Waste and Biomass Valorization (2020) 11:6793–6802
https://doi.org/10.1007/s12649-020-00936-3

ORIGINAL PAPER

# Co-production of Amylase and Protease by Locally Isolated Thermophilic Bacterium *Anoxybacillus rupiensis* T2 in Sterile and Non-sterile Media Using Waste Potato Peels as Substrate

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Received: 28 August 2019 / Accepted: 6 January 2020 / Published online: 14 January 2020


**Abstract**
The present study investigated the potential of thermophilic bacteria isolated from hot springs to simultaneously produce protease and amylase enzymes. Among ten isolates, the strain T2 was found to be more favorable for amylase and protease. This strain was identified as *Anoxybacillus rupiensis* (GenBank number: MN252572). Potato peel powder (PPP) was used as a substrate for co-production of amylase and protease from *A. rupiensis* T2. Experiments were performed under sterile and non-sterile culture conditions. The optimal parameters for co-production of these enzymes were a PPP concentration of 60 g/L, temperature of 50 °C, initial pH of 7.0 and incubation time of 48 h. Under these culture conditions, the amylase and protease activities were determined as 64.9 and 26.2 U/mL in sterile medium. Relatively lower amylase (41.1 U/mL) and protease (14.2 U/mL) activities were attained in non-sterile medium.

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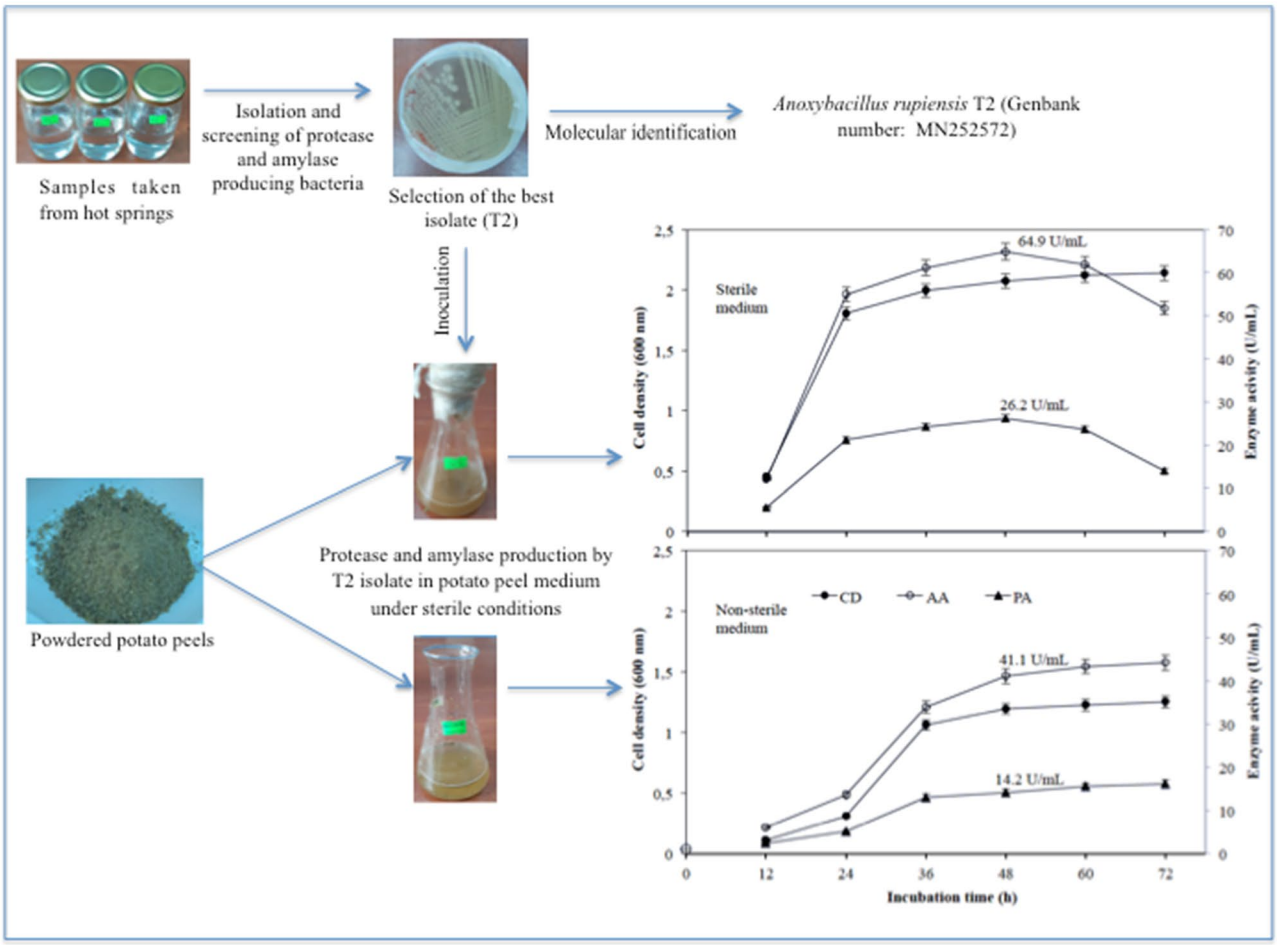
**Graphic Abstract**




**Keywords** *Anoxybacillus rupiensis* T2 · Thermophilic bacteria · Potato peel · Protease · Amylase · Co-production

## Statement of Novelty

This is the first report on the ability of *Anoxybacillus rupiensis* to produce protease or amylase. Furthermore, co-production of amylase and protease by *Anoxybacillus* species including *A. rupiensis* was demonstrated for the first time. On the other hand, waste potato peels were used for the first time as a substrate for co-production of amylase and protease.

## Introduction

Thermophilic bacteria are used as important sources of thermostable enzymes (such as amylases, proteases, lipases, cellulases, pectinases, xylanases and DNA polymerases), which find wide applications in various industries [1–5]. Especially thermostable proteases and amylases are extensively used together in detergent industry [6].

In industrial fermentation studies, cheap substrate selection is considered as an important criterion in reducing production costs. In this regard, it has been document that starch and protein-rich agricultural wastes or byproducts such as wheat straw, rice husk, sugar cane molasses can be utilized as complex substrates for co-production of amylases and proteases [7–10]. Conversely, there is no study on use of waste potato peels as substrate for co-production of amylase and protease even if this waste material has been used as a substrate for production of amylase or protease alone [11, 12]. Considering the knowledge that waste potato peels contain significant protein as well as starch [13, 14], it is possible to say that the peels may also function as a protease production substrate. On the other hand, high starch content

of potato peels may be also important to protease production because of the fact that bacterial proteases can be produced more when starch or starch-rich materials when used as carbon sources [15, 16].

Industrial production of thermostable proteases and amylases are usually carried out using thermophilic bacteria belonging to the genus *Bacillus* [17, 18]. Some bacterial species belonging to the genus *Anoxybacillus* have been also reported to possess high potential to produce amylases or proteases [19–22]. However, the potential of the species *Anoxybacillus rupiensis* to produce protease or amylase has not been investigated yet.

The simultaneous production of two or more substances in a microbial fermentation is accepted as a new technique. This technique can reduce time and energy consumption as well as labor [23]. Due to these advantages, several authors have used this technique in their studies for the simultaneous production of various microbial substances including enzymes [24–28]. However, the potential of not only the species *A. rupiensis* but also other *Anoxybacillus* species to simultaneously produce proteases and amylases has not been shown in the literature.

In summary, there are some gaps in the production of microbial proteases and amylases. The first is that there is no study on the protease or amylase production potential of the species *A. rupiensis*. The second is that the ability of the genera *Anoxybacillus* including the species *A. rupiensis* for the simultaneous production of amylases and proteases has not been investigated yet. The third is that there is no study on the use of potato peels as substrate for the simultaneous production of amylases and proteases.

Therefore, the aim of the present study to perform co-production of protease and amylase from locally isolated thermophlic bacterium *Anoxybacillus rupiensis* T2 in sterile and non-sterile media using waste potato peels as substrate.

## Materials and Methods

### Preparation of Potato Peel Powder

Potato peels were washed properly, followed by oven drying at 80 °C till constant weight. The dried peels were ground into fine particles and termed as Potato Peel Powder (PPP).

### Isolation of Thermophilic Bacteria

The bacteria used in the present study were isolated from hot springs. For this, 0.5 mL of samples taken from three different hot springs in Erzurum province (Turkey) were spread on the isolation medium (pH 8.0) containing soluble starch (10 g/L) and skim milk powder (2 g/L) as well as mineral salts (1.5 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4$, 0.01 g/L $CaCl_2$ and 0.003 g/L $FeSO_4$) and agar (20 g/L). After inoculation, the petri dishes were incubated at 55 °C. During incubation, the petri dishes were placed into plastic freezing bags to prevent drying of the media. At the end of 48-h incubation period, the colonies developing on the petri dishes were sub-cultured and purified on tyrptic soy agar (TSA) medium.

### Screening of Amylase and Protease Producing Isolates

In this stage, four bacteria were screened in terms of the ability to produce amylase and protease. The experiments were performed in 250 mL flaks containing 100 mL of the sterilized screening medium composed of 10 g/L PPP and some mineral salts (1.5 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4$, 0.01 g/L $CaCl_2$ and 0.003 g/L $FeSO_4$) (pH 8.0). To prepare the seed culture, the test bacteria activated on TSA were transferred into 250-mL Erlenmeyer flasks containing 100 mL of TSB, and the flasks were then left to the incubation in a shaker incubator at 150 rpm h at 55 °C for 24 h. One mL ($OD_{600nm} = 2.0$) of the prepared seed culture was then employed for the inoculation of the screening medium. After the flasks were inoculated, they were left to the incubation at 55 °C with agitation speed of 150 rpm. After 48 h, amylase and protease activities in the flasks were analyzed and the most productive strain was selected for subsequent experiments.

### Optimization of Culture Conditions for Amylase and Protease Production by the Best Strain

The amylase and protease production using the best strain was performed in 250 mL flasks containing 100 ml of sterilized potato peel medium (screening medium) described above. During the experiments, different PPP concentrations (10–70 g/L), temperatures (40–70 °C), pHs (4–10) and incubation times (with 12 h intervals upto 72 h) were tested to increase enzyme production. Under the optimized culture conditions, the enzyme production potential of the best srain was also tested in non-sterile PPP medium. For this purpose, PPP medium prepared in a non-sterile beaker was transferred into the non-sterile flasks. The flasks were not sterilized and directly inoculated with one mL of the seed culture. After inoculation, the flasks were not covered and left to incubation under the optimal culture conditions. To determine whether the undesired bacterial and/or fungal contamination occurred or not in the medium, 0.2 mL sample withdrawn from the culture medium at the end of appropriate incubation period was diluted and then spread on petri dishes containing TSA (pH 4.0–9.0) or potato dextrose agar (PDA) (pH 4.0–9.0). The inoculated petri dishes were then left to incubation for 24–72 h. The contamination was also examined using a microscope. In brief, 0.1 mL sample taken

from the culture medium was examined on a glass slide using a light microscope (Leica ICC50 HD) [29, 30].

### Molecular Identification of the Best Enzyme Producer Isolate

The isolation of total DNA from the best isolate was carried out by using the method described by Adiguzel et al. [31]. Amplification of 16S rRNA gene from purified genomic DNA was performed using oligonucleotide primers UNI16S-F(5′-ATTCTAGAGTTTGATCATGGCTCA-3′) and UNI16S-R (5′-ATGGTACCGTGTGACGGGCGGTGT GTA-3′). Then, it was cloned into pGEM-T Easy cloning vector (Promega, UK). As a consequence of cloning, the plasmids carrying 16 S rRNA gene region were selected and they were sent to Macrogen (Holland) for sequence analysis. The sequence obtained from Macrogen was compared with GenBank and EzTaxon data. The similarity rate between them was designated and GenBank accession number was received.

### Analysis of Enzyme Activities and Cell Growth

At the end of appropriate incubation period, one ml sample taken from culture was subjected to the centrifugation (10,000 rpm at 4 °C) and the obtained supernatant was employed as enzyme source. While amylase activity was determined, 0.1 mL of supernatant (enzyme source) was added to 1 mL of soluble starch solution (1%) prepared in 50 mM phosphate buffer (pH 7). The prepared mixture was left to the incubation at 50 °C for 10 min. Following this, 1 mL of 3,5-dinitro-salicylic acid (DNS) solution was added to the mixture, and the obtained final mixture was incubated in a water bath at 95 °C for 10 min. After the final reaction mixture was cooled to room temperature, the absorbance of the developed color (red-brown) was measured at 540 nm using spectrophotometer [32]. The standard curve was prepared using glucose. One unit of amylase activity was defined as the amount of enzyme that released 1 µmol glucose equivalent per minute from soluble starch under the assay conditions, and total amylase activity was expressed as U/mL of the fermented culture medium. Protease activity in the cultures was measured through the modification of the method described by Takami et al. [33]. In brief, 0.5 mL of supernatant was added to 2.5 mL of 0.6% casein solution in phosphate buffer (0.1 M, pH 7). The prepared mixture was incubated at 50 °C for 30 min. Following this, 2.5 mL of 0.11 M trichloroacetic acid solution was added to the mixture in order to stop the reaction. The prepared mixture was re-incubated at 50 °C for 30 min and then centrifuged at 10,000 rpm for 10 min. After the supernatant (0.5 mL) was mixed with 2.5 mL of 0.5 M $Na_2CO_3$ and 0.5 mL of 0.5 M Folin–Ciocalteau reagent, the prepared final mixture was re-incubated at 50 °C for 30 min. The absorbance of the developed blue color was measured at 660 nm using a spectrophotometer. Standard curve was prepared using tyrosine solution (0–1000 mg/L). One unit (U) of protease activity was defined as the amount of enzyme required to liberate 1 µg tyrosine per mL in 1 min under the defined assay conditions. All values were converted to U/mL.

### Statistical Analysis

Each experiment was repeated at least three times. The variance analysis was performed according to the one-way ANOVA test using SPSS 13.0 for Microsoft Windows, and the averages were compared with the Duncan test at a confidence level of 0.05.

## Results and Discussion

### Screening of Amylase and Protease Producer Isolates

Preliminary experiments were focused on isolating a thermophilic bacterium achieving the simultaneous production of protease and amylase. To do this, soluble starch and skim milk powder were used as carbon and nitrogen sources in the isolation medium, respectively. Three different hot springs were used as isolation source of enzyme producing thermophilic bacteria. Bacteria on isolation media were purified and those whose colony structure and morphology were thought to be different were selected. In this way, a total of ten thermophilic bacteria were isolated from samples taken from different hot springs. They were then screened for their amylase and protease production ability in the sterile medium containing powdered potato peels (PPP). Data presented in Table 1 demonstrates that although the maximum protease production (17.6 U/mL) was achieved in the culture medium of the strain T6, the lowest amylase activity (9.6 U/mL) was measured for the same strain. Among the tested 10 strains, the highest amylase activity (19.6 U/mL) and the second highest protease activity (12.6 U/mL) were reached in the culture medium of the strain T2 which was isolated from hot spring in Ilica district of Erzurum province (Turkey).

Since the present study focused on isolating a bacterium capable of producing both protease and amylase in high yield, the strain T2 strain was selected for subsequent experiments. The strain T2 was identified as *Anoxybacillus rupiensis* acccording to the 16S rRNA sequencing analysis. This strain (GenBank accession: MN252572) had approximately 1429 nucleotides (nt) and resembled to *Anoxybacillus* rupiensis a rate of 99% in GenBank database (Fig. 1).

**Table 1** Screening of amylase and protease producer bacteria

| Isolation source | Isolated strain | Cell growth (OD) | Amylase activity (U/L) | Protease activity (U/mL) |
|---|---|---|---|---|
| Ilıca Hot spring | T1 | 0.713 | 14.1 | 9.3 |
| | **T2** | **0.682** | **19.6** | **12.6** |
| | T3 | 0.544 | 13.2 | 9.4 |
| Pasinler Hot spring | T4 | 0.640 | 14.4 | 11.3 |
| | T5 | 0.725 | 18.4 | 6.9 |
| | T6 | 0.789 | 9.6 | 17.6 |
| | T7 | 0.690 | 14.3 | 6.9 |
| Horasan Hot spring | T8 | 0.650 | 10.2 | 11.4 |
| | T9 | 0.611 | 16.7 | 7.8 |
| | T10 | 0.733 | 13.2 | 10.9 |

Screening conditions: Temperature 55 °C, initial pH 8.0, PPP concentration 10 g/L, shaking speed 150 rpm and incubation time 24 h

Bold values indicate the T2 isolate to be used in the subsequent stages of the study and the protease and amylase activities obtained for this isolate

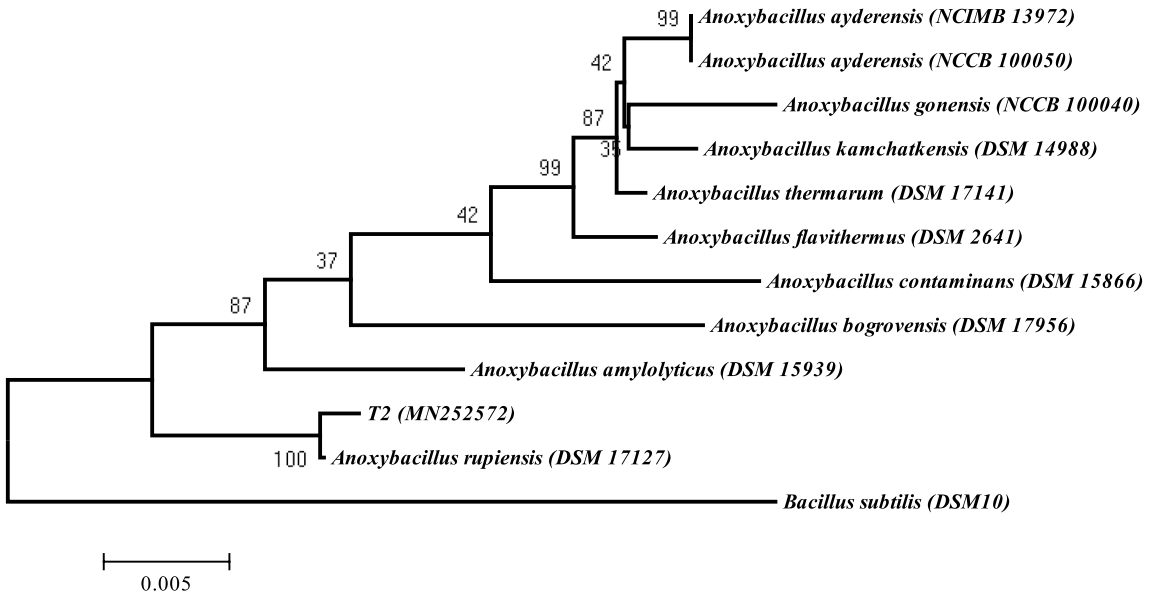



**Fig. 1** Neighbor joining phylogenetic tree on the basis of 16S rRNA gene sequence data of *Anoxybacillus rupiensis* T2 strain. Bootstrap values based on 1000 replications are listed as percentages at branching points. The accession numbers are given in parenthesis. Only bootstrap values > 50% are shown at nodes. The scale bar represented 1% divergence

## Optimization of Culture Conditions for Amylase and Protease Production by *Anoxybacillus rupiensis* T2

Different studies have shown that production of amylases and proteases is affected by a variety of physicochemical factors, including the type and composition of the substrate, incubation time and temperature, pH, agitation and the concentration and type of the carbon and nitrogen sources [7–9, 34]. Hence, in this study some culture parameters (substrate concentration, temperature, pH and incubation time) were optimized by following one-variable-at-a-time approach. Initially, different concentrations of PPP from10 to 70 g/L were tested, and it was observed that the maximal production of amylase (45.8 U/mL) and protease (20.4 U/mL) as well as the best cell growth ($1.675_{600\,nm}$) was achieved in the growth medium supplemented with 60 g/L PPP and further increase in PPP concentration gave rise to slight reduction in cell growth and enzyme production (Fig. 2). This might be due to the presence in PPP of some inhibitor compounds.

As seen from Fig. 3, although the bacterium could growth and and produce enzyme in a wide temperature range from

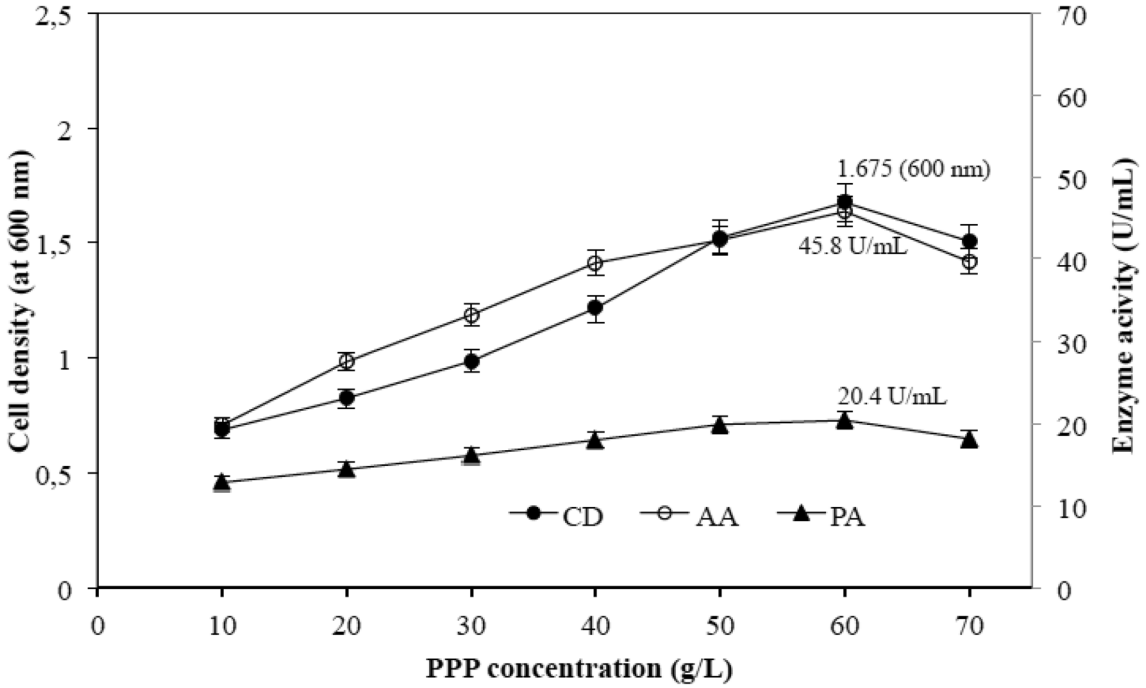



**Fig. 2** Effect of PPP concentration on cell growth and enzyme synthesis in *Anoxybacillus rupiensis* T2. Culture parameters: temperature 55 °C, initial pH 8.0, shaking speed 150 rpm and incubation time 24 h. *PPP* powdered potato peel, *AA* amylase activity, *PA* protease activity and *CD* cell density

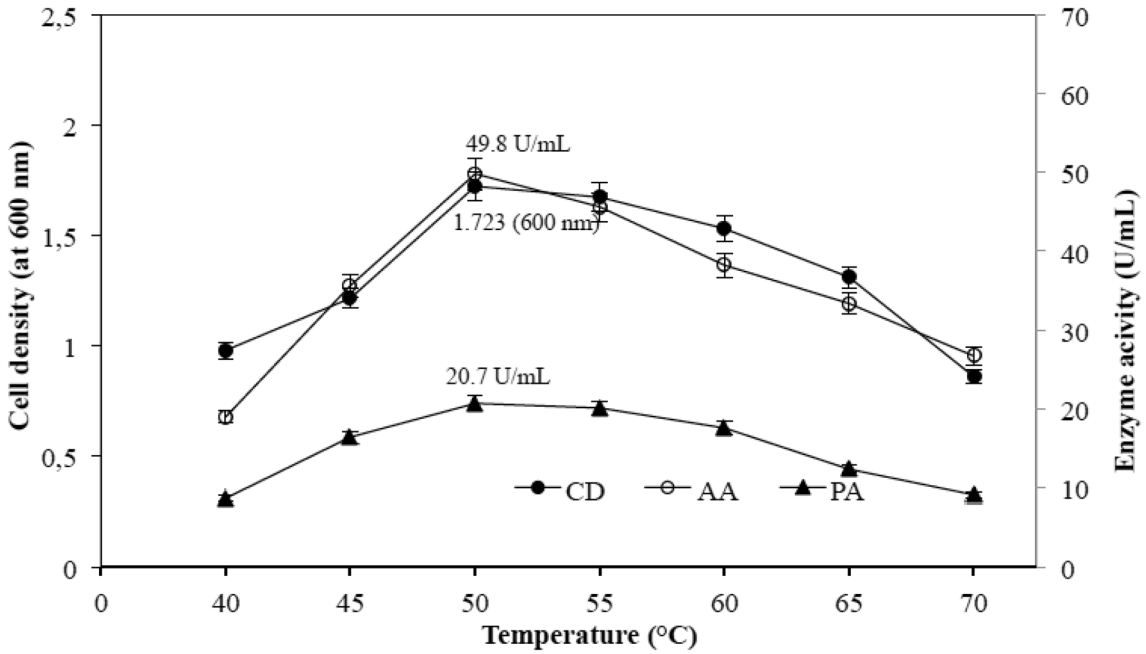



**Fig. 3** Effect of temperature on cell growth and amylase synthesis in *Anoxybacillus rupiensis* T2. Culture parameters: PPP concentration 60 g/L, shaking speed, 150 rpm, initial pH 8.0, shaking speed 150 rpm and incubation time 24 h. *AA* amylase activity, *PA* protease activity and *CD* cell density

40 to 70 °C, the amylase (49.8 U/mL) and protease (20.7 U/mL) production as well as cell growth ($1.723_{600\,nm}$) became maximum at 50 °C by followed at 55 °C. These results point that *A. rupiensis* T2 is a moderate thermophlic bacterium, since the optimal growth temperature for moderate thermophiles is accepted as 50–60 °C [35].

While the optimum pH for protease and amylase production was determined, a pH range from 4 to 10 at 50 °C was tested. Maximum amylase and protease production was achieved at pH 7.0; they were 54.7 and 21.3 U/mL, respectively. The second highest activities for protease and amylase were attained at pH 8.0. Furthermore, it was observed that the bacterium had the potential to produce protease and amylase in a wide pH range, especially pH 5–9 (Fig. 4). These results indicate that the purified enzymes of the bacterium may show high activity not only neutral pHs but also alkaline and acidic pHs.

The present experiments also focused on determining the optimal incubation duration for protease and amylase production in PPP medium. As seen from Fig. 5a, the most amylase and protease production were observed between 12 and 24 h of incubation, and the maximum activities of both enzymes could be reached at 48th h. They were 64.9 and 26.2 U/mL for amylase and protease, respectively. Incubation times over 48 h caused gradual decreases in activity of both enzymes. This result is good agreement with the fact that prolonged incubation times can reduce amounts of produced enzymes [7, 9, 29]. This decrease might be due to the exhaustion of some metal ions supporting enzyme activities as well as proteins and starch. Furthermore, long-term exposure to high temperatures could have denatured enzymes, resulting in reduced enzyme activity. On the other hand, the decrease in amylase activity might be due the proteolytic activity of the produced protease. In contrast to enzyme activities, bacterial growth showed small increases even after 48th h. This finding was attributed to the use of monomers (sugars and amino-acids) released by the hydrolytic activity of protease and amylase as growth substrates by bacterial cells.

Sterile and non-sterile culture techniques are used in microbial fermentations. In non-sterile culture technique, production medium and equipments are not subjected to a sterilization process and the culture is open to the environment during fermentation process. Since the non-sterile culture technique saves energy and time, this technique is considered to be more advantageous. In this technique, culture conditions (pH, temperature, inoculum volume) are selected which prevent unwanted contamination but make the target microorganism dominant [29, 36, 37]. For example, Smerilli et al. [38] aimed to produce lactic acid with thermophilic *Bacillus licheniformis* in a non-sterile medium prepared with potato wastes, and kept the temperature of the culture medium high in order to prevent unwanted contamination in this medium. These researchers demonstrated that the selected high temperature could hamper undesired microbial contaminants but thermophlic *B. licheniformis* could produce lactic acid at the same high temperature**.** Considering the fact that high temperature

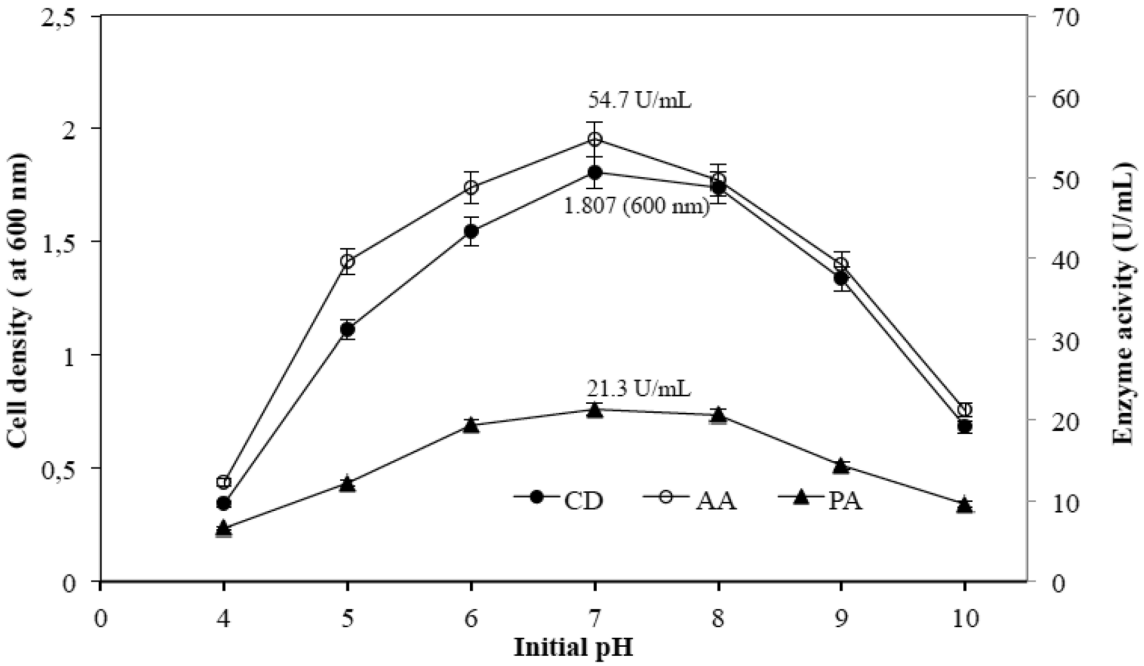



**Fig. 4** Effect of initial pH on cell growth and enzyme synthesis in *Anoxybacillus rupiensis* T2. Culture parameters: PPP concentration 60 g/L, temperature 50 °C, shaking speed 150 rpm and incubation time 24 h. *AA* amylase activity, *PA* protease activity and *CD* cell density

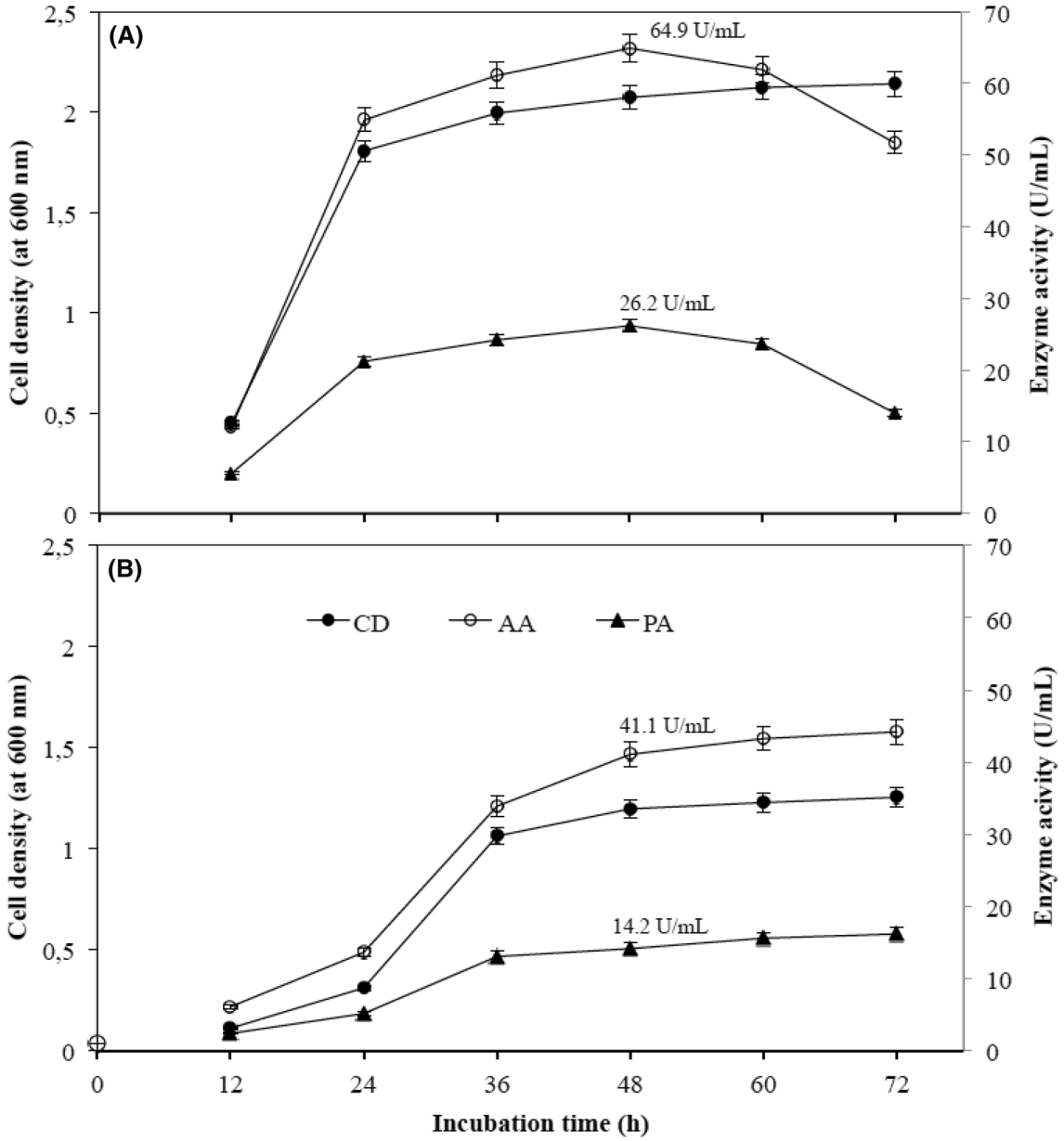



**Fig. 5** Time profile of cell growth and enzyme synthesis in sterile (**a**) and non-sterile (**b**) cultures of *Anoxybacillus rupiensis* T2. Culture parameters: PPP concentration 60 g/L, temperature 50 °C, initial pH 7.0, shaking speed 150 rpm and incubation time 24 h. *AA* amylase activity, *PA* protease activity and *CD* cell density

limits mesophilic or psychophilic contamination in the culture of thermophilic microorganisms, the ability of the thermophilic bacterium *A. rupiensis* T2 to produce amylase and protease was also investigated in a non-sterile PPP medium at 50 °C. However, contrary to expectations, low protease and amylase activities as well as poor cell growth were obtained in non-sterile culture medium during the inubation period (Fig. 4b). For example, amylase and protease activities were only 41.1 and 14.2 U/mL in non-sterile medium at 48th h, respectively. As described in methods section, the possible presence of bacterial or fungal contamination was investiagted on petri dishes or under microscope. After an icubation period of 24–72 h at 50 °C, no morphologically different bacterial or fungal colonies were observed on petri dishes (TSA or PDA media) except *A. rupiensis* T2. Moreover, during the cultivation period, only *A. rupiensis* T2 cells were detected in the culture when the culture medium was examined under a microscope. These results indicated that a temperature of 50 °C prevented undesired fungal and bacterial contamination in non-sterile PPP medium. If there is no microbial growth other than the strain T2 cells in the non-sterile PPP

medium, the question arises as to why low cell growth and enzyme activities are achieved in non-sterile medium compared to the sterile medium. The first possible answer is that PPP contain some substances that inhibit microbial growth and enzyme activities, but since these substances are broken down during the sterilization process in the autoclave, less inhibitory effects on cell growth and enzyme activities were seen in the sterilized PPP medium. For example, at the end of the first 12 h of incubation, the optical density of bacterial cells in sterilized PPP medium (Fig. 4a) was 0.450 but the optical density in non-sterile PPP medium (Fig. 4b) was only 0.112. On the other hand, the best bacterial growth in non-sterile PPP medium was observed between 24 and 36 h, whereas the bacterium in sterile PPP medium showed the best growth performance between 12 and 24 h. These results can also support that non-sterile PPP medium may contain some substances inhibiting the growth of *A. rupiensis* T2 cells. The second possibility was that the starch and protein in the structure of the peels was not sufficiently open to microbial or enzymatic attacks before autoclaving proces. But the high temperature and pressure applied during sterilization in the autoclave partially seperated the starch and protein from other components such as cellulose and lignin in the structure of the peels, making them more accessible to microbial attack. Achieving low yields under non-sterile conditions in the present study contradicts the results reported in previous studies [9, 30, 36, 39]. This contradiction may be due to the differences in the native of the selected microorganisms and/or the chemical composition of the used fermentation substrates.

## Conclusions

The present study revealed that *A. rupiensis* T2 isolated from hot spring could produce simultaneously amylase and protease enzyme on waste potato peels. By means of the optimization of the culture conditions, the production of both enzymes, in particular the amylase enzyme, can be significantly increased. It was shown that non-sterile culture technique was not suitable for amylase and protease production using potato peels as substrate and *A. rupiensis* T2 as microorganism.

**Funding** This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Complaince with Ethical Standards**

**Conflict of interest** All authors declare that there is no conflict of interest.

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