confirmed by sequencing. The resultant plasmid pMA5-AmySΔR179-G180 was transformed into *B. subtilis* 1A751 cells. The transformants were cultivated in 30 mL of SR medium in 250-mL shake flasks at 37 °C for 36 h. As shown in **Fig. 1a**, a protein band of approx. 58 kDa was present, which was consistent with the expected size of AmyS. In addition, the proteins of interest, AmyS and AmyS-ΔR179-G180, were purified to near homogeneity by saturated ammonium sulphate precipitation (**Fig. 1b**).

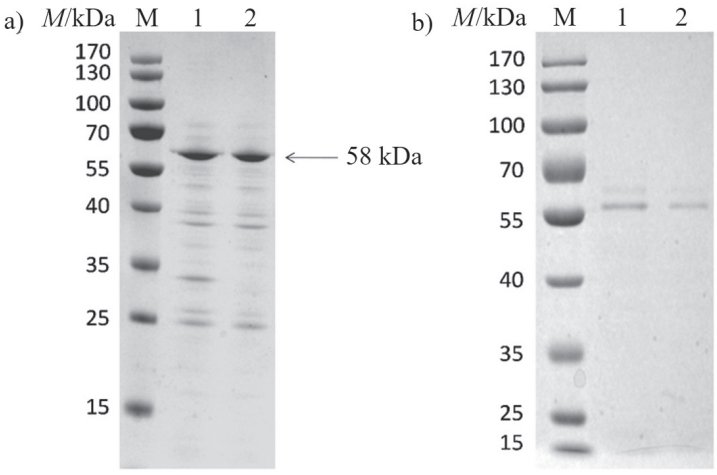

Fig. 1. SDS-PAGE analysis: a) secretion of AmyS and AmySΔR179-G180 in *Bacillus subtilis* 1A751, and b) purified AmyS and AmySΔR179-G180. Lane M=molecular mass marker, lane 1=AmyS, lane 2=AmySΔR179-G180

Effects of temperature and pH on AmyS and AmyS mutant properties

After purification of the enzymes, the enzymatic properties of AmyS and AmySΔR179-G180 were evaluated. The optimal temperature and pH for AmyS and AmySΔR179-G180 activity were assayed. As shown in **Fig. 2a**, wild type AmyS maintained biological activity within a temperature range of 50 to 90 °C (pH=6.0) and showed the highest α-amylase activity at 75 °C. The mutant AmySΔR179-G180 exhibited a similar temperature-activity curve to the wild type AmyS (pH=6.0), indicating that the mutation in AmyS had no significant effect on reaction temperature. In addition, the effect of pH on the wild type and mutant AmyS was measured (**Fig. 2b**). The wild type AmyS was active within a pH range of 4.0 to 7.5 (at 70 °C) and the optimal pH of AmyS was 6.0. However, it is worth noting that the mutant exhibited its highest activity at pH=5.5, suggesting that the optimal pH decreased by deleting R179-G180 in AmyS. Moreover, the mutant seemed to be more acid-resistant at pH=4.5-6.0. Considering that α-amylases ideally function at pH=4.5 in industry for the liquefaction of starch, the mutant AmySΔR179-G180 was, therefore, more suitable for future industrial applications.

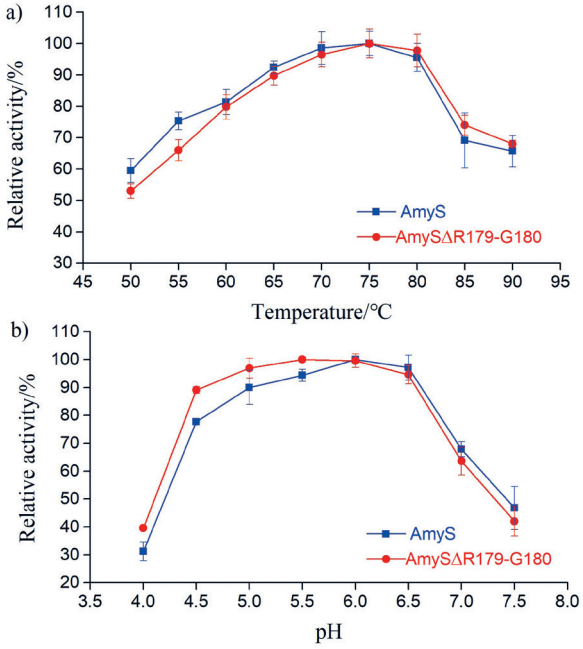

Fig. 2. Effects of: a) temperature, and b) pH on the activity of the wild type and mutant AmyS

Enhancement of AmyS thermostability by deleting residues R179-G180

In order to study the effect of deleting residues R179-G180 on AmyS thermostability, the half-lives of AmyS and AmySΔR179-G180 were determined at high temperatures. As shown in **Fig. 3**, AmyS showed a time-dependent decrease in amylolytic activity and the half-life was 24 min at 100 °C. However, AmyS mutant was found to exhibit increased thermostability and the half-life reached 33 min at the same temperature. In order to investigate the cause of the increased thermostability, we created a homology model of AmyS using Discovery Studio v. 4.1 (*15*) and compared the model with BLA (**Fig. 4**). We noticed a small extra loop containing Arg179--Gly180 located in domain B of AmyS. It has been reported

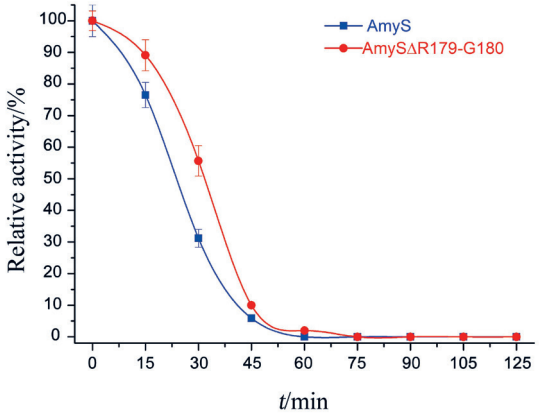

Fig. 3. Thermostability of AmyS and AmySΔR179-G180. The residual activity is shown as the relative activities after incubation at 100 °C for different times

that domain B is likely to be responsible for the differences in substrate specificity and thermostability between different α-amylases. In addition, Suzuki *et al.* (*7*) demonstrated that the thermostability of BAA was greatly improved by deletion of Arg176-Gly177, in a similar loop, along with the replacement of Ala269 with Lys269 using site-directed mutagenesis. According to sequence alignment and protein structure comparison, other α-amylases, including LAMY from *Bacillus* KSM-1378 (*8*), BStA from *B. stearothermophilus* US100 (*10*) and BACΔNC from *Bacillus* sp. strain TS-23 (*9*), also resulted in similar improvements in enzyme properties by the deletion of amino acids in the same region.

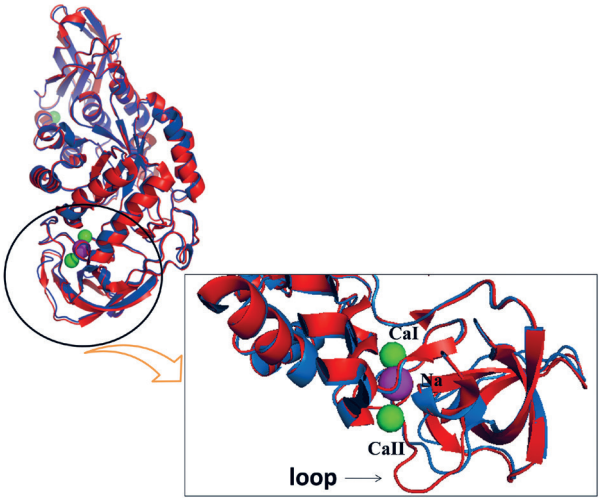

Fig. 4. Loop region with superimposition of AmyS and BLA, focusing on the loop region (Arg179-Gly180 using AmyS numbering). BLA (blue), AmyS (red), sodium ion (magenta sphere), and calcium ions (green spheres)

Decrease in AmySΔR179-G180 calcium requirements

We compared the structure of the AmyS model with that of the AmySΔR179-G180 model and found that the deletion of R179-G180 caused a slight structural rearrangement. Ali *et al.* (*10*) confirmed that the CaII site in the AmyUS100ΔI214-G215 model lost interactions with Asp105 and His238, which contributes to the maintenance of connections between the A and B domains. They also found that the deletion of I214-G215 in AmyUS100 not only increased enzyme thermostability, but also significantly decreased calcium requirements. It was thus possible that the deletion of R179-G180 affects the calcium-binding sites in AmyS. Therefore, calcium requirements of AmyS and AmySΔR179-G180 were tested through comparison of enzymatic activity of residue following incubation of the enzyme with different concentrations of calcium at 100 °C. As shown in **Fig. 5**, the maximal thermostability of AmyS (**Fig. 5a**) was obtained with 1 mmol/L of $CaCl_2$, however, the maximal thermostability of AmySΔR179-G180 (**Fig. 5b**) was obtained with only 0.25 mmol/L of $CaCl_2$. This observation confirmed the above hypothesis.

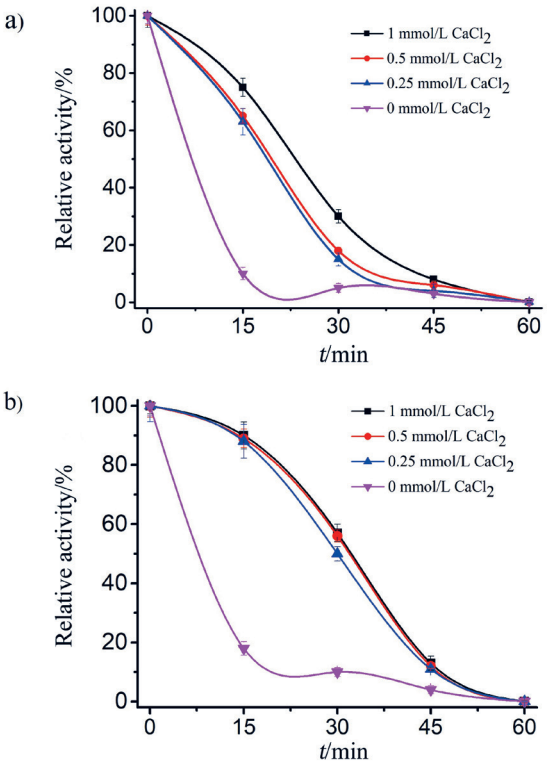

Fig. 5. Effect of R179-G180 deletion on calcium demand. Comparison of the thermostability of: a) AmyS and b) AmySΔR179-G180 at 100 °C and pH=5.5 with different $CaCl_2$ concentrations

It is well-known that α-amylases usually contain calcium-binding sites to stabilize the interface between the highly homologous domain A and the more variable domain B (*19*). Declerck *et al.* (*20*) reported that the interface was sensitive to structural disruption and confirmed that when BLA was exposed to high temperature, protein unfolding began in this region. Therefore, they speculated that any mutation that altered or reinforced stabilizing interactions in this region may influence the rate of thermal inactivation (*21*). There are several reasons to illustrate increased thermostability. Suzuki *et al.* (*7*) confirmed that increased thermostability of BAA by deletion of Arg176-Gly177 could be attributed to an increase in hydrophobicity and the charged residues changing into non-polar ones. Igarashi *et al.* (*8*) reported that the thermostability of LAMY improved with enhanced calcium binding of the enzyme. In the present study, the increased thermostability of AmySΔR179-G180 may be caused by changing the calcium-binding sites.

Production of AmySΔR179-G180 with fed-batch cultivation in a 7.5-litre fermentor

Since the AmySΔR179-G180 exhibited better enzymatic properties and was, thus, able to be applied in the industrial production of sugars, the expression efficiency of *B. subtilis* 1A751 containing the plasmid pMA5-AmySΔR179-G180 was evaluated for high density fermentation. A volume of 1 mL of frozen glycerol stock was inoculated into 100 mL of SR

medium and incubated for 18 h at 37 °C. Next, 5 % (by volume) of seed culture was inoculated into a 7.5-litre fermentor supplemented with fermentation medium. When the rate of cell growth became constant, fed-batch cultivation was initiated. Soluble starch (8 % by mass per volume) was added as the substrate at a constant flow rate until its final mass per volume ratio reached 4.0 %. For AmySΔR179-G180 production (**Fig. 6**), the maximum biomass concentration reached 32.3 g/L after 36 h. The maximum enzyme activity reached 3300 U/mL after 72 h and the productivity level reached 45.8 U/(mL·h).

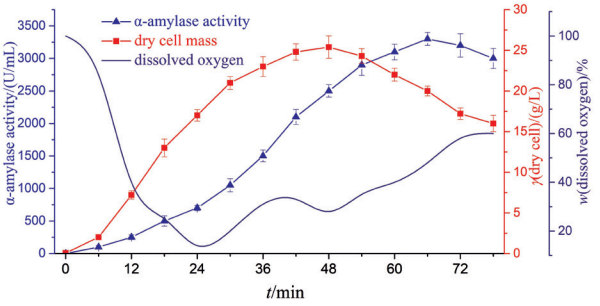

Fig. 6. Production of α-amylase in the recombinant strain 1A751 harbouring pMA5-AmySΔR179-G180 using a fed-batch strategy in a 7.5-litre fermentor

CONCLUSIONS

By deleting the amino acid residues arginine 179 and glycine 180, the thermostability of the α-amylase AmyS from *Bacillus stearothermophilus* was enhanced and the half-life at 100 °C was significantly increased. AmySΔR179-G180 acquired greater acid resistance and lower calcium requirements than the wild type AmyS to maintain α-amylase activity. Finally, we evaluated the secretory capacity of the recombinant strain in a 7.5-litre fermentor by fed-batch fermentation and observed a high level of α-amylase activity.

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