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Full Length Research Paper

Production of alkaline protease by *Bacillus cereus* and *Bacillus polymixa* in new industrial culture mediums and its immobilization

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After amylases, proteases are the most useful industrial enzymes that are produced 500 tones annually. In industry, proteases are produced from bacteria and fungi. The alkaline proteases, with an optimum activity in pH: 9 - 11, have numerous applications in daily life of peoples such as food complementary of beast and poultry, confectionary, bakery, fermentation industries, leathering, detergent industry, biotransformation and so on. In this research a wild strain of *Bacillus cereus* and *Bacillus polymixa*, isolated from the soils of Isfahan (Iran), showed the best alkaline protease activity in industrial mediums such as sweet sorghum extract and molasses. The maximum alkaline protease activity in productive culture mediums after 72 h in 30°C and with100 R.P.M. were measured. The results with *B. polymixa* were 46.7 (u/ml) in T.S.B, 57 (u/ml) in N.B, 60.22 (u/ml) in peptone solution, 67 (u/ml) in sweet sorghum solution, 216 (u/ml) in yeast extract solution, 358 (u/ml) in glucose solution, 413 (u/ml) in casamino acid solution, 418 (u/ml) in whey, 470.5 (u/ml) in casein solution, 490.5 (u/ml) in molasses, 525 (u/ml) in sweet sorghum extract and 580.5 (u/ml) in milk and with *B. cereus* were 34.72 (u/ml) in T.S.B, 39.85 (u/ml) in N.B, 39.1 (u/ml) in peptone solution, 58 (u/ml) in sweet sorghum solution, 200.65 (u/ml) in yeast extract solution, 363 (u/ml) in glucose solution, 191.5 (u/ml) in casamino acid solution, 302.5 (u/ml) in whey, 455.5 (u/ml) in casein solution, 485.5 (u/ml) in molasses, 510.5 (u/ml) in sweet sorghum extract and 535.5 (u/ml) in milk. The fixation experiments with alkaline protease from *B. polymixa* showed that this enzyme after 3 month in sawdust, perlit and silica has protected its stability and has 50, 100 and 100% of its primary activity respectively. In the case of immobilization with the same enzyme and the same bacterium, the examinations showed that the alkaline protease activity on perlit after one, two and three washing treatment were 52, 30 and 20% of its primary activity but in the case of silica were 93, 90 and 84% of its primary activity.

Keywords: Alkaline-protease, *Bacillus polymixa*, *Bacillus cereus*, sweet-sorghum, immobilization.

INTRODUCTION

The major microorganisms that were found as industrial protease producers exist in bacteria such as genera *Clostridium, Bacillus* and *Pseudomonas* and fungi such as genera *Aspergillus, Mucor* and *Rhizopus* (Aaslyng et al., 1990; Dosoretz et al., 1990). The *Bacillus spp,* as one of the best alkaline protease producers, have shown various physiological capabilities (Holt et al., 1994; Ram et al., 1994; Sneath et al., 1986). The detection and isolation methods of *Bacillus spp* are based on resistance of their endospores to high temperatures ranging 70 – 80°C. This condition destroys all vegetative forms of the other known bacteria and other endospores (Emtiazi et al., 2005; Holt et al., 1994; Sneath et al., 1986; Vela, 1974).

The most important aspect of proteases is their optimum pH of activity. The alkaline proteases, Max activity in pH = 9 - 11, hydrolyze extended spectrum of peptide bands (Emtiazi et al., 2005; Mao et al., 1992; Ram et al., 1994; Yoshimura et al., 1983). Alkaline proteases have numerous applications of daily life of peoples such as food complementary of beasts and poultries, confectionary, bakery, biotransformation, debittering of hydrolyzed pro-

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teins, detergent industries, waste water refinement, lea thering, oil manufacturing industries, alcohol production industries, beer production industries and so on (Emtiazi et al., 2005; Geweley, 1996; Godfrey et al., 1996; Heitmann and Meyer, 1981; Matoba et al., 1997).

The main goals of this research were comparison of *Bacillus cereus and Bacillus polymixa* regarding their capability of their alkaline protease production and finding the novel industrial culture mediums for the production of this enzyme.

MATERIALS AND METHODS

Microbiological culture media and main instruments

The main materials that we used in this research were skim milk medium, nutrient agar medium, nutrient broth medium (N.B.), tripticase soy broth (T.S.B), peptone, casein, casamino acid, yeast extract, glucose, L-tyrosine, folin ciocaltus phenol reagent, tricholoro acetic acid, (all from Merck), casein powder for enzyme assay and biochemistry (Merck, 2241), molasses, whey, sweet sorghum extract, and sweet sorghum solution (Biolab, Iran).

The major instruments that we used in this research were spectrophotometer (Milton Roy, Spectronic 1001, USA), high speed refrigerated centrifuge (Hitachi, CR21GIII, Japan) and shaker refrigerator incubator (Jahl, 1800M, Iran).

Isolation and characterization methods

The *Bacillus spp* were isolated from various soil samples with the specific methods and identified with specific biochemical tests (Henriette et al., 1993; Shah et al., 1986) then cultured in alkaline skim milk mediums with pH = 9, 10, 11 for the estimation of alkaline protease production.

Production of alkaline protease in biotechnological culture media

The best producers such as *B. polymixa* and *B. cereus* were screened from this stage and passaged to broth culture mediums such as T.S.B, N.B, yeast extract solution, peptone solution, casein solution, casamino acid solution, glucose solution, milk and four industrial mediums such as molasses, whey, sweet sorghum solution and sweet sorghum extract. These mediums were incubated in a shaker incubator (30℃ and 100 R.P.M) for 6 days and each 24 h were examined for alkaline protease production.

Alkaline protease assay procedure

The method which used for alkaline protease assay in all experiments was based on a colorimetric technique for assay of proteins and specifically for L-tyrosine, Lawry method (Atalo et al., 1993; Bierbaum et al., 1994). According to this method, one unit of alkaline protease activity is defined as amounts of enzyme which can release 1 µg L-tyrosine from alkaline casein substrate (pH = 11) under the assay condition (40℃ for 10´) and is expressed with u/ml. For all experiments we used a standard curve of L-tyrosine - OD (Atalo et al., 1993; Bierbaum et al., 1994; Knaysi, 1951; Lee et al., 1990; Moon et al., 1993).

Effects of various environmental conditions on enzyme activity

The effects of various conditions such as pH, temperature and aeration speed on alkaline protease production by *B. polymixa* and *B. cereus* were measured. For this reason one of the former mediums, glucose solution, was selected because of its simplicity and lack of obtrusive materials such as peptone, triptone and amino acids that could disorder the enzyme assay (Atalo et al., 1993; Emtiazi et al., 2005). The constituents of this medium were: glucose (6 g/l), (NH4)2SO4 (10 g/l), Na2HPO4 (8 g/l), KH2PO4 (4 g/l), MgSO4, 7H2O (0.5 g/l), CaCl2 (0.02 g/l) [all from Merck]. The pH of culture medium were regulated using specific buffers with pH 4, 5, 7, 8, 9, 10, 11 and 12. After injection with purified bacterium, all of the medium were incubated like previous experiment. The effects of temperature (10, 20, 30, 40, 50 and 60℃) and aeration speed (75, 100, 125, 150, 175 and 200 R.P.M.) on alkaline protease production by *B. polymixa* and *B. cereus* were measured using the same medium.

Fixation and immobilization of alkaline protease

The fixation and immobilization of the enzyme from *B. polymixa* on sawdust, perlit (an amorphous volcanic glass that has relatively high water content and a strong potential for filtration uses) and silica (sea sand) were investigated. Using mentioned glucose medium, the alkaline protease was produced by *B. polymixa*. For isolating the enzymatic solution from bacterial mass, the culture medium was centrifuged at 10000 R. P. M. for 10 min. The supernatant was then filtered through Watmann filtration paper No. one. Hundred millilitre of the filtrate was added to 50 g of each fixation substances include sawdust, silica and perlit. After complete mixing of enzymatic solution with each mentioned substances, they were incubated at 40℃ for 3 months. Then the alkaline protease activity was measured using 0.1 g of each substance by Lawry method. For evaluating the immobilization potentials of three mentioned substances, 0.1 g of each substance was washed one, two and three times using sterile water and after centrifugation at 5000 R. P. M. for 10 min, the activity of alkaline protease in sediment was measured by Lawry method.

RESULTS

The maximum alkaline protease activity in productive culture mediums after 72 h in 30℃ and with100 R.P.M were measured. The results with *B. polymixa* were 46.7 (u/ml) in T.S.B, 57 (u/ml) in N.B, 60.22 (u/ml) in peptone solution, 67 (u/ml) in sweet sorghum solution, 216 (u/ml) in yeast extract solution, 358 (u/ml) in glucose solution, 413 (u/ml) in casamino acid solution, 418 (u/ml) in whey, 470.5 (u/ml) in casein solution, 490.5 (u/ml) in molasses, 525 (u/ml) in sweet sorghum extract and 580.5 (u/ml) in milk and with *B. cereus* were 34.72 (u/ml) in T.S.B, 39.85 (u/ml) in N.B, 39.1 (u/ml) in peptone solution, 58 (u/ml) in sweet sorghum solution, 200.65 (u/ml) in yeast extract solution, 363 (u/ml) in glucose solution, 191.5 (u/ml) in casamino acid solution, 302.5 (u/ml) in whey, 455.5 (u/ml) in casein solution, 485.5 (u/ml) in molasses, 510.5 (u/ml) in sweet sorghum extract and 535.5 (u/ml) in milk. The comparisons of maximum alkaline protease production (72 h), by *B. polymixa* and by *B. cereus* in different culture mediums were shown in Figures 1 and 2 respectively.

The enzyme activity in glucose solution with *B. polymixa* at pH = 4, 5, 7, 8, 9, 10, 11, 12 were measured 6.97 (u/ml), 11.72 (u/ml), 358 (u/ml), 311.5 (u/ml), 285.5 (u/ml),

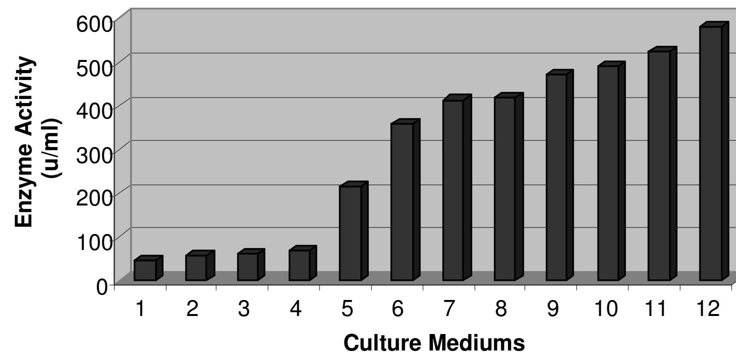

Figure 1. The effect of different cultures mediums in alkaline protease production by *Bacillus polymixa* after 72 h at 30°C and 100 RPM.

1: TSB, 2: NB, 3: Peptone, 4: Sorghum Solution, 5: Yeast Extract, 6: Glucose, 7: Casamino acid, 8: Whey, 9: Casein, 10: Molasses, 11: Sorghum Extract, 12: Milk.

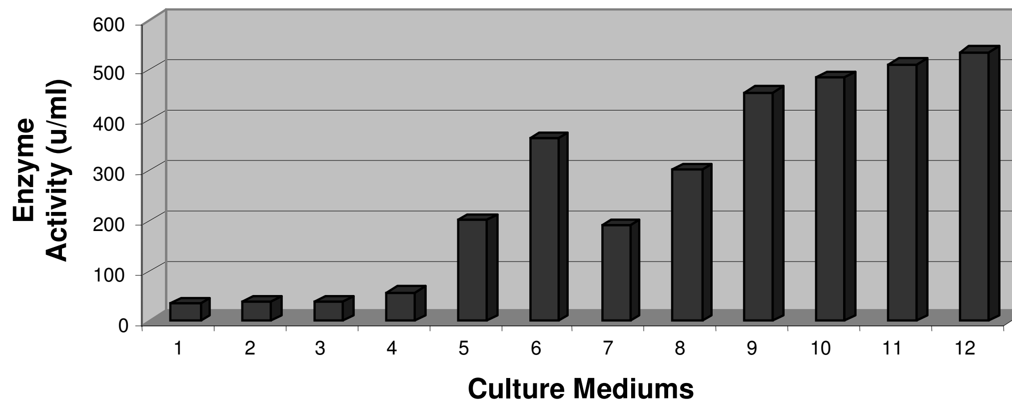

Figure 2. The effect of different cultures mediums in alkaline protease production by *Bacillus cereus* after 72 h at 30°C and 100 RPM.

1: TSB, 2: NB, 3: Peptone, 4: Sorghum solution, 5: Yeast extract, 6: Glucose, 7: Casamino acid, 8: Whey, 9: Casein, 10: Molasses, 11: Sorghum extract, 12: Milk.

232 (u/ml), 5.72 (u/ml) and 3.85 (u/ml) respectively and with *B. cereus* were measured 9.72 (u/ml), 13.72 (u/ml), 360.5 (u/ml), 343 (u/ml), 335.5 (u/ml), 14.6 (u/ml), 5.6 (u/ml) and 4.22 (u/ml) respectively. The optimum pH for maximum production of alkaline protease was pH 7. The results' comparison of this experiment in *B. polymixa* was shown in Figure 3 and in *B. cereus* was shown in Figure 4.

The results of various temperatures and aeration speeds effects on alkaline protease production by *B. polymixa* and *Bacillus cereus* were shown in Figures 5 to 8.

The Figure 5 shows that the production of this enzyme in *B. polymixa* increased from 10 to 60°C and the activities were 20.85 (u/ml) at 10°C, 163.5 (u/ml) at 20°C, 358 (u/ml) at 30°C, 365.5 (u/ml) at 40°C, 385.5 (u/ml) at 50°C and 410.5 (u/ml) at 60°C.

Figure 6 shows that the production of this enzyme in *B. cereus* increased from 10 to 60°C and the activities were 13.85 (u/ml) at 10°C, 111.5 (u/ml) at 20°C, 310.5 (u/ml) at 30°C, 335.5 (u/ml) at 40°C, 360.5 (u/ml) at 50°C and 381 (u/ml) at 60°C. The results showed that the optimum temperature of alkaline protease production in both spp was 60°C.

Figure 7 shows that increasing of aeration speed resulted in increasing of alkaline protease production by *B. polymixa*. For example the activities of enzyme in 75, 100, 125, 150, 175 and 200 R.P.M were measured 311.5 (u/ml), 358 (u/ml), 395.5 (u/ml), 414.5 (u/ml), 463 (u/ml) and 510 (u/ml) respectively.

Finally, Figure 8 shows that increasing of aeration speed resulted in increasing of alkaline protease production by

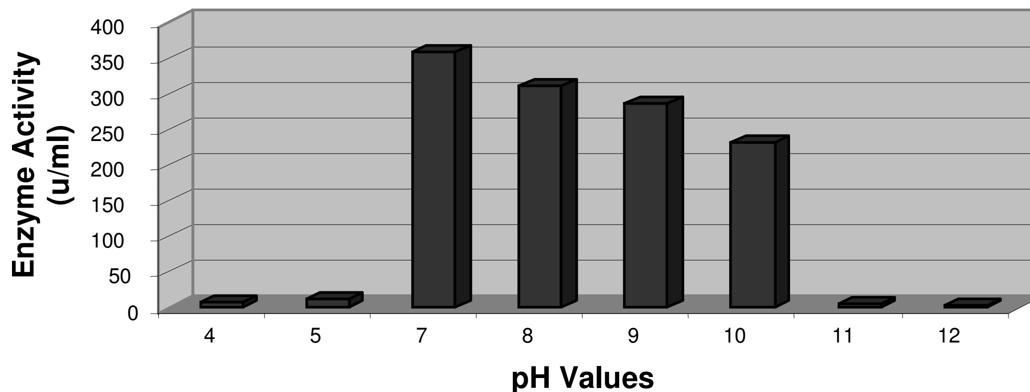

Figure 3. The effect of pH values in alkaline protease production by *Bacillus polymixa* after 72 h at 30°C and 100 RPM in glucose medium.

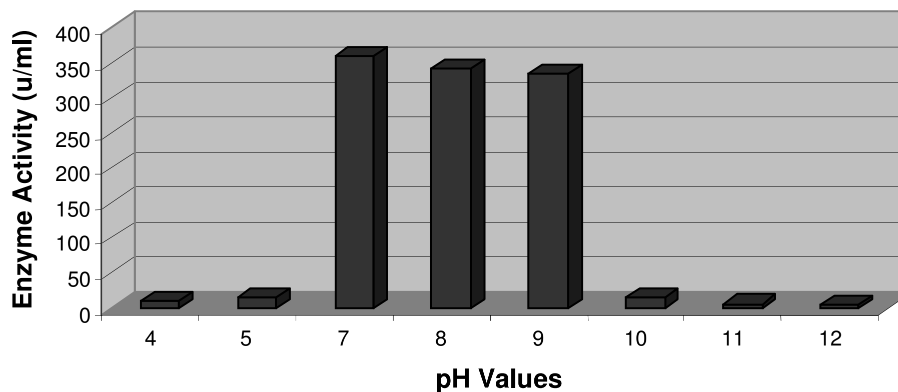

Figure 4. The effect of pH values in alkaline protease production by *Bacillus cereus* after 72 h at 30°C and 100 RPM in glucose medium.

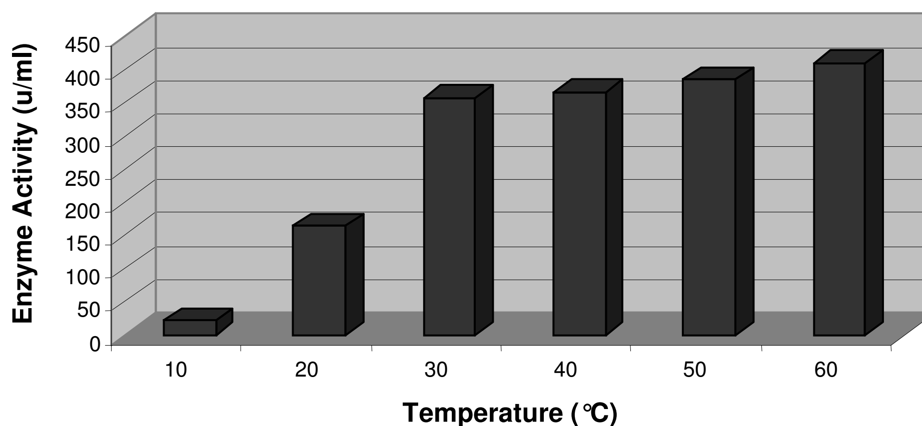

Figure 5. The effect of different temperatures on alkaline protease production by *Bacillus polymixa* after 72 h at 100 RPM in glucose medium at pH = 7.

Bacillus cereus too. For example the activities of enzyme in 75, 100, 125, 150, 175 and 200 R.P.M were measured 308 (u/ml), 311.5 (u/ml), 345.5 (u/ml), 370.5 (u/ml), 395.5 (u/ml) and 436.5 (u/ml) respectively. The results showed

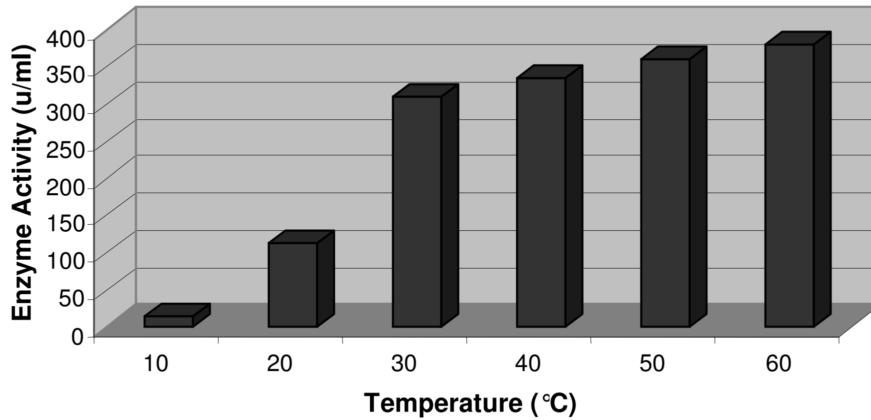

Figure 6. The effect of different temperatures on alkaline protease production by *Bacillus cereus* after 72 h at 100 RPM in glucose medium at pH = 7.

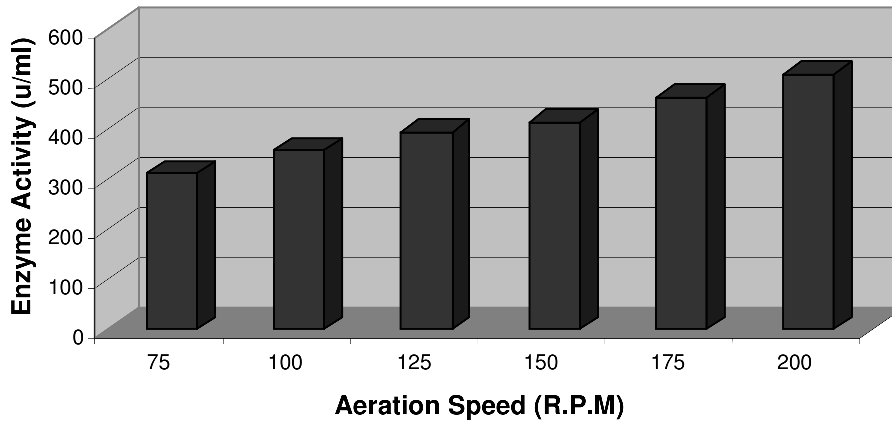

Figure 7. The effect of aeration speeds (RPM) on alkaline protease production by *Bacillus polymixa* after 72 h at 30°C in glucose medium at pH = 7.

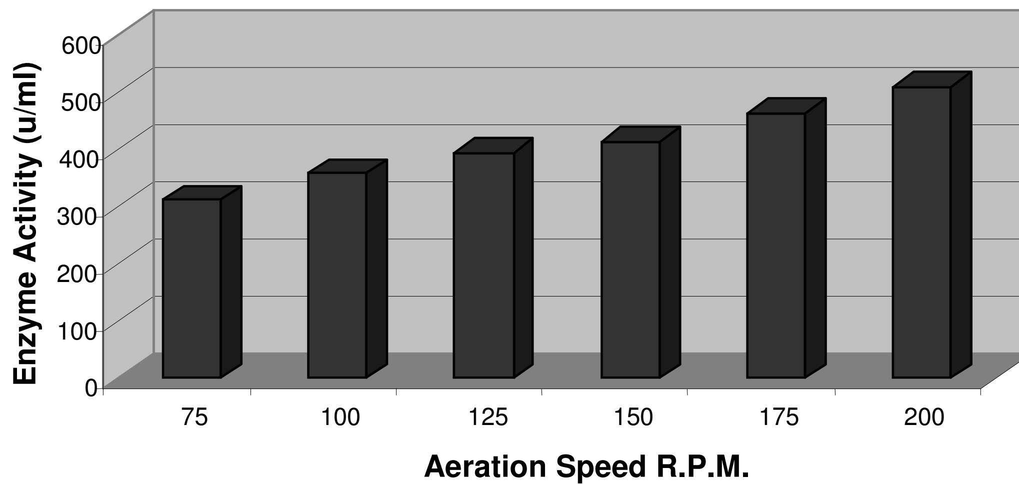

Figure 8. The effect of aeration speeds (R.P.M) on alkaline protease production by *Bacillus cereus* after 72 h at 30°C in glucose medium at pH = 7.

that the optimum aeration speed for production of alkaline protease by both *spp* was 200 R.P.M.

The fixation experiments with alkaline protease from *B. polymixa* showed that this enzyme after 3 month in sawdust, perlit and silica has protected its stability and has 50, 100 and 100% of its primary activity respectively. In the case of immobilization with the same enzyme and the same bacterium, the examinations showed that the alkaline protease activity on perlit after one, two and three washing treatment were 52, 30 and 20% of its primary activity but in the case of silica were 93, 90 and 84% of its primary activity.

DISCUSSION

Shah et al. (1986) used mutant strains of *Bacillus licheniformis* to produce alkaline protease in a two-phase system. Atalo et al. (1993) produced an alkaline protease using a thermophilic *Bacillus* strain P-001A isolated from a hot alkaline water geyser. Abo-Aba et al. (2006) produced alkaline protease from *Bacillus circulance*, *Bacillus alvei, Bacillus sphaericus* and *Bacillus pumilus* using Luria – Bertani Broth, Luria – Bertani Agar and Luria – Bertani Agar plus 1% skim milk and measured the maximum activity at 30℃ and after 40 h. We isolated wilds strains of *B. cereus* and *B. polymixa* from Isfahan soil, Iran and produced alkaline protease using new industrial culture media with comparable activity. Nilegaonkar et al. (2002) used peptone, trypton, yeast extract and casein as substrates for alkaline protease production. Atalo et al. (1993) showed that yeast extract and peptone can induce the alkaline protease production in glucose medium. Although yeast extract solution medium and peptone solution medium were rich in proteins and amino acids components, but didn’t induce production of alkaline protease in *B. polymixa* and *B. cereus* substantially. We suggest that this phenomenon could be related to poor growth of these spp. in yeast extract and peptone as only carbon and nitrogen source. It seems that our spp. were fastidious and needed other carbon sources for their growth such as carbohydrates and several salts as growth factors. Although the maximum of alkaline protease production in our approach was observed in milk with 580.5 (u/ml) in *B. polymixa* and 535.5 (u/ml) in *B. cereus*, the best industrial culture mediums for production of alkaline protease by *B. polymixa* were sweet sorghum extract (525 u/ml), molasses with brix 2 (490.5 u/ml) and whey (418 u/ml) respectively and by *B.* cereus were sweet sorghum extract (510.5 u/ml), molasses with brix 2 (485.5 u/ml) and whey (302.5 u/ml) respectively. We applied these three novel Industrial culture mediums for production of alkaline protease by *B. polymixa* and *B. cereus* and obtained suitable results. While the whey is an obtrusive material in ecosystem and its penetration to water streams can lead to elevation of Biological Oxygen Demand (BOD) values and increase the perils associated with such a condition, utilization of whey and biotransformation of it to a significant product such as alkaline protease can participate to control environmental contaminations and pollutions. Giesecke et al. (1991) produced of alkaline protease with by *B. licheniformis* in a controlled fed-batch process. They isolated the alkaline protease producers using calcium caseinate agar but this medium has not been effective in alkaline protease production. We used alkaline casein agar and milk agar with alkaline pHs 9, 10 and 11. We suggest that using this approach could be useful for initial evaluating of strains in production of alkaline protease. Mattern et al. (1992) indicated that the casein concentration has an effect in inducing alkaline protease production by *Aspergillus niger*. In *B. polymixa* and *B. cereus* increasing of casein concentrations led to promotion of alkaline protease activity but addition of casein more than a recognized limit has no effect on production. It suggests that these two spp. behave like *A. niger* at least in this case. While the enzyme activity in casein solution medium and casamino acid solution medium were relatively high, however due to their routine laboratorial applications there are no biotechnological and economical advantage using these two substrates in large scale fermentation. Olajuyigbe et al. (2008) selected the strains of *B. licheniformis* using skim milk agar and nutrient broth culture mediums and indicated that optimum temperature for protease activity was 60℃. We showed that the best environmental conditions for production of alkaline protease by *B. polymixa* and *B. cereus* are Ph = 7, temperature of 60℃ and aeration speed of 200 R.P.M in glucose solution medium. Angelova et al. (1995) immobilized an acid proteinase from *Humicola lutea*. The fixation of alkaline protease of *B. polymixa* suggested that this enzyme after 3 months has been fixed and preserved its activity in sawdust partially but in perlit and silica completely so the perlit and silica could be suitable materials for fixing alkaline protease in large scale production also these latter substances could be used for immobilization of alkaline protease of *B. polymixa* in an acceptable manner.

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