Supplementary Material

Exoproduction and biochemical characterization of a novel thermophilic serine protease from *Ornithinibacillus caprae* L9^T with hide-dehairing activity

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Methods

Selection of medium components

TSB (15 g tryptone, 5 g soy peptone, 5 g NaCl, 1 L H_2O, pH 7.2) was selected as the basal medium to optimize the production of extracellular protease from *Ornithinibacillus caprae* $L9^T$. Firstly, protease production was carried out in 250 mL Erlenmeyer flasks to evaluate the influence of varying NaCl concentrations (0, 10, 30, 50, 80, 100, 130, 150 and 170 g/L) while keeping other factors constant. Thenceforth, tryptone was replaced by glucose, sucrose, lactose, maltose, fructose, glycerol, soluble starch and yeast extract for screening carbon source. The effect of optimal carbon source concentration on protease production was studied with a range of 0–30 g/L. Similarly, organic nitrogen sources such as soy peptone, tryptone, peptone, beef extract, casein peptone as well as inorganic nitrogen sources including ammonium sulfate, potassium nitrate and urea were supplemented separately in the fermentation medium for extracellular protease production. And the nitrogen source with the most positive effect was optimized by varying of concentration from 0 to 20 g/L (at intervals of 5 g/L).

Optimization of fermentation conditions

To investigate the effect of incubation time, samples were withdrawn aseptically and monitored for caseinolytic activity at 10 different points in time: 24, 36, 48, 60, 72, 84, 96, 108, 120 h. Individually, the initial fermentation pH was adjusted to the range 5–10 (at intervals of 0.5 unit) with HCl or NaOH. And the effect of incubation temperature on protease production was measured with a range of 25 to 45 ^oC under previously optimized conditions.

Results and discussion

Statistical optimization of protease production

Selection of medium compositions

Generally, the lower yield of protease will bring huge obstacles to subsequent studies, including electrophoresis analyses, biochemical characterization and industrial applications. Thus, the fermentation parameters were optimized using the one factor at-a-time approach to allow the protease hyperproduction. In these studies, the secretion of protease was monitored by measuring the protease activity of the cell-free supernatant. As shown in Fig. S1, strain $L9^T$ was able to secrete protease at a wide range

of salinities varying from 30 to 150 g/L NaCl. The protease activity progressively increased with the increase of salt, reached a maximal value at 130 g/L NaCl, and then decreased with 150 g/L NaCl. The results are consistent with other reports, *Bacillus subtilis* BLK-1.5 (Ali et al. 2016) and *Halobacterium* sp. HP25 (Elbanna et al. 2015) showed maximum protease yield in the presence of 70 g/L and 250 g/L NaCl, respectively. This phenomenon might be attributed to the fact that these halophilic/halotolerant microbes could alter the polar lipid composition of cell membranes with increase in salt concentration, thereby further regulating the growth rate and enzyme production (Mokashe et al. 2018).

The study of protease production was investigated by culturing strain L9^T in high-salt TSB medium containing different saccharides and organic carbon sources, the results showed that glucose, sucrose, lactose, maltose, fructose and glycerol repressed production of the enzyme when compared with the control (Fig. S2a). This is probably due to the fact that saccharides could act as catabolic repressor or the synthesis of protease is inhibited when the energy status of the cell is high at the tested sugar concentration (Sharma et al. 2017; Zambare et al. 2011;). Protease production by strain L9^T in the media supplemented with soluble starch or yeast extract was significant enhanced. Many investigators (Khosravi-Darani et al. 2088; Prakasham et al. 2006) have reported that yeast extract and starch are superior to other substrates in producing proteases. In view of the operability of yeast extract, and it has been well documented as good carbon source for protease production from halophilic microorganisms (D'Alessandro et al. 2007), yeast extract was selected as the best carbon source and the optimal concentration was determined to be 15 g/L in present study.

The results for the screening of nitrogen source show that urea notablely increased the protease production with the concentration of 5 g/L (Fig. S2b). Similarly, Wang et al. (2005) indicated urea could effectively promote the protease production by *Aspergillus oryzae*. Perhaps, as a quick-acting nitrogen source, urea can be directly absorbed and used by bacteria, which is beneficial to the early growth and enzyme production of microorganism.

Optimization of fermentation conditions

The study strain was able to grow and secrete the extracellular protease over a wide range of pH (5–10). The protease production was optimum at pH 9, and decreased drastically with the increasing alkalinity above pH 9 (Fig. S3a). It is speculated that the

weak alkaline environment is more conducive for some nutrients and the protease to across the cell membrane (Mokashe et al. 2018). Usually, incubation temperature is an important physical factor, greatly affects the metabolic transactions of growing cells and protein synthesis. The optimal temperature for protease production was 37 ^{o}C (Fig. S3b), and the optimum fermentation time was concluded to be 72 h (Fig. S1).

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Table S1. Response surface experimental design factors and levels

Factor	Units	Symbol	Range of Levels		
			-1	0	1
Yeast extract	g/L	A	10	15	20
Urea	g/L	B	0	5	10
pH		C	8.5	9	9.5

Table S2. The Box-Behnken design of RSM for optimization of the protease production by *O. caprae* L9^T

Laboratory No.	A: Yeast extract	B: Urea	C: pH	Proteolytic activity (±SD, U/ml)
1	1	-1	0	157.58 ±1.50
2	0	0	0	240.32 ±1.82
3	-1	-1	0	181.19 ±1.19
4	0	-1	-1	124.25 ±2.25
5	1	0	1	60.16 ±8.59
6	1	1	0	90.52 ±4.47
7	0	1	-1	77.82 ±2.09
8	-1	0	1	163.73 ±3.59
9	0	1	1	58.97 ±6.11
10	-1	0	-1	117.70 ±4.39
11	-1	1	0	112.74 ±0.60
12	0	0	0	253.61 ±17.35
13	0	-1	1	140.12 ±3.31
14	0	0	0	250.44 ±12.03
15	0	0	0	239.92 ±5.66
16	1	0	-1	163.33 ±8.18
17	0	0	0	246.47 ±3.59

Table S3. Details of the peptide sequences with the unused ProtScore ≥ 1.3

Number	Unused ProtScore	Description	Sequence
1	10.23	S8 family serine peptidase	VFGNDPEMQYTYGDVYIK
2	10.23	S8 family serine peptidase	NYYDGGLAPYYSLDPAR
3	10.23	S8 family serine peptidase	IVDFDGAPVSPR
4	10.23	S8 family serine peptidase	KNYYDGGLAPYYSLDPAR
5	10.23	S8 family serine peptidase	FNVLDENGNLLTTLTEESDVR
6	10.23	S8 family serine peptidase	YALVQR
7	7.42	S8 family serine peptidase	SYQQVGGVWSPSPAAEGNYMIR
8	7.42	S8 family serine peptidase	LAGPIDAEAIR
9	7.42	S8 family serine peptidase	VVDDYGNETR

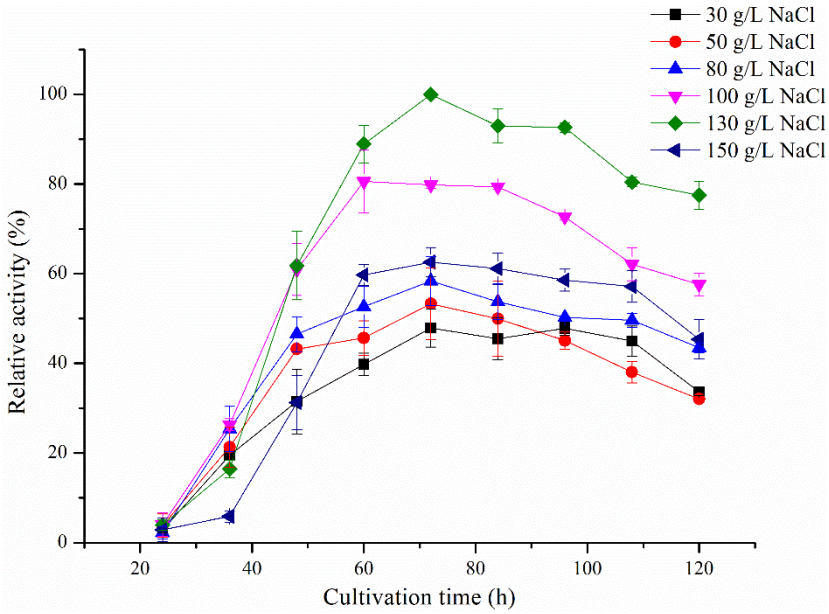

Fig. S1 Effect of fermentation period and salt concentration on the protease production of *Ornithinibacillus caprae* L9^{T}. Values are represented as mean ± standard deviation of triplicates.

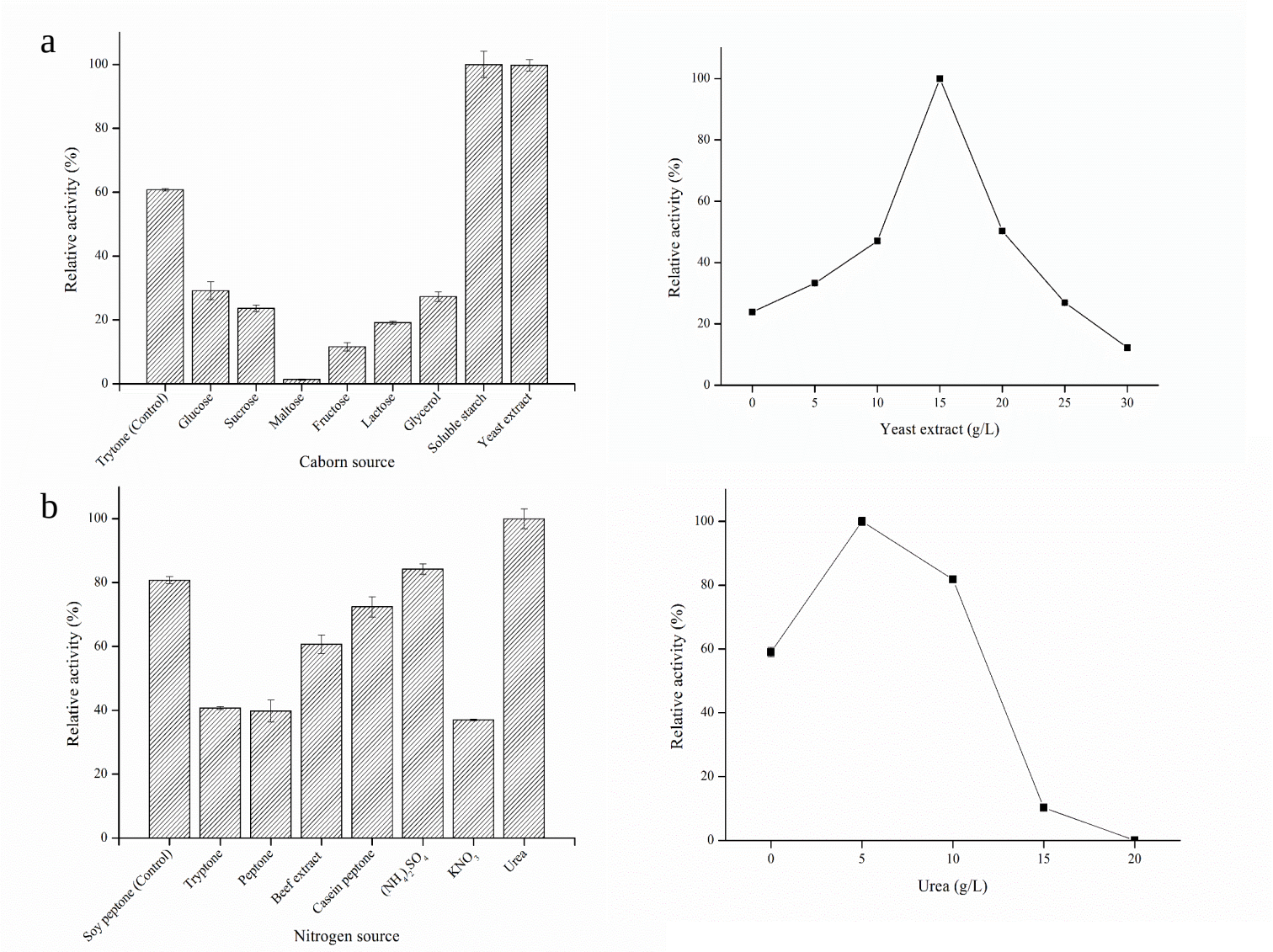

Fig. S2 Effect of nutritional parameters (a, carbon sources; b, nitrogen sources) on protease production from *O. caprae* L9^T. Values are represented as mean ± standard deviation of triplicates.

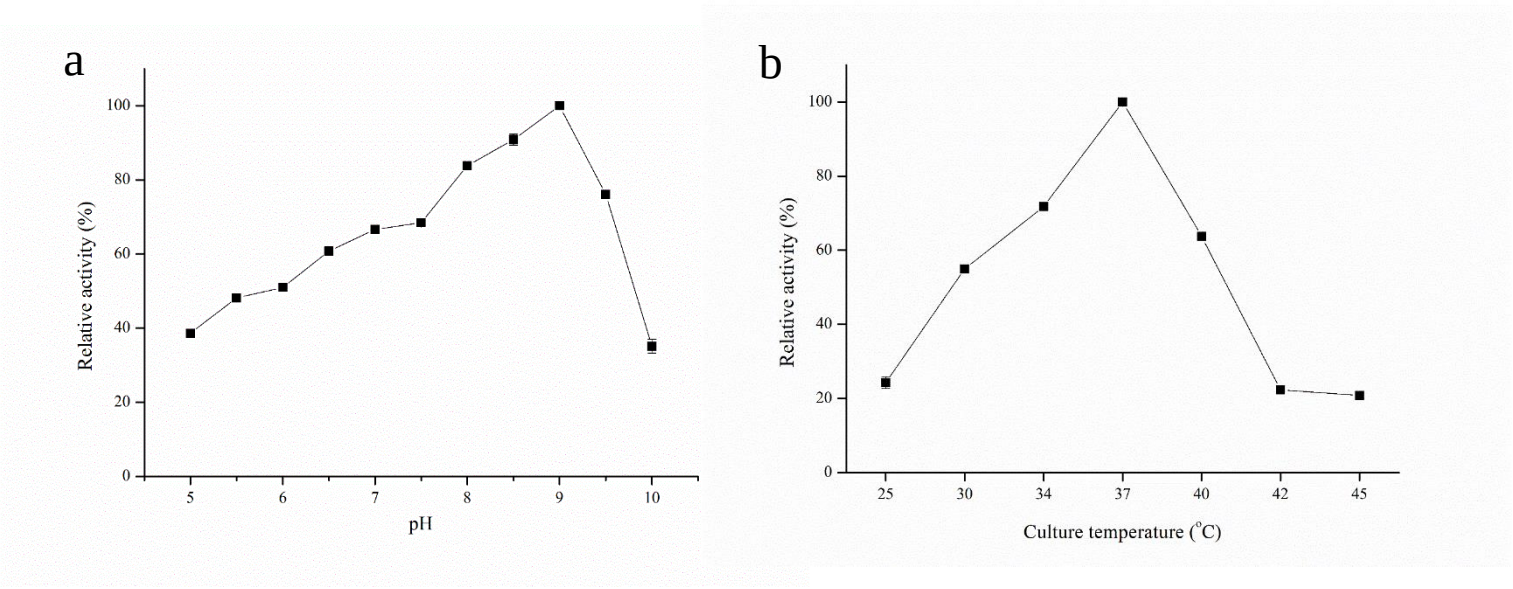

Fig. S3 Effect of fermentation pH (a) and culture temperature (b) on the protease secretion of strain *O. caprae* L9^{T}. Values are represented as mean ± standard deviation of triplicates.

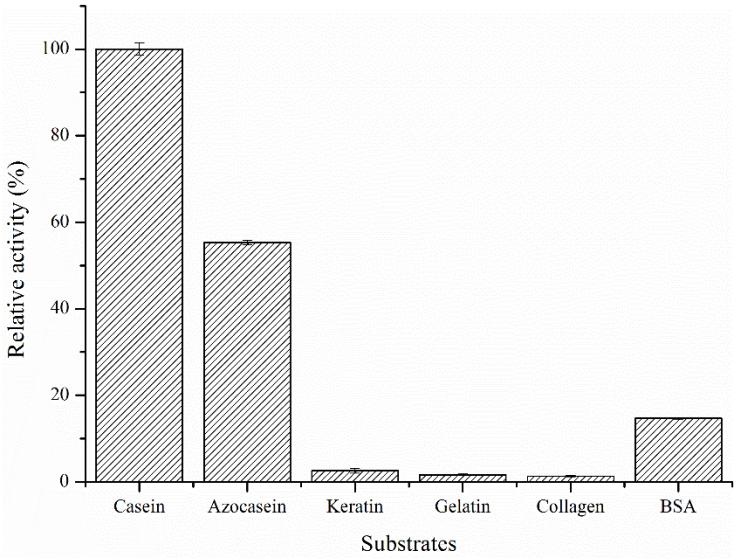

Fig. S4 Effect of substrate specificity on L9^{T} protease activity.

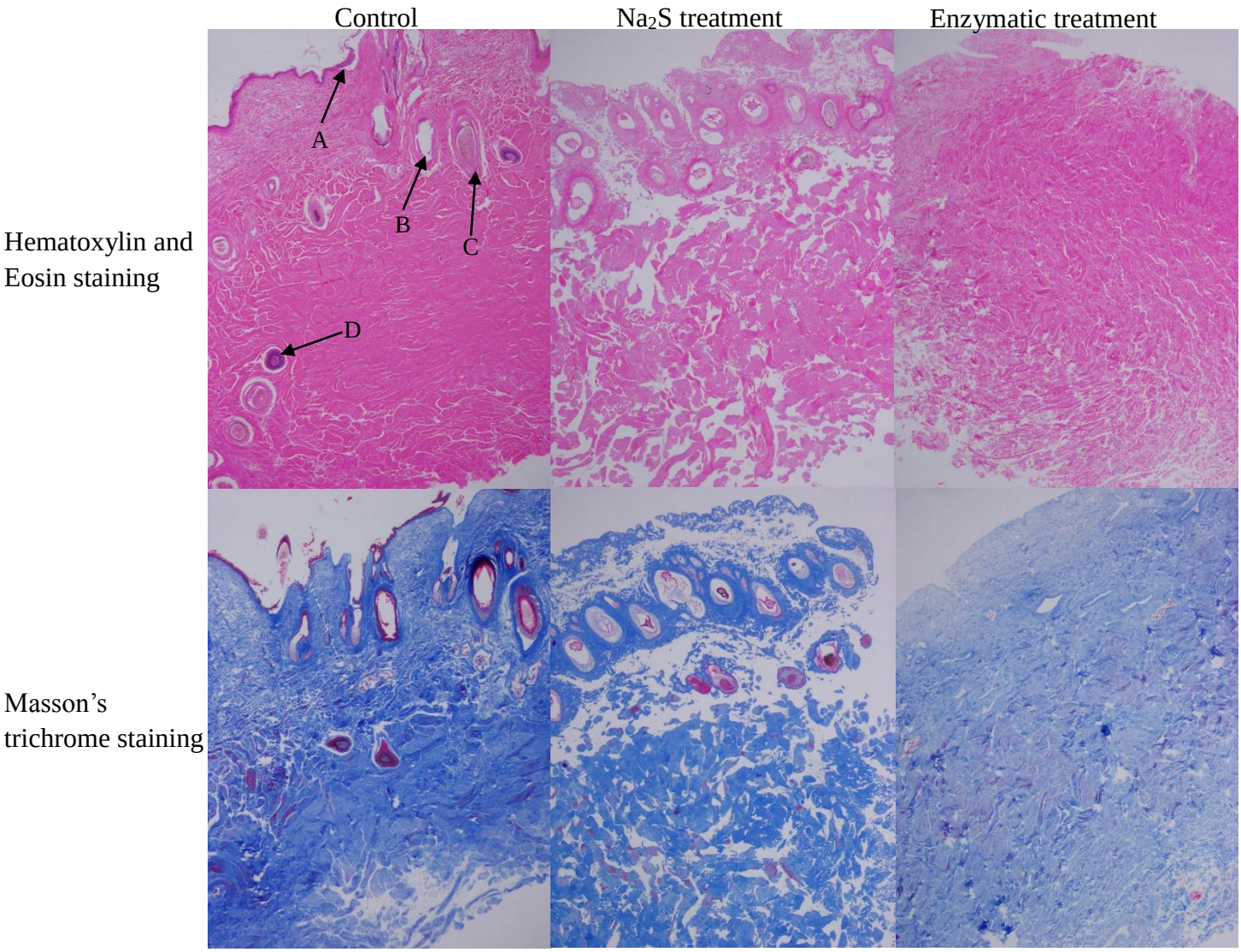

Fig. S5 Images of histology sections of dehaired goatskins in 40× stained with Hematoxylin and eosin and Masson's trichrome staining. A, epidermis; B, hair pore; C, hair follicle; D, hair shaft.