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Response Surface Methodology for Enhanced Keratinase and Disulphide Reductase Production by *Bacillus subtilis* CFB-09 for Cow Hoof Biodegradation

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ABSTRACT

Keratinous wastes are resistant to biodegradation due to disulphide bonds present in keratin. Despite extensive work on keratinolytic microorganisms and keratinase involved in biodegradation, there is dearth of information on disulphide reductase responsible for breaking disulphide bonds. Hence, the need to investigate simultaneous production of keratinase and disulfide reductase by keratin degrading microorganisms, and to optimize biodegradation of keratinous wastes. In this study, *Bacillus subtilis* CFB-09 was screened for proteolytic and keratinolytic abilities using skimmed milk and cow hoof. Enzyme production was optimized using Box Benkhen Design response surface methodology (BBD-RSM) for enhanced degradation of cow hoof. Qualitative screening confirmed proteolytic and keratinolytic abilities of *B. subtilis* CFB-09 with clearance zones of 26 mm and 30 mm, respectively. Results revealed optimum enzyme production (39.37 U/mL) at 48 h of incubation of *B. subtilis* CFB-09. Optimal conditions for biodegradation of cow hoof were 37.5 °C, pH 8.0, and 1.0 % (w/v) for keratinase and 40 °C, pH 8.0, 1.5 % (w/v) for disulphide reductase, having activities of 406.04 U/mL and 139.71 U/mL, respectively. Remarkably, the best numerical solution from RSM for maximum enzyme production revealed a temperature of 35 °C, pH 8.2, and substrate concentration of 0.5 % (w/v) with desirability of 0.80. These numerical solutions yielded 526.85 U/mL and 151.14 U/mL of keratinase and disulphide reductase, respectively. The optimised conditions offer insight into enhanced degradation of keratin-rich cow hoof through improved enzymes co-production. These results provide sustainable tool for keratinous waste management, with applications in leather, detergent, and animal feed industries.

Keywords: *Bacillus subtilis*, Cow hoof, Disulphide reductase, Keratinase, Optimization, Response surface methodology

Introduction

The generation of keratinous waste caused by increased nutritional requirements of protein content is due to ever increasing human population (Shalaby *et al*., 2021). These wastes contain keratin that serve as a potent source of proteins, smaller peptides, amino acids, and nitrogen utilized in several applications such as animal feed and fertilizer production (Sharma

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& Gupta 2016; Emran *et al*., 2020). Remarkably, keratin is a tough compound containing protein found in nature that is difficult for many microorganisms to completely enzymatically degrade due to presence of large amount of cysteine and disulphide bonds (Abdelmoteleeb *et al*., 2023).

Mechanism of keratin hydrolysis involves protein unfolding, breakdown, and then transamination (Wang *et al.*, 2023). The tightly bonded structure is weakened and converted to a degraded form through the reduction of disulphide bond from cystine (-S-S-) to cysteine (-SH) (Shavandi et al., 2017). Keratin is then hydrolysed into polypeptides, short peptides, and free amino acids (Fitriyanto *et al*., 2022). Due to the potent applications of keratinase and disulphide reductase in biotechnological industries, it has become essential to explore various conditions necessary to produce novel enzymes for enhanced biodegradation (Nnolim *et al*., 2020). Reports have shown low reductive activity of keratinase in the breakdown of disulphide bond. These indicate reduction of disulphide bond as key rate-limiting step necessitating the importance of disulphide reductase in the degradative process (Ji *et al*., 2024).

Temperature, pH, size of inoculum, incubation time, carbon, and nitrogen sources are parameters crucial for keratinolytic enzyme production (Rehman *et al*., 2019). These parameters and medium composition largely influence microbial growth and metabolite production. Hence, optimization of the variables are essential for optimum enzyme yield (Elmansy *et al*., 2018). Optimization is typically carried out using 'One Variable at a Time' (OVAT) which involve several experimentations and consume time. Consequently, the development of response surface methodology statistical approaches, a faster and more concise process. Among these approaches, Central Composite and Box-Behnken Designs (BBD) have been extensively applied in biological optimization processes including enzyme production. The BBD is often employed to determine optimal condition for the various factor and analyse interaction effects using minimal experimental runs (Szpisják-Gulyás *et al.,* 2023).

Increasing demand for biodegradation of recalcitrant keratin waste has led to more studies on optimizing bacterial keratinases and disulphide reductases with optimal activity. In this study, a novel strain identified as *B. subtilis* with ability to produce extracellular enzymes such as cellulase (Olajuyigbe *et al*., 2025) with potential industrial applications was investigated for its ability to produce keratinase and disulphide reductase. The production of keratinase and disulphide reductase by *B. subtilis* CFB-09 was optimized using statistical approach of response surface methodology-Box-Behnken Design.

Materials and Methods

Qualitative Screening of *Bacillus subtilis* CFB-09 for Proteolytic and Keratinolytic Activities.

B. subtilis CFB-09, a bacterium whose genome sequence has been identified by Olajuyigbe *et al*., (2025) was gotten from the Department of Biochemistry, Enzyme and Microbial Technology Laboratory, Federal University of Technology, Akure, Nigeria. Proteolytic screening, comprised of medium of pH 7.0, 1% skimmed milk powder, 0.9% NaCl, and 2.0% agar according to Jeong *et al*., (2010). A clear zone around the colony showed proteolytic activity. The bacterium was further screened for keratinolytic potential by inoculating five microliters of the overnight grown culture using the spot plate method on modified hoof agar consisting of (g/L): K_2HPO_4 0.3; KH_2PO_4 0.4; NaCl 0.5; $MgCl_2$ 0.1; cow hoof 10; Agar 20 and pH 7.0.

Seed Culture Preparation for Keratinase and Disulphide Production

Seed culture of *Bacillus subtilis* CFB-09 was prepared by inoculating a culture into sterilized medium of peptone (5 g/L), NaCl (5 g/L), beef extract (1.5 g/L) and yeast extract (1.5 g/L) as previously described by Olajuyigbe and Ogunyewo (2018). This was incubated in a rotatory incubator at 150 rpm for 24 h ata temperature of 37 °C. Five percent (5%) of the 24

h bacterial culture was used as innoculum for further studies.

Microbial Growth and Incubation Time Determination

The seed culture was inoculated into a cow hoof medium comprising (10 g/L): $CaCl_2$ 0.22, $MgSO_4 \cdot 7H_2O$ 0.2, K_2HPO_4 0.3, KH_2PO_4 0.4, cow hoof 10.00. This was incubated at 37 °C at 150 rpm in a shaking incubator (Stuart, UK) for 5 days. Fifteen millilitre (15 mL) was aseptically collected at 24-hour intervals to determine microbial growth at 600 nm using spectrophotometer and enzyme activity for optimal incubation time (Olajuyigbe and Ogunyewo, 2018).

Keratinase Activity Assay

Keratinase activity was measured according to Abdelmoteleb *et al*., (2023). One millilitre (1 mL) each of crude enzyme and 2% substrate (casein) in 50 mM phosphate buffer (pH 7), was incubated at 40 °C for 20 min and terminated with 2 mL of 0.4 M trichloroacetic acid. The mixture was centrifuged at 12,000 rpm and 4 °C for 10 min to remove precipitated proteins. Supernatant (1 mL) was thoroughly mixed with 5 mL of 0.4 M Na_2CO_3 and 1.5 mL of Folin–Ciocalteu-phenol and kept in the dark at room temperature for 30 min. The absorbance of the developed colour was determined at 660 nm. A unit of keratinase activity was expressed as the amount of enzyme required to liberate 1 µM of tyrosine per minute under defined assay conditions.

Assay for Disulfide Reductase Activity

Disulfide reductase activity was measured by the method of Abdelmoteleb *et al*., (2023). One millitre of Ellman's reagent was mixed with 0.5 mL of the crude enzyme, and the mixture was kept at room temperature for 5 min. Absorbance of the resulting yellow 2-nitro-5-thiobenzoic acid (TNB), resulting from DTNB reduction, was measured at 412 nm confirming occurrence of thiolysis. One unit of disulfide reductase activity was defined as an increase of 0.01 in absorbance under standard conditions

Statistical optimization for keratinase and disulphide reductase from *Bacillus subtilis* CFB-09

Optimization medium was prepared in 100 ml Erlenmayer flask and autoclaved at 121 °C for 15 mins, allowed to cool and 5 % inoculum was added under aseptic condition. Optimum levels of three important variables (temperature, pH, and substrate concentration) which significantly influenced keratinase and disulphide reductase production by *B. subtilis* CFB-09 strain were determined using Box-Behnken design (Box and Behnken, 1960). Selected variables were examined in a total of the seventeen experimental runs at three different levels (−1, 0 and + 1). The experiments were designed using Design Expert software (13.0 version). Keratinase and disulphide activities were determined by coefficient of determination (R^2), analysis of variance (ANOVA), and 3D plots. A second-order polynomial equation (Eq. 1) was employed to calculate the responses and identify the significant model terms.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where: Y is the predicted response;
β_0 is constant-coefficient;
β_i is the linear coefficient;
β_{ii} is the quadratic coefficient;
βij is the interaction coefficient; and
$X_i X_j$ are the independent variables influencing the response value (Y).

Enzyme Production

Keratinase and disulphide reductase were produced from *B. subtilis* CFB-09 strain using the optimized culture conditions, temperature of 35 °C, pH 8.2, and substrate concentration of 0.5 % (w/v) and enzyme activity was measured at 660 nm.

Statistical Analysis

The experiments were carried out in duplicate, and the statistical analysis was performed using the mean ± standard deviation. Additionally, Design Expert13 software was used to implement the BBD Response

Surface Methodology (RSM) for point analysis. This software facilitated the generation of fit summaries and statistics, including, analysis of variance (ANOVA), and model graphs.

Results and Discussions

Qualitative Screening Result of Proteolytic Keratinolytic Property by *B. subtilis* CFB-09 on Agar Plates Using Hoof.

Proteolytic screening of *B. subtilis* CFB-09 on agar plate showed a significant clearance zone of 26.0 mm in diameter. In addition, keratinolytic screening of *the bacterium* using cow hoof showed clearance zone with diameter of 30.0 mm. Proteolytic bacteria exhibit clear zones around their colonies, indicating ability of a bacterium to secrete extracellular proteases to hydrolyse keratin-rich proteins present in nutrient medium into smaller peptides and amino acids (Jeong *et al*., 2010). Visible zones of clearance around bacterial growth on agar plate containing hoof as substrate indicated degradation of keratin. Similar report was observed by Gupta and Singh (2019) and Ire and Ahluwalia (2020) when keratin was used as substrate.

Effect of Cultivation Time on Enzyme Production

Keratinase from *B. subtilis* was produced over 120 h cultivation period. Optimal enzyme production was 33.97 U/mL at 48h (Figure 1). There was an exponential increase in enzyme production from 24–48 h indicating that cultivation above 48h results in decreased keratinase production. This decrease was due to factors such as nutrient depletion, accumulation of inhibitory by-products, or changes in the microorganism's physiological state (Olajuyigbe *et al*., 2018). The exact timing of keratinase production vary depending on factors such as the growth conditions, and the presence of inducing agents (Nnolim *et al*., 2020).

Figure 1. Effect of cultivation time on enzyme production

Design of Box-Behnken Showing Experimental and Predicted Values of Keratinase and Disulphide Reductase Activity Produced from *B. subtilis*

Statistical analysis of significantly independent variables; temperature (35 °C - 40 °C), pH (7 - 9), hoof meal (0.5 g - 1.5 g per 100 mL)] in 17 experimental runs showed that 404.36 U/mL was observed at 37.5 °C, 1 g/mL substrate concentration, and pH 8 for keratinase and 139.71 U/mL at 40 °C, 1.5 g/mL and pH 8 for disulphide reductase as shown in Table 1. This optimization process is essential for enhancing enzyme production efficiency, particularly in biotechnology and bioremediation applications where keratinase and disulphide reductase are crucial enzyme for biodegradation (Kumar *et al*., 2022).

The following second-order polynomial was derived through multiple regression analysis equation showing the relationship between variables for keratinase and disulphide reductase respectively regardless of significance of coefficients:

$$\mathbf{Y} = 402.35 - 18.67\,\mathbf{A} - 33.20\,\mathbf{B} - 25.73\,\mathbf{C} - 2.52\,\mathbf{AB} - 26.43\,\mathbf{AC} - 16.65\,\mathbf{BC} - 62.06\,\mathbf{A^2} - 12.27\,\mathbf{B^2} - 33.66\,\mathbf{C^2} \quad (2)$$

$$\mathbf{Y} = 72.04 + 4.60\,\mathbf{A} - 6.41\,\mathbf{B} + 5.97\,\mathbf{C} + 44.13\,\mathbf{AB} + 2.86\,\mathbf{AC} + 9.08\,\mathbf{BC} + 31.99\,\mathbf{A^2} - 12.27\,\mathbf{B^2} - 21.80\,\mathbf{C^2} \quad (3)$$

Where Y= keratinase activity/disulphide reductase activity, A= Temperature, B= pH and C= Substrate concentration.

Table 1. Box-Behnken Design (BBD) showing experimental and predicted values for keratinase and disulfide reductase production.

Std	Run	A:Temp (°C)	B:Sub. Conc. (g/mL)	C:pH	Experimental-Ker (U/mL)	Predicted Ker Activity (U/mL)	Experimental-Dsr (U/mL)	Predicted Dsr Activity (U/mL)
5	1	35	1	7	328.86	324.60	73.66	74.52
8	2	40	1	9	231.54	235.80	96.52	95.67
15	3	37.5	1	8	397.65	402.35	72.39	72.04
6	4	40	1	7	347.32	340.12	76.84	78.01
7	5	35	1	9	318.79	325.99	81.92	80.74
3	6	35	1.5	8	317.11	316.00	41.91	36.61
16	**7**	**37.5**	**1**	**8**	**406.04**	**402.35**	74.93	72.04
2	8	40	0.5	8	343.96	345.07	53.34	58.64
17	9	37.5	1	8	397.65	402.35	71.12	72.04
1	10	35	0.5	8	379.20	377.37	132.08	137.70
14	11	37.5	1	8	404.36	402.35	63.66	72.04
12	12	37.5	1.5	9	286.91	280.83	40.13	46.61
10	13	37.5	1.5	7	360.22	365.59	12.07	16.51
11	14	37.5	0.5	9	385.91	380.53	45.72	41.28
9	15	37.5	0.5	7	392.62	398.70	53.98	47.50
4	**16**	**40**	**1.5**	**8**	271.81	273.63	**139.71**	**134.08**
13	17	37.5	1	8	406.04	402.35	78.12	72.04

Regression model and analysis of variance for keratinase and disulphide reductase

ANOVA showed the model was highly significant, with interactions between all variables and influence enzyme yield for keratinase and disulphide reductase with p-values of less than 0.0001 (Table 2a and b). However, p-values for interaction of temperature with substrate concentration (AB) for keratinase and temperature with pH in disulphide reductase were non-significant ($p < 0.05$). In addition, the model had an overall F-value of 96.33 and 33.25 for keratinase and disulphide reductase, respectively. These values also indicated significant quadratic model for enzyme production. The likelihood of obtaining an F-value this high due to random noise is 0.01%. Statistical lack of fit for keratinase and disulphide reductase of

4.96 and 2.83, respectively, suggested that it is not significant relative to pure error, indicating a good model fit.

Table 2a. ANOVA of Quadratic Model for Keratinase Production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	44114.89	9	4901.65	96.33	< 0.0001	significant
A-Temp	2787.39	1	2787.39	54.78	0.0001	
B-Sub. Conc.	8819.51	1	8819.51	173.33	< 0.0001	
C-pH	5296.83	1	5296.83	104.10	< 0.0001	
AB	25.34	1	25.34	0.4979	0.5032	
AC	2793.33	1	2793.33	54.90	0.0001	
BC	1108.59	1	1108.59	21.79	0.0023	
A^2	16214.98	1	16214.98	318.67	< 0.0001	
B^2	634.08	1	634.08	12.46	0.0096	
C^2	4771.76	1	4771.76	93.78	< 0.0001	
Residual	356.18	7	50.88			
Lack of Fit	280.74	3	93.58	4.96	0.0779	not significant
Pure Error	75.44	4	18.86			
Cor Total	44471.07	16				

Table 2b. ANOVA of Quadratic Model for Disulphide Reductase Production

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	15534.67	9	1726.07	33.25	< 0.0001	Significant
A-Temp	169.57	1	169.57	3.27	0.1137	
B-Sub. Conc.	329.12	1	329.12	6.34	0.0399	
C-pH	285.02	1	285.02	5.49	0.0516	
AB	7791.24	1	7791.24	150.07	< 0.0001	
AC	32.66	1	32.66	0.6291	0.4537	
BC	329.80	1	329.80	6.35	0.0398	
A^2	4308.71	1	4308.71	82.99	< 0.0001	
B^2	634.14	1	634.14	12.21	0.0101	
C^2	2000.57	1	2000.57	38.53	0.0004	
Residual	363.42	7	51.92			
Lack of Fit	247.09	3	82.36	2.83	0.1702	not significant
Pure Error	116.33	4	29.08			
Cor Total	15898.10	16				

Model for the 3D Response Surface

Three dimension (3D) plot for interaction between the variables on keratinase and disulphide reductase production by *B. subtilis* CFB-09 is shown in Figure 2. The interactions were highly significant for keratinase but non-significant for temperature and substrate concentration, as well as temperature and pH, and for disulphide reductase. This suggests that the effect of the variables on enzyme production is strongly dependent on one another. The pH for keratinase and disulphide reductase production was at 8.22, which was consistent with the optimal pH range for many bacterial enzymes. *B. subtilis* is known to thrive in slightly alkaline conditions, with maximum enzyme production often occurring at pH levels between 7.5 and 8.5. This was supported by the findings of Nnolim *et al*. (2020), who noted that keratinase production was optimal at pH 8.0 in a *Bacillus* strain. Li *et al*. (2024) reported that keratinases from *Bacillus* species exhibit optimal activity in slightly alkaline environments, with the pH range of 7.5–8.0 being particularly favourable for keratin degradation.

The different conditions used to optimise keratinase and disulphide reductase production indicated a desirability score that reflects the best optimization. The best result occurred at 35 °C, 0.5 g/mL substrate concentration, and pH 8.22, with predicted values of 379.58 U/mL for keratinase and 135.19 U/ml for

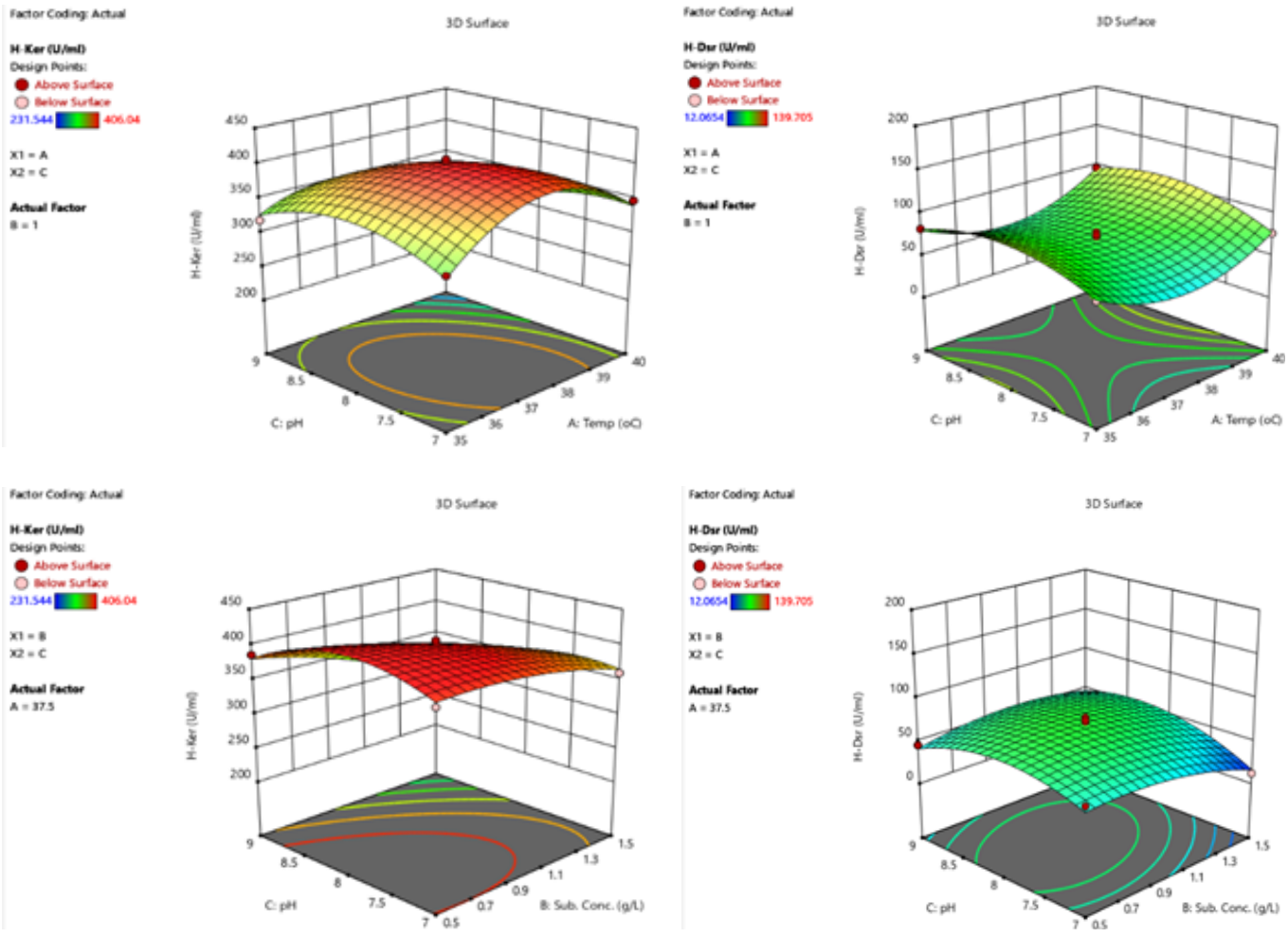

Figure 2. Response surface in three dimensions (3D) plot for temperature, substrate concentration and pH interactions for keratinase and disulphide reductase.

disulphide reductase, and a desirability score of 0.80. These numerical solutions yielded 526.85 U/ml and 151.14 U/ml of keratinase and disulphide reductase, respectively as shown in **Table 3**. This solution was considered ideal for maximizing enzyme production.

Conclusion

Optimization of production of keratinase and disulphide reductase enabled thorough investigation of effects and interactions of temperature, substrate concentration, and pH on enzyme activity. This study remarkably demonstrated potential of keratinase and disulphide reductase from *Bacillus subtilis* CFB-09 for biodegradation of keratinous waste, such as hoof, which holds significant promise for environmental and industrial applications. This research supports the emerging knowledge in the field of bioremediation and demonstrates capability of microbial enzymes for industrial applications. The enzymatic breakdown of keratinous waste through this enzymatic process, therefore offers a sustainable solution for waste management, reducing environmental pollution and potentially generating valuable by-products such as amino acids and peptides.

Table 3. Desirability table for Optimization

Number	Temp	Sub. Conc.	pH	H-Ker	H-Dsr	Desirability	
1	**35.000**	**0.500**	**8.229**	**379.579**	**135.192**	**0.801**	**Selected**
2	35.000	0.500	8.237	379.594	135.069	0.801	
3	35.000	0.500	8.221	379.561	135.327	0.801	
4	35.000	0.500	8.256	379.606	134.744	0.801	
5	35.000	0.500	8.200	379.495	135.639	0.801	
6	35.000	0.500	8.210	379.522	135.476	0.801	
7	35.000	0.502	8.179	379.364	135.835	0.800	
8	35.000	0.503	8.192	379.409	135.604	0.800	
9	35.000	0.500	8.330	379.431	133.363	0.799	
10	35.079	0.500	8.209	382.686	132.307	0.799	
11	35.083	0.500	8.252	382.911	131.426	0.799	
12	35.000	0.500	8.351	379.310	132.915	0.799	
13	35.340	0.500	8.174	392.271	122.604	0.790	
14	35.227	0.500	8.029	387.070	128.388	0.790	
15	35.393	0.500	8.103	393.829	121.404	0.787	
16	39.589	1.188	7.729	340.587	103.158	0.643	
17	37.944	0.902	7.877	406.040	72.210	0.633	

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