



ISSN : 2347 - 2243

Indo - American Journal of Life Sciences and Biotechnology



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Purification and Characterisation of an Alkaline Protease From *Bacillus subtilis* Isolated From Tannery Effluent

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Received: August 10, 2012 Accepted: November 18, 2012 Published: March 9, 2013

DOI: [10.62644/iajlb.2013.v10.i1.pp9-18](https://doi.org/10.62644/iajlb.2013.v10.i1.pp9-18)

ABSTRACT

Alkaline proteases from *Bacillus* species are among the most widely used industrial enzymes, and tanneries, whose effluent is rich in protein, salt, sulfide and chromium, are an obvious place to look for strains that work under harsh conditions. This narrative review asks what the published literature says about isolating *Bacillus subtilis* from such effluent, purifying its alkaline protease and characterising the enzyme, and how much confidence each step deserves. It draws on work on the subtilisin family, on *Bacillus* biology, on tannery wastewater and on enzyme methods, and it reports no new experimental data. The recurring pattern is that a clear zone on a plate and a good crude activity are easy to obtain, whereas a defensible purification table, an honest assay description and a kinetic analysis that respects its own assumptions are much rarer. Reported pH and temperature optima depend on substrate, buffer and incubation time, so numbers from different laboratories are seldom comparable. Inhibitor profiles identify the catalytic class only when controls are included, and claims of stability in detergent or in the presence of metals need matching negative controls. The review closes with a short list of what a convincing study of a tannery isolate would report.

Keywords: Alkaline protease; *Bacillus subtilis*; Tannery effluent; Enzyme purification; Subtilisin; Enzyme kinetics; Narrative review

Citation: Correia, Fauvel and Kuipers [2013]. Purification and Characterisation of an Alkaline Protease From *Bacillus subtilis* Isolated From Tannery Effluent. *Indo-Am. J. Life Sci. Biotechnol.* 10(1):9-18. DOI: [10.62644/iajlb.2013.v10.i1.pp9-18](https://doi.org/10.62644/iajlb.2013.v10.i1.pp9-18)

Article Class: Narrative Review

1. Introduction

Proteases are enzymes that cut peptide bonds, and a large share of the enzyme sold for industrial use is protease. Of these, the alkaline proteases, which work best above neutral pH, have been the

commercial workhorses for decades, mainly because of their use in laundry detergents but also in leather processing, protein hydrolysis and waste treatment (1, 2). Most of them come from species of *Bacillus*, a genus that grows readily,



secretes proteins into its medium and has a long industrial record (3).

Bacillus subtilis sits at the centre of this story. Its genome was one of the first to be completed for any bacterium (4), its secretion systems are well studied, and it is a familiar host for enzyme production. A tannery is, on the face of it, an unlikely and a promising place to look for new strains. The effluent carries protein from hides, salt from curing, sulfide from unhairing and chromium from tanning (5, 6), so an organism that grows well there has already passed a demanding screen. The same conditions, however, make the enzyme harder to interpret, because a strain that tolerates the effluent is not automatically a source of an enzyme that tolerates it.

This article reviews what the published literature says about isolating *Bacillus subtilis* from tannery effluent, producing and purifying an alkaline protease from it, and characterising the enzyme. It is not a report of a local study. No samples were collected, no enzyme was purified and no data are presented here from any particular tannery, region or laboratory; where the title of the paper points to a specific isolate, we write about the topic as it appears in the literature generally. The question we try to answer is practical: at each stage, what do published studies show, and how far should a reader trust the figures they quote? The aim is to separate what has been shown from what is routinely repeated.

2. Literature Review

2.1 Alkaline Proteases and the Subtilisin Family

Microbial proteases have been classified for decades by the chemistry of their active site, and the early surveys already described serine, cysteine, aspartic and metalloproteases as the main groups (1, 7). The alkaline proteases of *Bacillus* are

mostly serine proteases belonging to the subtilisin family, a very large superfamily whose members share a catalytic triad and a common fold (8). The family is catalogued, with its substrates and inhibitors, in the MEROPS database (9), which is now the usual reference for naming a protease and placing it among its relatives.

Subtilisins have also been among the most intensively engineered enzymes. Decades of site-directed work on stability, specificity and oxidative resistance have shown how small changes in sequence alter behaviour in detergent and at high temperature (10). That history matters for anyone isolating a new strain, because it means a protease described as novel will usually turn out to be a variant of a familiar scaffold, and the useful question is how it differs, not whether it is a subtilisin.

2.2 *Bacillus subtilis* as a Producer

Bacillus species suit industrial production for plain reasons. They tolerate simple media, form spores that survive storage, and release their proteins into the culture fluid, which saves the effort of breaking cells (3). Alkaline protease production is usually regulated by the growth phase and by the nature of the carbon and nitrogen sources, and optimisation studies have varied both together, sometimes with statistical designs (11, 12). Low-cost substrates, such as agricultural residues in solid-state culture, have been explored for the same reason (13).

One complication is that *B. subtilis* makes several extracellular proteases at once. Work on the cell-wall-associated protease, for example, showed how an enzyme made by the host can degrade other secreted proteins and so alter what is recovered (14). A crude culture fluid labelled alkaline protease activity is therefore usually a mixture, and the properties measured on it describe the mixture. Purification is the



point at which the question of which enzyme is being studied has to be answered. An enzyme assayed once under one set of conditions has been described, not characterised.

2.3 Tannery Effluent as a Source Habitat

Tannery wastewater is not a single material. It combines the liquors from soaking, liming and unhairing, from deliming and bating, and from tanning, so its composition changes through the process cycle and between plants (5). Reviews of its treatment emphasise high organic load, high salinity, sulfide and chromium as the features that complicate biological treatment (6). Protein-rich fractions from hide and hair make the effluent a natural niche for proteolytic bacteria, and the leather literature has long been interested in enzymes that could reduce the chemical load in processing (15).

Bacteria that live in such an environment meet several pressures at once. Resistance to metals involves efflux, reduction and sequestration mechanisms that are well described in general (16), and chromium-resistant isolates able to reduce chromate have been recovered from contaminated soils (17). Whether a metal-tolerant isolate secretes an enzyme that also tolerates metals is a separate question, and one that the literature rarely tests directly. The ionic strength of the liquors, the alkaline conditions of liming and the presence of sulfide each affect the stability of proteins, so an enzyme that works well in a clean laboratory buffer is not guaranteed to work in the habitat it came from. Few studies of tannery isolates test the purified enzyme under anything resembling these conditions, and the point is easy to overlook when the isolate itself grows well. Figure 3 sketches the layers of constraint that the habitat imposes.



Figure 3. Layers of constraint that tannery effluent places on an enzyme-producing isolate (schematic, not data).

2.4 Where the Enzymes Are Used

Detergents remain the largest market for alkaline proteases, and the demands are specific: activity at alkaline pH, tolerance of surfactants and builders, and stability toward oxidants (18, 19). Several well-known reports describe enzymes selected or engineered for those traits, including a protease from *Bacillus clausii* noted for stability toward oxidants and sodium dodecyl sulfate (20) and a thermostable enzyme from *Bacillus brevis* tested as a laundry additive (21). Reviews of the field from a bioindustrial viewpoint show how long this list of requirements has been stable (22). Broader accounts of alkaliphiles and their enzymes place these uses within a wider biotechnology (23, 24).

Leather processing is the field that most directly connects to a tannery isolate. Enzymatic soaking and dehairing are meant to replace part of the sulfide and lime used in conventional unhairing, and the case for them is largely environmental (15, 25). A dehairing protease from *Bacillus cereus* and a keratinolytic serine protease from *B. subtilis* have both been evaluated for this purpose (26, 27). Table 1 collects the main fields of use and the



question each one raises. A reader of an enzyme paper wants to know one thing above all: could I repeat this and get the same enzyme?

A protease must do more than hydrolyse protein: it must be cheap to make, stable on storage and compatible with the rest of the product (29), and organic-solvent-tolerant enzymes form a niche of their own (28).

Table 1. Main fields of use for microbial alkaline proteases and the questions each raises.

Field	Role of the enzyme	Main question
Detergents	Removes protein stains at alkaline pH (18, 19)	Stability with surfactant and bleach (20)
Leather	Soaking and dehairing in place of harsh chemicals (15, 25)	Selectivity: hair removal without damage to the hide
Waste and feed	Breaks down keratin and other protein waste (27)	Cost and consistency of supply
Synthesis	Peptide work in organic media (28)	Solvent tolerance
General industry	Food, pharmaceutical and other processing (24, 29)	Purity and regulatory status

Note: Qualitative summary of the cited reviews; no performance figures are given because they depend on enzyme, formulation and process.

3. Materials and Methods

3.1 Scope and Question

The review asks one question in three parts. What does the literature show about obtaining *Bacillus subtilis* from an effluent such as tannery wastewater? How is an alkaline protease from such a strain usually produced and purified? And how is it characterised, with what methods, and with what weaknesses? The questions were set out in this order because the quality of each stage limits what can be said at the next. None of these steps is hard, but each

is easy to skip when a bench is busy.

3.2 Literature Assembly

The review draws on peer-reviewed work in four areas: protease biochemistry and the subtilisin family; the biology and industrial use of *Bacillus*; the composition and microbiology of tannery wastewater; and the methods commonly used to measure, purify and characterise enzymes. Sources were chosen for relevance, standing in the field and clarity of method. Reports on other bacterial species, and on fungal or animal proteases, are used only for what they show about methods and limits, and not as evidence about *B. subtilis*.

No formal systematic search was run and none is claimed. This is a narrative synthesis, and the choice of sources reflects the authors' judgement. The practical consequence is that the review is a better guide to what a sound study should contain than to the exact value of any reported optimum, and it deliberately avoids quoting numerical results from individual papers that could not be rechecked against the primary reports for this article.

3.3 Appraisal Framework

To judge how far a report can be trusted we asked four questions of each stage. Was the material described well enough to be obtained again? Were the methods standard, and were departures stated (30, 31, 32)? Did the study include the controls needed to read its result, such as an inhibitor-free sample or a heat-treated blank? And was the conclusion no larger than the data? Where a paper was silent on a point, we treated the point as unreported and not as satisfied. The appraisal is descriptive and carries no scoring, so it should not be read as a quality ranking of any paper. Table 2 lists the purification



stages we looked for.

Table 2. Typical stages in purifying an alkaline protease and what each can and cannot show.

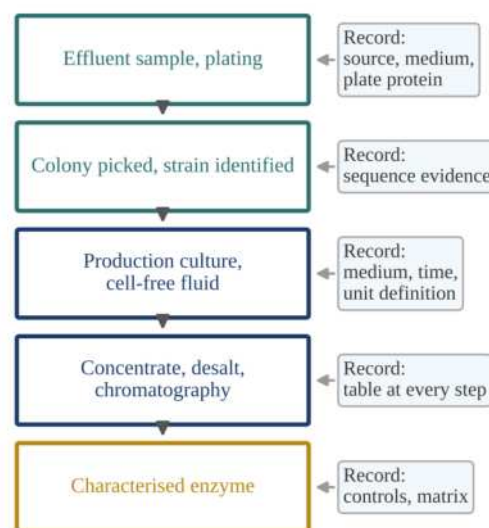
Stage	Purpose	Caution
Cell-free culture fluid	Starting material; the enzyme is secreted (3)	Other proteases may be present (14)
Salt precipitation	Concentrates protein and removes some contaminants	Enzyme can lose activity on handling
Desalting or dialysis	Removes salt before chromatography	Dilution; activity loss on long runs
Chromatography	Separates by charge or size (33, 34)	Report every step in a table
Gel check	Estimates purity and mass (35, 36)	Mass from a gel is approximate

Note: Stages are those described in the cited purification reports; their order and number vary between studies.

4. Results

4.1 From Effluent to a Purified Enzyme

The reports that describe the full path share a recognisable outline (Figure 1). An effluent sample is plated on protein agar, where clear zones, sharpened by tannic acid in some methods (37), flag protease-positive colonies. The best producer is picked, identified with sequence evidence (3, 4), grown in a production medium and separated from its cells. The cell-free fluid is concentrated, usually by salt precipitation or ultrafiltration, desalted, and passed through one or more chromatographic steps (2, 38). Several studies obtained an alkaline protease from halotolerant or haloalkaliphilic *Bacillus* in a few steps, in one case in a single step (33, 34). The number of steps is less important than whether each one is accounted for, and a short route can hide as much as a long one.



Schematic, not data

Figure 1. Outline of the path from effluent sample to a characterised enzyme and the records each stage should leave (schematic, not data).

What distinguishes a persuasive purification from a weak one is the table that accompanies it. Each stage should show total activity, total protein, specific activity, yield and fold purification, so that a reader can see where enzyme was lost and whether the final preparation is really enriched. This matters particularly for proteases, which can degrade themselves and each other during concentration and storage, and for *B. subtilis*, which makes more than one (7, 14). Read this way, a poor yield from a purification is information and not merely a disappointment.

Purity is usually judged by gel electrophoresis, most often in the denaturing system described by Laemmli (35). A single band is encouraging, though not proof. The mass read from the gel is an estimate, and subtilisin-type enzymes are often in the same size range, so mass alone does not identify the enzyme. Activity staining in a gel containing substrate, usually called zymography, adds the useful information that the band actually has



protease activity (36).

4.2 What Characterisation Usually Reports

Characterisation papers follow a familiar order: pH and temperature optima, stability, effects of metal ions and inhibitors, substrate specificity and kinetic constants (Table 3). The activity assay is most often a variant of the caseinolytic or haemoglobin method, in which acid-soluble products are measured after a fixed time (30), and protein is measured by a dye-binding or phenol-reagent method (31, 32). These choices have consequences. Substrate, buffer, incubation time and the definition of a unit all differ from paper to paper, and a unit defined one way cannot be compared with a unit defined another. The same isolate can therefore look excellent in one paper and unremarkable in the next.

Table 3. Properties usually reported for an alkaline protease and common pitfalls in reading them.

Property	How it is found	Pitfall
Activity	Release of acid-soluble products from protein (30)	Substrate and time differ between laboratories
Protein content	Colour-based assays (31, 32)	Assays respond differently to different proteins
pH and temperature optimum	Activity across a range of conditions (21, 39)	Optimum is not stability; buffer changes with pH
Kinetic constants	Rates at several substrate levels (40)	Linear plots distort errors
Inhibitor profile	Activity with class-specific inhibitors (41, 42)	Needs controls and one inhibitor per class

Note: Pitfalls are those discussed in the cited methods literature; they are not attributed to any single study.

Optimum pH and optimum temperature are the most quoted and the most

misunderstood numbers. An optimum describes the condition at which a short assay gave the highest rate, and it says nothing about how long the enzyme survives there. Stability needs its own experiment, in which the enzyme is held under the condition and residual activity is measured afterwards. Papers on *B. subtilis* proteases that combine salt tolerance with thermostability, such as the early work on a strain from a culture collection (39), illustrate why the two are reported separately. Detergent-oriented work does the same, adding tests with surfactants and oxidants (20, 21). The point is not that a number is wrong, only that it travels less far than its abstract suggests.

Inhibitors are used to place the enzyme in a catalytic class (Figure 2). Strong inhibition by a serine-protease inhibitor, with little effect from inhibitors of the other classes, fits the subtilisin family (8, 41). Two cautions apply. A single inhibitor at a single concentration is weak evidence, and some enzymes need a metal ion for stability even when the active site is serine, so a chelator can reduce activity without implying a metalloprotease. The table of inhibitor effects should therefore be read with the controls in mind.

	Protease class			
	Serine	Metallo	Cysteine	Aspartic
Serine inhibitor	strong	little	little	little
Metal chelator	little	strong	little	little
Thiol reagent	little	little	strong	little
Pepstatin-type	little	little	little	strong

Expected pattern only; schematic, not data

Figure 2. Qualitative grid of how inhibitor classes are expected to affect the main protease classes (schematic of the expected pattern, not data).



Kinetic constants are usually derived from a double-reciprocal plot (40). The approach is old and simple, and it has well-known weaknesses: the transformation magnifies the error at low substrate concentration, and a handful of points can give an unreliable line. Inhibition constants are likewise often read from graphical methods (42). Nothing is wrong with these tools when used with care, but the numbers they produce are best reported with their uncertainty, and fitted directly to the rate equation where possible.

4.3 Behaviour in Metal-Rich and Saline Conditions

Studies of *Bacillus* proteases in the presence of metal ions report a mixed picture. Some enzymes are stabilised by calcium, as is typical of the subtilisins, and some are inhibited by heavy metals, which is expected for proteins with exposed thiol or histidine groups (10, 16). For an enzyme from a tannery isolate the interesting question is chromium and salt, since both are present in the source. The literature on resistant bacteria shows tolerance in the cell (17), but cell tolerance and enzyme tolerance are different properties, and papers sometimes blur them. Salt-tolerant enzymes have been described (33, 34), but a buffer with a metal salt is not an effluent. It is a mistake that costs little to avoid and a great deal to correct once it is published.

4.4 Reading a Claim of Novelty

A reader meeting a claim that a new protease has been purified and characterised can put a short list of questions to it. How was the organism identified, and by what evidence (3)? Is there a purification table, and does the final specific activity exceed the first by a believable margin? Were the inhibitors tested with controls (8)? And is the enzyme compared with a known reference such as a commercial subtilisin, or only

with itself? A report that answers all four is uncommon, and one that answers none should be read as an observation and not as a characterisation. It is a habit worth keeping even when the answer seems obvious.

5. Discussion

5.1 Interpretation

The main finding of this review is a cautious one. Alkaline protease from *Bacillus* is a thoroughly studied enzyme class, a tannery isolate is a plausible source of it, and the methods for purifying and characterising it are standard. What the literature does not supply is a body of results comparable enough to say how much a given isolate adds. The weak point is rarely the biochemistry; it is the reporting, which leaves out the table, the control or the description that would allow a second laboratory to confirm the result.

5.2 Limitations

This is a narrative review and shares the limitations of the form. The selection of sources reflects judgement, no formal search or quality scoring was done, and the review does not quote numerical results from individual studies, since these could not be rechecked against the primary reports for this article. A reader who needs a particular optimum or kinetic constant should go to the original papers and apply the questions above. No data from any tannery are reported. Reviews of this kind are more useful when they say where the evidence stops than when they smooth it over.

5.3 Directions for Future Work

Three needs stand out. The first is complete reporting of purification, with a table at every step, the unit definition and the assay conditions, so that results can be compared (30, 31). The second is characterisation that tests the enzyme in



the matrix for which it is proposed, including effluent-like mixtures and leather or detergent trials (18, 25). The third is identification that goes beyond colony morphology, supported by sequence data and comparison with the MEROPS record of related enzymes (9). A study that did these three things would add more than another isolate with another optimum. These points apply to any newly isolated enzyme, not only to this one.

6. Conclusion

Alkaline proteases from *Bacillus subtilis* are well understood as a class, and a tannery is a reasonable place to look for new producers. The published literature supports a steady method for isolating, purifying and characterising such an enzyme, but it supports far less confidence in the numbers that those studies report, because assays, units and controls differ so much. A convincing study of a tannery isolate would identify the strain with sequence evidence, present a complete purification table, define its assay and unit, include controls for inhibitor and stability experiments, and test the enzyme in conditions that resemble the source and the intended use. That is a modest conclusion, and it is the one the literature will currently bear.

For the practitioner the message is practical and not discouraging. Isolates from harsh effluents deserve study, provided the enzyme is described in a way that others can check. For the reader of the literature, a sensible habit is to ask what each stage of a report would look like if it were wrong, and whether the paper would show it.

7. Acknowledgements

The authors thank colleagues who commented on earlier drafts of this review.

8. Funding

This work received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

9. Conflict of Interest

The authors declare no conflict of interest.

10. Author Contributions

D.C. conceived the review and wrote the sections on *Bacillus* biology and enzyme production. S.F. wrote the sections on purification and characterisation methods. M.K. wrote the sections on tannery effluent and applications. All authors revised the manuscript and approved the final version.

11. Ethics Statement

This is a review of published literature. No human participants or animals were involved and no ethical approval was required.

12. Data Availability

No new data were generated. All sources are cited in the reference list.

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