Optimization of cell permeabilization conditions for improved isolation of horseradish peroxidase from horseradish roots

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INTRODUCTION

Horseradish peroxidase (HRP) is an **important biocatalyst** widely used in biotechnology, analytical chemistry, and environmental applications. Efficient **recovery** of HRP from plant tissues **depends** strongly **on cell permeabilization,** which facilitates enzyme release while preserving catalytic activity. Although commercial HRP is obtained from roots, extraction protocols reported in the literature vary considerably and are often poorly optimized.

Ultrasound-assisted extraction (UAE) represents an attractive approach for enzyme recovery because it can enhance cell disruption while reducing extraction time. However, excessive sonication may simultaneously promote enzyme inactivation.

This study **aimed** to optimize cell permeabilization conditions to improve enzyme recovery and activity.

MATERIALS AND METHODS

Plant material

Fresh horseradish roots

Experimental design

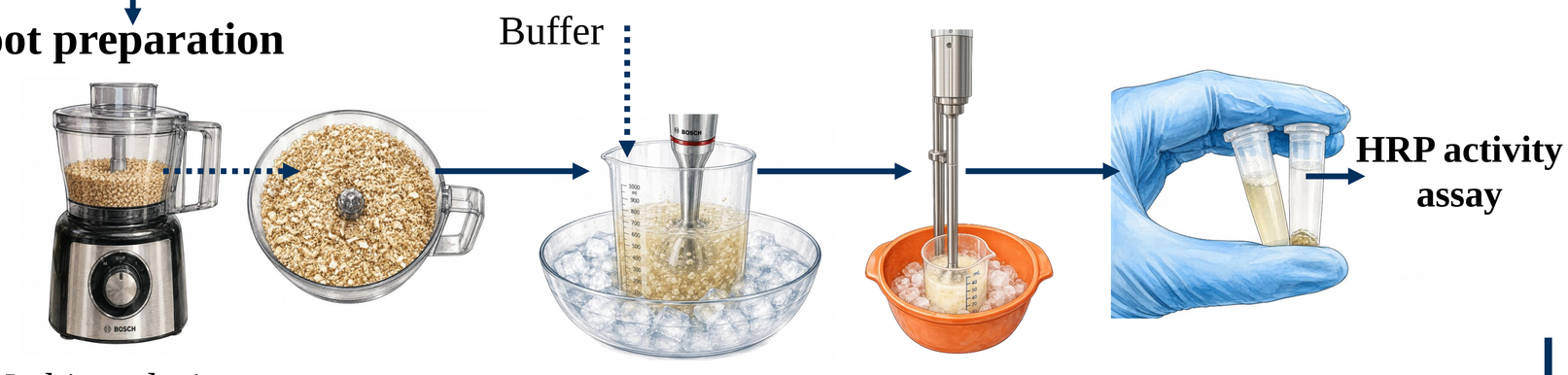
Stage I: Initial optimization (Box-Behnken design)

Conditions
- pH (X_1)
- Liquid-to-solid ratio (mL/$g_{fresh\ weight}$) (X_2)
- Homogenization time (min) (X_3)
- Ultrasound treatment time (min) (X_4)
- Ultrasound amplitude (%) (X_5)

→ **Response**: HRP specific activity (U/$g_{dry\ weight}$)

Root preparation → Washing, drying, cutting

Buffer → **Homogenization** → **Ultrasound treatment** → **Centrifugation** 14,000 rpm, 4 °C, 2 min → **HRP activity assay**



Stage II: Further optimization
- pH (X_1)
- Liquid-to-solid ratio (mL/$g_{fresh\ weight}$) (X_2)
- Homogenization time (min) (X_3)

→ **Optimal conditions**

RESULTS

Stage I

A **Box-Behnken** design comprising **56 experiments**, including center-point replicates, was used to optimize HRP extraction(Table 1). The measured specific activities showed considerable variation among the experiments, indicating that the extraction conditions strongly influenced enzyme recovery (Figure 1).

Table 1. Experimental conditions and their coded levels used in the Box-Behnken design for the optimization of HRP extraction during Stage I.

Condition	Low level (−1)	Centre level (0)	High level (+1)
pH	6	7	8
Liquid-to-solid ratio (mL/$g_{fresh\ weight}$)	5	10	15
Homogenization time (min)	0.17	0.5	0.83
Ultrasound time (min)	1	3	5
Ultrasound amplitude (%)	20	50	80

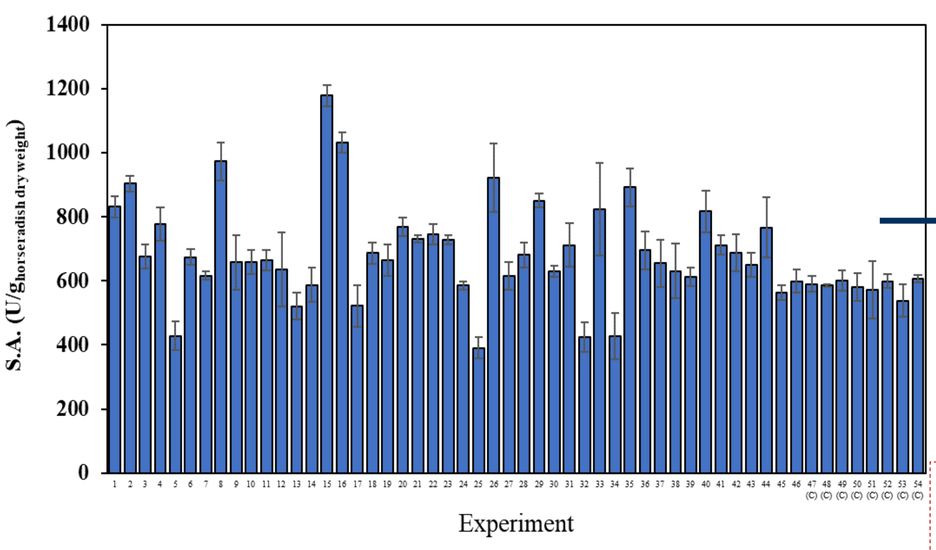


Figure 1. Effect of extraction conditions on HRP specific activity during Stage I optimization.

Effects of extraction conditions

ANOVA results

Not significant ($p > 0.05$)
- Ultrasound treatment time (min) (X_4)
- Ultrasound amplitude (%) (X_5)

Significant ($p < 0.05$)
- pH (X_1)
- Liquid/solid ratio (mL/$g_{fresh\ weight}$) (X_2)
- Homogenization time (min) (X_3)

Table 2. Optimal extraction conditions for HRP identified during Stage I.

Condition	Optimal value
pH	6
Liquid-to-solid ratio	5 mL/$g_{fresh\ weight}$
Homogenization time	0.83 min
Ultrasound time	5 min
Ultrasound amplitude	50 %

PROBLEM: The initial optimization (Table 2) revealed that the optimal values for pH, the liquid-to-solid ratio, and homogenization time were located at the boundaries of the investigated design space, suggesting that the true optimum had not yet been reached. *Therefore, a second optimization stage comprising 17 experiments and expanded factor ranges was conducted* (Table 3).

Stage II

Table 3. Experimental conditions and their coded levels used in the Box-Behnken design for the optimization of HRP extraction during Stage II.

Condition	Low level (−1)	Centre level (0)	High level (+1)
pH	5.0	5.5	6.0
Liquid-to-solid ratio (mL/$g_{fresh\ weight}$)	1	3	5
Homogenization time (min)	0.83	2.92	5.00

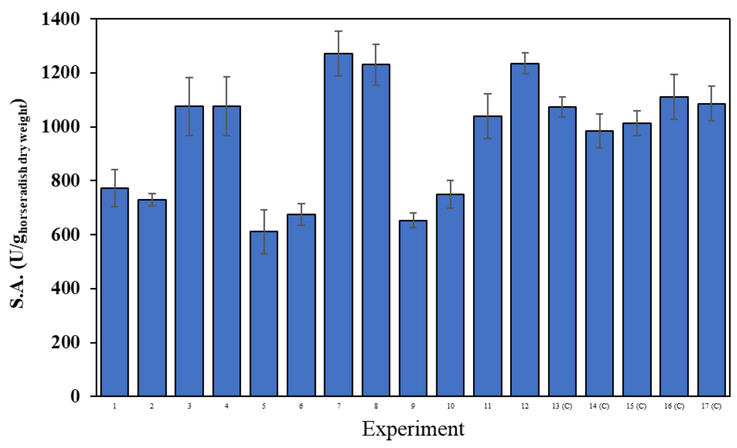


Figure 3. Effect of extraction conditions on HRP specific activity during Stage II optimization.

Table 4. Optimal extraction conditions for HRP identified during Stage II.

Condition	Optimal value
pH	5.75
Liquid-to-solid ratio	5 mL/$g_{fresh\ weight}$
Homogenization time	5 min
Ultrasound time	5 min
Ultrasound amplitude	50 %

CONCLUSION: Stage II optimization successfully identified the optimal conditions for HRP extraction (Figure 3). The highest specific activity was achieved at **pH 5.75, using a liquid-to-solid ratio of 5 mL/$g_{fresh\ weight}$, a homogenization time of 5 min, an ultrasound treatment time of 5 min, and an ultrasound amplitude of 50 %** (Table 4).

FINAL HRP SPECIFIC ACTIVITY

1617.20 ± 126.54 U/$g_{dry\ weight}$

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