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Short Communication

بهبود تولید پروتئاز *کریزوباکتریوم ایندولوجنسیس* BYK27 به منظور رنگ زدایی خون از لباس ها

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چکیده

پروتئازها یکی از مهم ترین آنزیم های صنعتی هستند که حدود ۶۰ درصد از فروش جهانی آنزیم ها را به خود اختصاص داده اند. پروتئاز خارج سلولی کاربردهای زیادی در صنایع مختلف مانند چرم سازی، دترجنت، آب میوه گیری، نانوایی و صنایع گوشت دارند. با این وجود، اصلی ترین چالش تولید صنعتی آنزیم ها، میزان تولید اندک شان است. این مطالعه با هدف بهینه سازی شرایط تولید آنزیم پروتئاز و ارزیابی کاربردشان در حذف لکه های خونی انجام شد. در ابتدا، نمونه های فاضلاب بر روی محیط اسکیم میلک آگار کشت داده شدند. جدایه ای که بیشترین هاله را در اطراف کلنی داشت، به منظورمطالعات بعدی انتخاب گردید. بهینه سازی عوامل موثر در تولید پروتئاز *کریزو باکتریوم ایندوجنسیس* BYK27 با روش تاگوچی انجام پذیرفت. همچنین قدرت پاک کنندگی پروتئاز نیز با تیمار پارچه های خونی با این آنزیم مورد بررسی قرار گرفت. پارامترهای بهینه برای تولید پروتئاز *کریزو باکتریوم ایندوجنسیس* BYK27، شامل گلوکز (۱ درصد)، عصاره مخمر (۰/۰۶ درصد)، دمای ۴۰ درجه سلسیوس و pH ۹ بود. میزان تولید آنزیم پروتئاز در شرایط بهینه ۵۹۰ U/ml بود. این مقدار در مقایسه با محیط پایه معادل ۶۳ درصد افزایش داشت. فعالیت پروتئازی و پایداری آنزیم توسط بتا-مرکاپتواتانل ۵۰ درصد افزایش داشت. اما این فعالیت توسط DMF موجب مهار ۸۸ درصدی آنزیم گردید. همچنین پروتئاز سویه BYK27 قادر بود پس از ۲۰ دقیقه گرماگذاری، رنگ بری لکه‌های خونی را به طور کامل انجام دهد. نتایج این مطالعه نشان می دهد که پروتئاز تولید شده توسط سویه BYK27 قابلیت استفاده در بیوتکنولوژی به ویژه در صنایع شوینده و ساخت ترکیبات با ارزش را دارا می باشد.

واژگان کلیدی: پروتئاز، بهینه سازی، *کریزو باکتریوم ایندوجنس*، حلال های آلی.

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Short Communication

Improvement of protease production by *Chryseobacterium indologenes* BYK27 and its application in de-colorization of blood on clothes

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Abstract

Protease is one of the most important industrial enzymes occupying nearly 60% of global enzyme sales. Extracellular protease finds numerous applications in industrial processes like in leather tanning, detergents, dairy, brewery as well as meat tenderization industries. In spite of that, the low level of enzyme production is the main challenge of industrial production of enzyme. Therefore, optimization of industrial protease production and its application in blood de-staining were the aims of this study. The sewage samples were cultivated on the skim milk agar. BYK27 isolates with the highest clear halo around the colonies were selected for further studies. Optimization of parameters affecting protease production by *Chryseobacterium indologenes* BYK27 was studied by Taguchi approach. De-staining ability of protease was also investigated by de-colorization of bloody cotton cloth. The optimal factors for protease production by *Ch. indologenes* BYK27 were found to be the temperature of 40 ˚C, pH of 9.0, 0.06% yeast extract and 1% glucose supplements. Protease production under optimal condition was found to be 590 (U/ml) which was improved by 63%, as compared to the basal medium. The protease activity and stability were increased 50% by beta-mercaptoethanol but inhibited about 88% by DMF. In addition, BYK27 protease was able to completely de-stain blood after 20 min of incubation. The results of this study indicate that BYK27 protease has biotechnological potential, specifically in the detergent industry and provision of valuable compounds.

Keywords: Protease, Optimization, *Chryseobacterium indologenes,* Organic solvents.
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Introduction

Proteases are one of the three largest groups of industrial enzymes and account for approximately 60% of the total enzyme market in the world (1).

They have different applications in a wide variety of industries like food, detergent, pharmaceutical, leather and recovery of silver from used X-ray films (2). Proteases can be found in a wide variety of sources such as plants, animals, and microorganisms.

Microorganisms are preferable sources for enzyme production because of biochemical diversity and genetic manipulation potential (3). Microbial proteases can be produced in large amounts (4) and can be active and stable at different temperatures and pH conditions and also in the presence of solvents (2).

Bacilli are one of the most important thermostable and alkaline protease producers (4) and *Bacillus* species that produce extra-cellular protease are *Bacillus cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. subtilis*, and *B. megaterium* (5).

Solvent-tolerant proteases are produced by genera *Pseudomonas* species like *P. aeruginosa* (6). Fungal species found to produce alkaline protease are *Trichoderma harzianum*, *Arthrobotrys olgospora*, and *Aspergillus clavatus* (7). *Chryseobacterium* sp. is a keratinolytic protease producer that can degrade insoluble and stable proteins like a feather, hair, wool, and nail (8-12).

Enzyme production is regulated by microbial cell metabolism, which depends on media composition, metal ion, and their concentration. As well, microbial cell metabolism is also dependent on the factors controlling its environment, such as pH, incubation, temperature, aeration, and agitation during the fermentation process (13).

Therefore, to obtain the maximum enzyme production, the fermentation medium of microorganism must be optimized. There are two ways to optimize the media components: conventional and statistical.

Conventional optimization, in which altering each factor by another, is a time-consuming and hard process which does not provide the combined effects of different factors, on the other hand, statistical, approached mathematics and analytical tools (13).

This goal is supplied by the Taguchi (DOE) approach. Taguchi is applied to design experimental sets, study the interaction of various parameters and select the best condition to produce the products. In general, proteases have activity in aqueous media. However, sometimes solvent-stable proteases may become active in organic media and can produce some particular peptides. Use of this sort of protease instead of chemical agents is preferred (10).

In this study, Taguchi design of orthogonal array experimental design was used for the optimization of cultural conditions to develop protease production by *Chryseobacterium indologenes* BYK27 isolated from Kerman's dairy industry sewage.

Material and methods

Microorganism

A bacterial strain was isolated from Kerman's dairy industry sewage. Sampling was done at 6 different position of refinement system and transferred to the laboratory at -20 ˚C.

The microbial strain was biochemically and molecularly characterized as *Chryseobacterium indologenes* strain BYK27 (14). The casein and yeast extract were procured from Sigma Aldrich (USA). Skimmed milk powder was purchased from Quilab (Canada). Some other material like glucose, ammonium sulfate, Tris buffer, phosphate buffer, toluene, chloroform, methanol, butanol, cyclohexane, dimethyl formamide and 2β mercaptoethanol were purchased from Merck (Germany). Dye and reagents of molecular identification (SDS-PAGE) were procured from Bio-Rad (USA).

Molecular identification

Genomic DNA was extracted and its purity was checked by the A260/A280. Universal *16S rRNA* PCR forward primer (5'-AGTTTGATCCTGGCTCAG-3') and reverse primer (5'-GGC/T ACCTTGTTAC-GACTT-3') were used for the amplification of *16S rRNA* genes (15). PCR program was performed as follows: 94 ˚C for 5 min as initial temperature, a run of 33 cycles with each cycle consisting of 45 s at 94 ˚C, 45 s at 48 ˚C and 90 s at 72 ˚C and 5 min at 72 ˚C to permit for the extension of any incomplete products.

PCR products were electrophoresed on agarose gel (0.7%) and subsequently amplified *16S rRNA* bands were purified by DNA extraction kit (Cinaclone) and then DNA sequencing was performed on both strands directly by SEQ-LAB (Germany).

Screening of protease production

Chryseabacterium indologenes BYK27 was selected for its proteolytic activity. This was done by incubating the organisms on agar plates containing casein (1%) and skim milk powder (5%). After 24 h of incubation at 40˚C, the formation of a clear zone around the colonies was studied (15).

Cultivation conditions for protease production

Protease production from *Chryseabacterium indologenes* BYK27 was carried out in 250 ml Erlenmeyer's flask containing: Yeast extract 0.02%, Inorganic nitrogen source (NaNO3) 0.5%, NaCl 0.15%, Glucose 1% at 30 ˚C and pH 7.0. The flasks were inoculated with 25 ml of 18h culture in Nutrient broth and incubated at 40 ˚C for 48h. Agitation speed was 150 rpm. Then, the whole fermentation mixture was centrifuged at 10000 rpm and the clear supernatant was recovered (15, 16).

Protease assay

200 µl of supernatant cell-free as the crude enzyme was added to 100µl of casein 1% and incubated at 37 ˚C for 20 min. The reaction was terminated by the addition of 300 µl of TCA solution (10%). The solution was allowed to stand for 10 min in 4 ˚C. The precipitated unhydrolyzed casein was removed by centrifuging at 12000 rpm and the absorbance of the supernatant was measured at 280 nm.

The protease activity was measured in terms of tyrosine released using a tyrosine standard curve. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine under experimental conditions (17, 18).

Optimization methodology

In this study, Qualitek-4 software was used for automatic design of experiments by Taguchi

Table 1. Variables and their levels employed in Taguchi's robust design method for optimal protease production by *Chryseabacterium iumindologenes* BYK27.

Factors	Level 1	Level 2	Level 3	Level 4
Temperature	25	30	35	40
pH	6	7	8	9
Yeast extract (% w/v)	0.01	0.02	0.04	0.06
Inorganic nitrogen source (0.5 % w/v)	$NaNO_3$	NH_3SO_4	NH_4Cl	-
NaCl (% w/v)	0.05	0.1	0.15	0.2
Glucose (% w/v)	0.25	0.5	0.75	1

approach. This software could be used in L-4 to L-64 arrays and for designing experiments with 2-63 factors with 2, 3 and 4 levels for each factor.

According to Taguchi, OA which is a standard orthogonal array L32 (64) with 31 degrees of freedom, was used to examine six factors in four levels. Table 1 presents factors and levels. The decision on the factors and their levels was based on literature data used for these types of productions (13, 17, 19-20).

Experiments were done according to an experimental plan presented in Table 2. In this study, all the graphs were represented in terms of signal-to-noise ratios value of the factors that could not be controlled. The quality characteristics of the program were set as bigger is better. Based on this method, biggest S/N ratio was optimum. The analysis of variance (*ANOVA*) technique determines which factors were statistically significant.

Protease activity in the presence of organic solvents

To investigate the effect of organic solvents on protease activity, the following organic solvents were applied (40% V/V): 2ME, chloroform, diethyl ether, methanol, butanol, cyclohexane, isopropanol, toluene, dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO). The organic solvents added to reaction mixture and the protease activity was measured. The effect of following organic solvents on protease stability was studied by pre-incubating the enzyme and chemical for 1 hour at room temperature, the residual activity was determined. A control was incubated without any organic solvent (16).

Table 2. L32 orthogonal array of Taguchi design of experiments for protease production.

Trial No.	A	B	C	D	E	F	Activity (U/ml)
1	1	1	1	1	1	1	15
2	1	2	2	2	2	2	26
3	1	3	3	3	3	3	98
4	1	4	4	4	4	4	191
5	2	1	1	2	2	3	80
6	2	2	2	1	1	4	85
7	2	3	3	4	4	1	127
8	2	4	4	3	3	2	159
9	3	1	2	3	4	1	71
10	3	2	1	4	3	2	121
11	3	3	4	1	2	3	215
12	3	4	3	2	1	4	269
13	4	1	2	4	3	3	219
14	4	2	1	3	4	4	258
15	4	3	4	2	1	1	277
16	4	4	3	1	2	2	336
17	1	1	4	1	4	2	124
18	1	2	3	2	3	1	90
19	1	3	2	3	2	4	149
20	1	4	1	4	1	3	121
21	2	1	4	2	3	4	166
22	2	2	3	1	4	3	124
23	2	3	2	4	1	2	62
24	2	4	1	3	2	1	91
25	3	1	3	3	1	2	135
26	3	2	4	4	2	1	170
27	3	3	1	1	3	4	283
28	3	4	2	2	4	3	260
29	4	1	3	4	2	4	326
30	4	2	4	3	1	3	291
31	4	3	1	2	4	2	239
32	4	4	2	1	3	1	254

Wash performance of protease activity

To determine the stain elimination ability of this protease, white cotton clothes pieces (4×4 cm) were blotted with blood stain and then dried. The stain pieces were treated with phosphate buffer, detergent solution, and purified enzyme. Dimension of de-staining was determined by comparison of clothes (21).

Results

Chryseabacterium indologenes strain BYK27 created a clear zone around colony on the agar plate with casein and skim milk that showed its proteolytic activity. To find the maximum protease production by *Ch. indologenes* strain BYK27, the following parameters were optimized: temperature and pH of medium, yeast extract as organic nitrogen source (w/v %), nitrogen salts as inorganic nitrogen source (w/v %), NaCl (w/v %) and glucose (w/v %).

Each factor assigned four levels. From Table 2, it can be concluded that experiment No. 1 gave minimum protease activity and trial No. 16 gave maximum protease activity. Individually, temperature and pH showed the highest effect at level 4, yeast extract had the most influence at level 3 and glucose is effective at level 2.

The medium temperature had the strongest impact on protease production and inorganic nitrogen source and NaCl showed the least influence among the selected factors.

The order of impact of the selected optimization parameters was observed to be temperature> glucose> pH> yeast extract at the assigned levels, so temperature had an important and vital role in protease production in this bacterium.

Figure 1 shows the average effect of individual factors on protease activity. It is demonstrated that protease activity was maximum at the higher level of temperature (40 ˚C), glucose (1%) pH (9.0) and yeast extract (0.06%). However, NaCl and inorganic nitrogen source had an insignificant effect on protease activity.

Taguchi (DOE) provides an opportunity to understand the interaction between two factors. Estimated interaction severity index (SI) of the factors shows the effect of the two individual factors at various levels of interaction.

It can be deduced that inorganic nitrogen source and NaCl had the highest SI value (98.78%) followed by pH and NaCl with 72.42% SI value. In addition, the least effective factors had a major role in the production of protease compared with their contribution at individual levels, demonstrating that the effect of each factor on production depends on the level and condition of the other factors in the process. In the other words, these factors (NaCl and nitrogen salts) were effective on enzyme production, but their concentrations did not influence the process.

The total contribution of all factors was 221.125, and the current grand average performance of the experimental factors was 169.75. Increasing the temperature of the medium proved to be more effective for protease production by *Ch. indologenes* strain BYK27. Fig. 2 demonstrates that conditions suggested for optimum medium increases protease production and bacterial growth in different period times by *Ch. indologenes* strain BYK27. At 48 h hour of growth, protease activity under optimal condition increased 63% in comparison to the basal medium. At 36 h

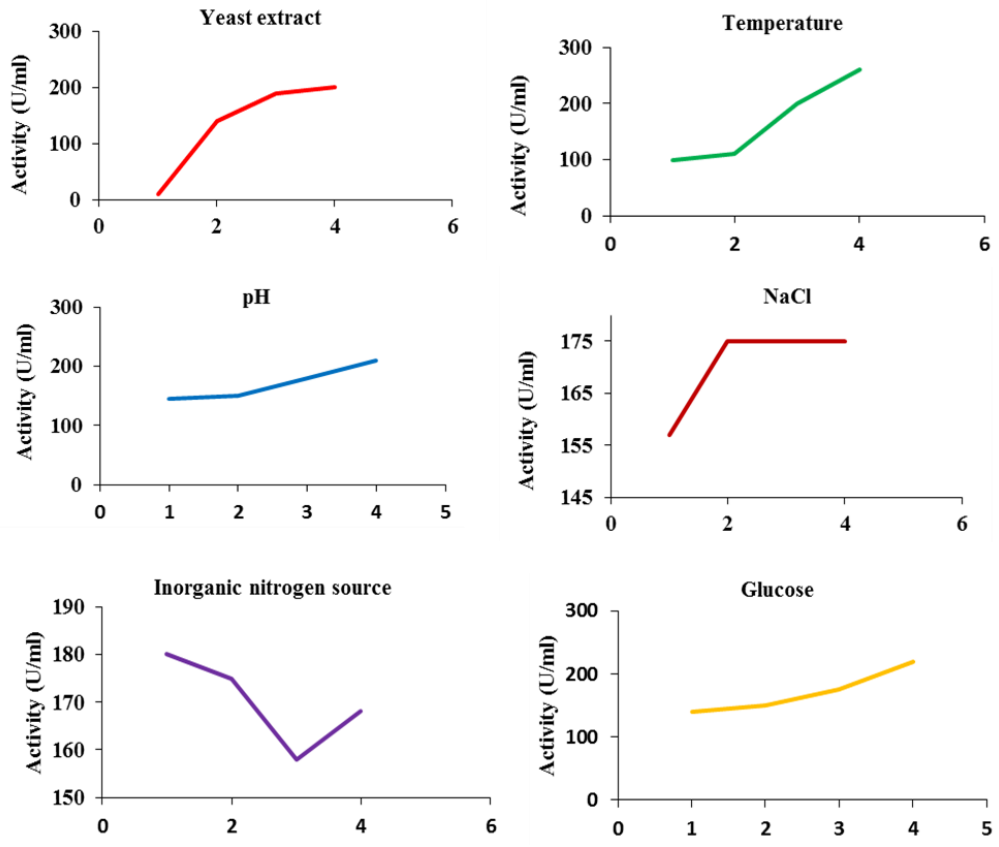

Fig. 1. Average effect of individual factors (temperature, pH, yeast extract, NaCl, glucose and inorganic nitrogen source) performance at different levels.

hour of life period, biomass production improved by about 25%.

Protease synthesis begins in late exponential stage and follows medium component and environmental factors condition (15). The different condition of the *Ch. indologenes* in comparison with other strains correlates diversity in their nature.

Effect of organic solvents

To measure protease activity, chemicals (40%) were added to reaction mixture and then

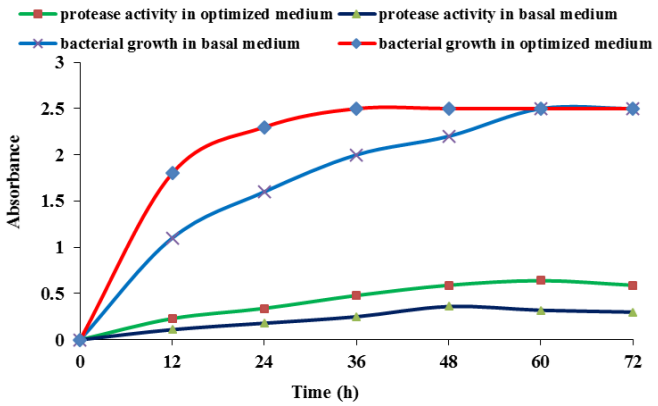

Fig. 2. Time profile protease production (280 nm) and bacterial growth (600 nm) by using *C. indologenes* BYK27 in basal medium and optimized medium.

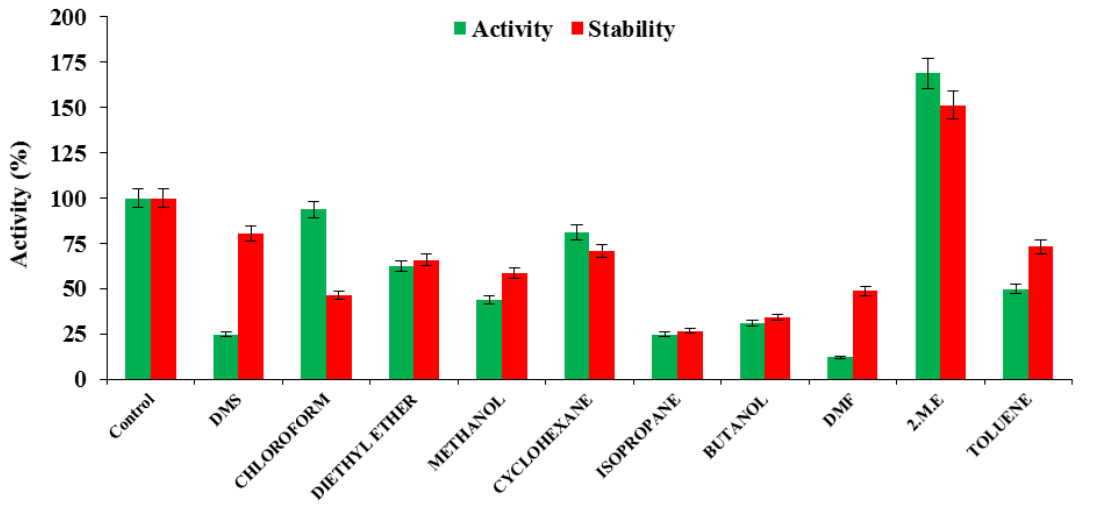

Fig. 3. Effect of organic solvents on the protease activity and stability. Each value represents the means of three experiments and the error bars indicate ± SD.

protease activity was measured. 2ME had an additive effect and increased the activity by about 68%. Other chemicals like chloroform, diethyl ether, methanol, cyclohexane, butanol, isopropanol, toluene, and DMS decreased the activity about 19-75 % and DMF showed the most inhibition about 88%.

Some enzymes were inactivated and denatured in the presence of chemicals but some organic solvents were replaced with water molecules of protein and stabilize enzyme. This stability enables the enzyme to shift the equilibrium of reversible reaction between hydrolysis and synthesis of protein to complement the hydrolysis. The enzyme which has resistance to organic solvent could be used instead of the chemical catalyzer in the synthesis reaction.

In this study, residual enzyme activity was measured after staying at 30 ˚C for 5 days in the presence of an organic solvent. The stability of protease was found 151% in the treatment with 2ME. But, other chemicals reduced the enzyme stability (Fig. 3).

Wash performance

Due to the stain removal activity, proteases can be used as additive in commercial detergents. This ability is measured by cotton cloth blotted with blood. The blotted cloth pieces were treated with phosphate buffer (pH 7.0 and 0.1 mM) as control (A), detergent (B) and detergent supplement with the crude enzyme (C) at 30 ˚C. After 20 minutes of incubation, the blood stain was completely removed (Fig. 4).

Phylogenic tree

16S rRNA gene was sequenced (1400 bp) and submitted to NCBI GenBank (accession number: KM878674). The isolate was phylogenetically characterized and indicated that the strain BYK27 belonged to *Chryseobacterium* genus and displayed the closest relationship and sequence similarity (98%) with *Ch. indologenes* (Fig. 5).

Discussion

The optimized culture condition showed an

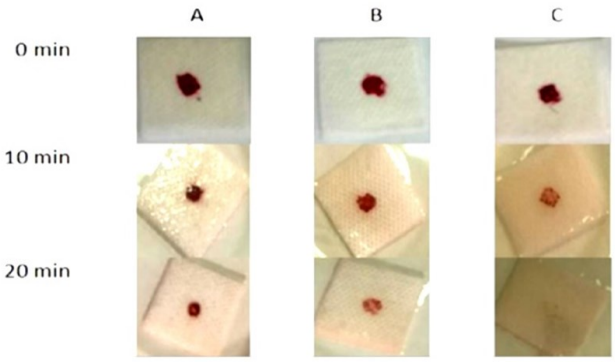

Fig. 4. Wash performance of the *Ch. indologenes* BYK27. (A) Stained cloth washed with phosphate buffer, (B) stained cloth washed with detergent only, and (C) stained cloth washed with detergent supplemented with the crude enzyme of *Ch. indologenes* BYK27.

increased enzyme production of about 63%. In this research, 28% enhancement of protease expression of *Pseudomonas aeruginosa* was reported (17).

In other research done by Prakasham, protease production improved about 55%. A recent investigation demonstrated that protease production followed the change in primary pH of the medium (19, 20). In this study, the optimal pH and temperature were maintained at 9.0 and 40 ˚C, respectively. Prakasham et al. reported that the optimal pH for protease production is 12.0 (13).

Venil and Lakshmana proved the maximum protease production occurred at 30 ˚C by Taguchi design (22). The suggested culture contained yeast extract (0.06%) and glucose (1%), whereas the optimal concentration of yeast extract for protease expression by *B. clausii* was reported (1%) (23). Similar to these findings, optimized amount of glucose was found 1% by Prakasham (13).

Wang et al. reported that ethanol (25% v/v for 4 days) decreased 30% of initial stability of TKU014 protease and isopropanol had the most negative effect on the protease stability (-90%) (11). Butanol, ethanol, and methanol decreased the protease stability of *Saccharopolyspora* (24). Annamalai et al. showed isopropanol enhanced 15% activity of *Bacillus firmus*

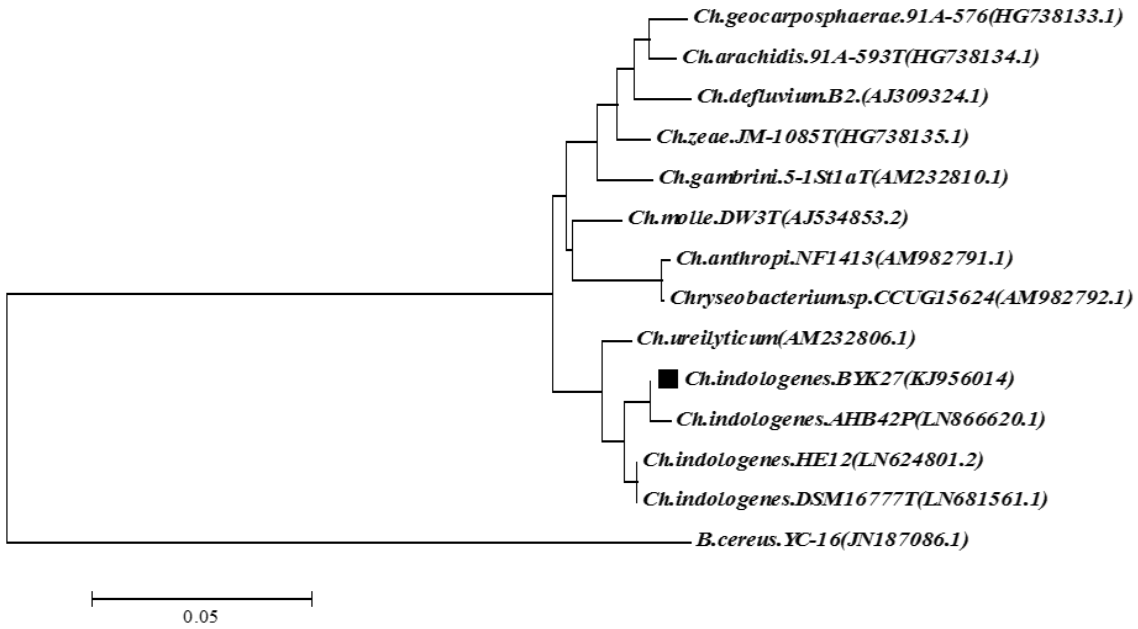

Fig. 5. Phylogenic tree was drawn by Mega5 software.

CAS7 protease (25). 2ME stimulated the protease activity due to the reduction of disulfide bridges of protein and generation more available substrate (8).

Paul et al. showed protease from *Paenibacillus* at 50 ˚C could remove blood stain (26). The purified enzyme from *Bacillus cereus* SIU1 in combination with a detergent eliminated stain at 30 minutes (21).

Subbarao et al. studied an alkaline protease from *Bacillus circulans* and demonstrated it was able to remove blood stain alone and with detergent at 30 minutes (27). Our results proved worthiness use of BYK27 protease in the detergent industry.

Chryseabacterium indologenes strain BYK27 isolated from Kerman's dairy industry sewage and its proteolytic activity was determined by clear zone on casein and skim milk agar plate. The maximum protease activity was found with optimization conditions including yeast extract (0.06%), glucose (1%), temperature (40 ˚C) and pH (9.0); different concentrations of NaCl solution and inorganic nitrogen source had a poor effect on the protease activity.

Optimum medium increased the enzyme activity to 60% more than the basal medium. Taguchi experimental design investigated individual effect and combined parameters' effect on the protease production. The temperature was the most significant factor followed by glucose and pH. Interaction between inorganic nitrogen source and NaCl had highest SI value (98.78%); interaction between pH and NaCl was the next high SI value (72.42%). Due to its resistance to organic solvent like 2ME, it can be used in the production of feedstuff, fertilizer and rare amino acids like proline, serine, and cysteine; it is a suitable alternative for hazardous chemicals in the peptide synthetic and treatment of aviculture effluent including feather and leather process with low pollution and eco-friendly characteristic.

Conclusion

Although bacterial protease finds numerous applications in industrial processes, low level of enzyme production was the main challenge of using the enzyme. Taguchi experimental design investigated the individual effect and combined parameters' effect on the protease production. In this study, under optimal condition protease production was increased 63 %, in comparison to the basal medium. It is mentioned that, based on results of wash performance, this protease can be used as a complement to the current detergent, for improving the quality of wash performance.

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