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# Improving thermostable α–amylase production by *bacillus* $RB_4$

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**ABSTRACT**

*This study was aimed at improving α- amylase production by Bacillus $RB_4$. Bacillus $RB_4$ isolated from rice broth was grown in a medium containing ($gL^{-1}$) starch, 2.0; $CaCl_2.2H_2O$, 0.01; $MgCl_2.6H_2O$, 0.01; $FeCl_2$, 0.01; $K_2HPO_4$, 2.5; $KH_2PO_4$, 10.0; peptone, 4.0; NaCl, 2.0 and $(NH_4)_2SO_4$, 4.0. Decrease in the phosphate ion concentration in the fermentation medium decreased the enzyme production. Sodium dodecyl sulphate (0.05% w/v) and succinic acid (0.1 and 0.5$gL^{-1}$) reduced α–amylase production while Tween 80 (0.1, 1.0 and 5.0% v/v) did not improve α–amylase production. Soluble starch (4.5$gL^{-1}$) increased the enzyme production by 1.5 times. Two-fold increase in the enzyme production was observed with sesame oil (18$mLL^{-1}$) supplementation in addition to 4.5$gL^{-1}$ soluble starch while coconut oil (3.0$mLL^{1}$) completely stopped α–amylase production. Thus by optimizing the medium 4.26 fold increase in α–amylase production by Bacillus $RB_4$ was achieved.*

**Key words:** α–Amylase, *Bacillus spp.*, improving, additives and sesame oil.

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## INTRODUCTION

The amylases of microorganisms have a broad spectrum of industrial applications, as they are more stable than plant and animal α–amylases [1, 2]. Microbial production of enzymes is expensive [3]. Different studies have been reported for the improvement of α–amylase production by altering the nitrogen sources and other nutrients in the used [4, 5]. Including fatty acids have been reported to improve the membrane permeability [6, 7] and improving the growth of microorganisms [8 - 10]. Further Surfactants have been reported to improve α–amylase production [5, 11, 12]. To reduce the production cost of glucose syrup the enzyme production has o be improved and at the same time thermostable enzyme is also essential. As the thermophilic *Bacillus* $RB_4$ has been isolated and found to produce thermostble α–amylase [13] this study is aimed to increase the thermostable α–amylase production by *Bacillus* $RB_4$.

## MATERIALS AND METHODS

### Materials, Strain and Media used

The chemicals and media used were from standard sources. *Bacillus* $RB_4$ isolated in the Department of Biochemistry was used [13]. Nutrient broth (25$gL^{-1}$) with 2.0$gL^{-1}$ starch at pH 7.0 was used as the activation medium. The fermentation medium contained ($gL^{-1}$) starch, 2.0; $CaCl_2.2H_2O$, 0.01; $MgCl_2.6H_2O$, 0.01; $FeCl_2$, 0.01; $K_2HPO_4$, 2.5; $KH_2PO_4$, 10.0; peptone, 4.0; NaCl, 2.0 and $(NH_4)_2SO_4$, 4.0.

### Estimation of α–amylase activity

The supernatant (centrifuged for 15 min at 5000rpm) of the spent medium was used as the enzyme source and the activity of α–amylase was determined [3]. One unit of α–amylase activity was defined as the amount of enzyme that released 1μmole of reducing sugars from 20$gL^{-1}$ starch solution in one minute at 85°C.

**Preparation of inocula and Cultivation of the selected best strain in fermentation medium**
To 25mL of activation medium, 2 loops full of strain from bacterial colonies was inoculated and incubated at 45°C for 8h and then at 50°C for another 10 hours in a shaker water bath (100rpm). The fermentation medium (800mL) was inoculated with inoculum (20%, v/v) of the strain and incubated in an orbital shaker water bath at 50°C (100rpm). The α–amylase production and growth (OD at 600nm) were monitored.

**Effect of different concentrations of $K_2HPO_4$ and $KH_2PO_4$ on α–amylase production**
To the fermentation medium different amounts of $K_2HPO_4$ and $KH_2PO_4$ (1.25 & 5.0; 0.625 & 2.5 and 0.0 & 0.0 $gL^{-1}$) were added and the fermentation medium with ($gL^{-1}$) 2.5 $K_2HPO_4$, and 10.0 $KH_2PO_4$, was used as the control. α–Amylase production and cell growth were monitored.

**Effect of different additives on α–amylase production**
***Effect of sodium dodecyl sulphate***
To the fermentation medium (at pH 7.0) 0.05% (w/v) of sodium dodecyl sulphate was added. The medium without sodium dodecyl sulphate was used as the control.

***Effect of different concentrations of Tween -80***
To the fermentation medium (at pH 7.0) different amounts of Tween -80 **(**0.1, 1.0 and 5.0, v/v**)** was added. Control had no Tween-80.

***Effect of different concentrations of succinic acid***
To the fermentation medium (at pH 7.0) different amounts of succinic acid **(**0.01 and 0.5, **v/v)** was added. Control contained no succinic acid.

***Effect of different concentrations of soluble starch***
To the fermentation medium (at pH 7.0) different amounts of soluble starch **(**2.0 to 10.0 $gL^{-1}$**)** was added. The medium with 2.0 $gL^{-1}$ soluble starch was used as the control.

***Effect of different concentrations of sesame oil***
To the fermentation medium (at pH 7.0) different amounts of sesame oil **(**2.25 to 27.0 $mLL^{-1}$**)** was added. The fermentation medium without the oil was used as the control.

***Effect of coconut oil***
To the fermentation medium (at pH 7.0) coconut oil (3.0$mLL^{-1}$) was added. The medium without the oil was used as the control.

***Effect of optimized concentrations of sesame oil and soluble starch***
To the fermentation medium (at pH 7.0) optimized amounts of sesame oil and soluble starch were added and the enzyme production was monitored.

## RESULTS AND DISCUSSION

**Effect of different concentrations of $KH_2PO_4$ and $KH_2PO_4$ on α–amylase production by *Bacillus* $RB_4$**
Phosphate ion is essential for the growth of bacterial strains and $KH_2PO_4$ & $KH_2PO_4$ were added to the fermentation medium to maintain or reduce the changes in the pH. When the amount of $KH_2PO_4$ and $KH_2PO_4$ were decreased from 2.5 & 10.0 to 1.25 & 5.0; 0.625 & 2.5 and 0.0 & 0.0 $gL^{-1}$ the α–amylase activity obtained was 20, 13, 3.3 and 2.4 U $mL^{-1}$ at 48h (Table 1). This study indicated the importance of phosphate ions for the metabolism of *Bacillus* $RB_4$ and the concentration of phosphate ions, which have been used, was the minimal optimum concentration. Further reduction in the phosphate ion concentration can affect the enzyme production. Phosphate serves as the construction material of cellular components such as nucleic acids, phospholipids, nucleotides and coenzymes. α–Amylase synthesis was stimulated by phosphate. An increase of α–amylase production from 52 772 to 55 070 U/g with 0.01M $KH_2PO_4$ has been reported [14]. Therefore the phosphate ion concentration cannot be reduced or omitted from the medium. Hence it was decided not to decrease the concentrations of these salts in the fermentation medium. As an alternative to avoid the enzyme precipitation along with calcium phosphate, the spent medium was dialyzed to eliminate the phosphate ions present in the spent medium before the addition of $Ca^{2+}$ for the stability studies.

**Table 1: Effect of different concentrations of $K_2HPO_4$ and $KH_2PO_4$ on α–amylase production by *Bacillus* $RB_4$ at 48h. Here the concentration of $K_2HPO_4$ and $KH_2PO_4$ were selected to have pH 7.0. In medium D the initial pH was 7.0**

| Medium | $K_2HPO_4$ ($gL^{-1}$) | $KH_2PO_4$ ($gL^{-1}$) | α–Amylase activity (U $mL^{-1}$) |
|---|---|---|---|
| A (Control) | 2.5 | 10.0 | 20.03 |
| B | 1.25 | 5.0 | 12.00 |
| C | 0.625 | 2.5 | 3.30 |
| D | 0.0 | 0.0 | 2.40 |

## Effect of different additives on α–amylase production

### *Effect of sodium dodecyl sulphate on α–amylase production*

Protein leakage into the medium can be enhanced by the addition of detergents and hence the fermentation medium was supplemented with different detergents to improve the α–amylase release into the medium. α–Amylase production was inhibited by sodium dodecyl sulphate (0.05%, w/v) in the fermentation medium at pH 7.0 and at 50°C. The enzyme activity decreased to 1.68U $mL^{-1}$ at 48h compared to the control (20U $mL^{-1}$, Table 2). Previous studies have indicated that slight inhibition [15] or repression of production [14, 16] or destabilization of α–amylase resulted in a decrease in the temperature of unfolding with an increase in SDS concentration [17]. Surfactant applied to the medium at the initial stage of the fermentation must still be present and thus influencing the structure of proteins and therefore the activity of enzyme. This may decrease both enzyme activity as well as the secretion. In addition, the effects of the membrane and on the protein export mechanism may contribute to a decreased α–amylase production [5]. Thus addition of SDS is of no use to improve the α–amylase release into the medium because it has either repressed α–amylase production or destabilized the α–amylase protein or both.

**Table 2: Effect of different additives on α–amylase production by *Bacillus* $RB_4$ at 48h**

| Additives | Concentration | α–Amylase activity (U $mL^{-1}$) |
|---|---|---|
| Control | Nil | 20.03 |
| Sodium Dodecyl Sulphate (%, w/v) | 0.05 | 1.68 |
| Tween-80 (%, v/v) | 0.1 | 19.06 |
| | 1.0 | 17.82 |
| | 5.0 | 18.11 |
| Succinic acid (%, w/v) | 0.01 | 18.49 |
| | 0.05 | 14.5 |

### *Effect of Tween -80 on α–amylase production*

Tween-80 itself is stimulatory because it is a derivative of oleic acid. The enzyme production was slightly decreased by Tween 80 at all the concentrations considered in this studies (Table 3). Enzyme production in the culture supernatants of surfactants containing Tween 40 was not detectable while growth was accelerated [5]. Production of α–amylase by *A. flavus* increased with the addition of surfactants into the growth medium, whereas non –ionic surfactants enhanced the enzyme production as compared to that of anionic surfactants. When the medium was supplemented with either 1.0% (w/v) Tween 80 or Tween 20, higher enzyme titer was obtained with 1.0% (w/v) Tween 80. The effect of synthetic surfactants is dependent upon the characteristics of the applied microbial strain [12]. Surfactant, Tween-80, at 0.02, 0.002 and 0.0002% concentration were most effective for enhancement of α–amylase production [18]. Tween 80 decreased the enzyme production when different surfactants were tested for enzyme production [11]. Tween 80 enhanced the activity of α–amylase [19] or the α–amylase production [20]. Thus Tween 80 has shown different effects on different bacterial strains and it was not useful to *Bacillus* $RB_4$.

### *Effect of different concentrations of succinic acid on α–amylase production*

Succinic acid is an intermediate of citric acid cycle and said to be activating citrate synthase and hence it was expected that it can improve the α–amylase production by *Bacillus* $RB_4$. Succinic acid at 0.01 and 0.05% (w/v) concentrations inhibited the enzyme production to 92.5 and 72.5% respectively of the activity obtained in the control medium (Table 2). Citrate or glutamate was better carbon sources than soluble starch for α–amylase production by *Bacillus licheniformis* [21]. Thus succinic acid also has not improved or induced α–amylase production by *Bacillus* $RB_4$.

### *Effect of different concentrations of soluble starch on α–amylase production*

When the concentration of soluble starch in the fermentation medium was varied from 2.0 to 10.0 $gL^{-1}$ the α–amylase production from 22.5 to 1.46 $UmL^{-1}$ through 38$gL^{-1}$ with 4.5 $gL^{-1}$ soluble starch at 48h (Table 3). Increase in soluble starch concentration beyond 5$gL^{-1}$ inhibited the α–amylase production and 10$gL^{-1}$ has tremendously decreased the α–amylase production by *Bacillus* $RB_4$. The result indicated that higher starch concentrations have inhibited the α–amylase production by *Bacillus* $RB_4$. Hydrolysed starch and glucose repressed

the enzyme yield while 1.5% soluble starch induced α–amylase production but further increase in soluble starch resulted in gradual decrease in enzyme titer [22]. In presence of glucose α–amylase production was repressed but rapid growth was observed [21]. Addition of starch to media containing different types of oil seed cakes did not improve α–amylase production [4]. The decrease in α–amylase production beyond 4.5gL$^{-1}$ soluble starch could be due to the decrease in the solubility of starch and formation of clumps. Further the α–amylase could have got adsorbed to starch [23] and led to decreased availability of free α–amylase for the activity measurements.

***Effect of different concentrations of sesame oil on α–amylase production***

When sesame oil was added, the highest enzyme activity (65.75 UmL$^{-1}$) was obtained with 18 mL L$^{-1}$ sesame oil (Table 3). The cell division needs phospholipids and triacyl glycerol for the membrane formation. The growth temperature influences the membrane lipid composition. Cells at 75$^{o}$C contained 70% more total fatty acids than cells grown at 50$^{o}$C [21]. Membranes from the obligatory alkalophilic species contained a high concentration of branched-chin fatty acids as well as a relatively high content of unsaturated fatty acids, comparable to that in the membrane of *B. subtilis*. The facultative alkalophilic strains contained almost no unsaturated fatty acids and a lower concentration of branched-chain fatty acids than either the obligate alkalophiles or *B. subtilis* [23]. Rumen bacteria growth was stimulated by low concentrations of olecic (0-1gL$^{-1}$), lauric (0- gL$^{-1}$) or capric (<0- gL$^{-1}$) acids while higher concentrations of these acids were inhibitory. Myristic, palmitic and stearic acids were inhibitory at all concentrations tested [11]. As the environmental temperature increased, the proportion of saturated fatty acids found in the membrane lipids also markedly increased with a concomitant decrease in the proportion of unsaturated and branched chain fatty acids [7, 10]. Oleic acid has biotin like activity. Fatty acids are stimulatory only when biotin was absent or present in suboptimal concentration [8]. The importance of the unsaturation of fatty acid with respect to growth promoting ability is demonstrated by the fact that with more double bonds in the molecule the stimulating effect becomes less [8]. The fact that the fatty acids particularly unsaturated ones, are able to both inhibit and stimulate growth of microorganisms was called 'double action' an inhibitory effect might change into a stimulatory one depending on (a) the presence of a detoxicant, (b) the concentration of the fatty acid in the medium and (c) duration of the incubation [8]. Effect of fatty on microbial growth depends on the bacterial species; fatty acid structure; neutralization of inhibition by antagonists, dualistic character (depends on the presence of detoxicants; concentration of fatty acids in the medium and Length of incubation time) and substitution of biotin [8]. Sesame oil is rich in unsaturated fatty acids and hence the *Bacillus* $RB_4$ is of the type, which requires unsaturated fatty acids for its growth. Hence 18mL L$^{-1}$ sesame oil was selected for further studies.

**Table 3: Effect of different concentrations of soluble starch, sesame oil, coconut oil and optimized amount of soluble starch and sesame oil on α–amylase production by *Bacillus* $RB_4$ at 48h**

| Additives | Concentration | α–Amylase activity(U mL$^{-1}$) |
|---|---|---|
| Control | Nil | 20.03 |
| Soluble starch (gL$^{-1}$) | 2.0 | 20.0 |
| | 3.0 | 24.0 |
| | 3.5 | 25.0 |
| | 4.0 | 28.0 |
| | 4.5 | 38.0 |
| | 5.0 | 28.3 |
| | 10.0 | 1.46 |
| Sesame oil (%, v/v) | 2.25 | 31.9 |
| | 4.5 | 53.0 |
| | 9.0 | 55.8 |
| | 18.0 | 65.8 |
| | 22.5 | 58.3 |
| | 27.0 | 22.9 |
| Coconut oil (%, v/v) | 3.0 | 4.92* |
| Soluble starch (gL$^{-1}$) and Sesame oil (%, v/v) | 4.5 | 85.4 |
| | 18.0 | |

**Highest activity obtained at 72h.*

***Effect of coconut oil on α–amylase production***

Addition of coconut oil (3.0mLL$^{-1}$) to the fermentation medium decreased the enzyme production to 4.92 UmL$^{-1}$ with the delay in the maximum enzyme production to 72h, i.e. by one day (Table 4). Although in general, the bacterial activity increases with unsaturation, saturated fatty acids can also act as growth inhibitors, the antibacterial properties being optimal for the substances with a chain length of about 12 carbon atoms [8]. Spore germination is unaffected by saturated fatty acids but affected by unsaturated fatty acids [8]. Antibacterial activity increases with unsaturation, saturated fatty acids can also act as growth inhibitors [6, 8, 24]. The observation made with *Bacillus* $RB_4$ indicated that the growth of the organism is inhibited by saturated fatty acids and coconut oil is a rich source of saturated fatty acids.

***Effect of optimized concentration of sesame oil and soluble starch***

When the optimized amounts of sesame oil (18mLL$^{-1}$) and soluble starch (4.5 gL$^{-1}$) were added highest α–amylase activity obtained was 85.4UmL$^{-1}$ at 48h. In the media either with the optimized amount sesame oil (18mLL$^{-1}$) or optimized amount soluble starch (4.5 gL$^{-1}$), the α–amylase activities obtained were 65.8 and 38.0 UmL$^{-1}$ respectively (Table 3). Antibacterial activity increases with unsaturation while saturated fatty acids can also act as growth inhibitors [8].

## CONCLUSION

This study has shown that *Bacillus* RB$_4$ needed phosphate ions. Sodium dodecyl sulphate and succinic acid reduced α–amylase production while Tween 80 did not improve α–amylase production. Soluble starch and sesame oil increased the enzyme production while coconut oil completely stopped α–amylase production. Thus optimizing the concentrations of soluble starch and sesame oil increased the α–amylase production by 4.26 fold by *Bacillus* RB$_4$.

## REFERENCES

[1] S. Sriramakrishnan, D. Gangadharan, K. M. Nampoothiri, C. R. Soccol, A. Pandey, *Food Technology and Biotechnology.* 44(2), 2006, 173-184.
[2] P. M. de Souza, P. O. Magalhaes, *Brazilian Journal of Microbiology*. 2010, 41, 850-861.
[3] V. Arasaratnam, K. Thayaananthan, *Malaysian Journal of Biochemistry and Molecular Biology.* 2009, 17*(*1), 1-4.
[4] A. Venkadaramana, S. Balakumar, V. Arasaratnam, *Archives of Applied Science Research.* 2011, 3 (6), 87-97.
[5] Z. Baysal, F. Uyar, C. Aytekin, *Annals Microbiology*. 2003, 53(3), 323-328.
[6] P. H. Ray, D. C. White, T. D. Brock, *Journal of Bacteriology*. 1971, 106(1), 25-30.
[7] R. N. Mc Elhaney, K. A. Souza, *Biochimica et Biophysica Acta- Biomembranes.* 1976, 443(3), 348-359.
[8] C. Nieman, *Bacteriological Review.* 1954, 18, 147-163.
[9] S. Clejan, T. A. Krulwich, K. R. Mondrus, D. Seto-Young, *Journal of Bacteriology.* 1986, 168(1), 334-340.
[10] E. E. Allen, D. Facciotti, D. H. Bartlett, *Applied Environmental Microbiology.* 1999, 65(40), 1710-1720.
[11] Ikram-Ul-Haq, N. Shamim, H. Ashraf, S. Ali, M. A. Qadeer, *Pakistan Journal of Botany*. 2005, 37(2), 373-379.
[12] S. Bhardwaj, A. B. Vedamurthy, S. Battacharya, A. Das, *Journal of Chemical Biological Physical Sciences.* 2012, 2(3): 1390-1397.
[13] V. Arasaratnam, K. Thayaananthan, *European Journal of Experimental Biology*. 2014, 4 (3): 576-582.
[14] N. Ogbonnaya, A. Odiase, *Acta Scientiarum Polonorum Technologia Alimentaria.* 2012, 11(3), 231-238.
[15] M. Y. Yoon, Y. J. Yoo, T. W. Cadman, *Biotechnology Letters.* 1989, 11, 57-60.
[16] B. Arikan, *Bioresource Technology*. 2008, 99 (8), 3071-3076.
[17] B. Shareghi, and M. Arabi, *Indian Journal of Science Technology.* 2008, 32(A2), 135-140.
[18] A. Gupta, N. Gautam, D. R. Modi, *Journal of Biotechnology and Pharmaceutical Research.* 2010, 1(1), 1-8.
[19] L. Gajjar, R. S. Dubey, R. C. Srivastava, *Applied Biochemistry and Biotechnology*. 1994, 49(2), 101-112.
[20] T. Sivakumar, T. Shankar, P. Vijayabaskar, J. Muthukumar, E. Nagendrakannan, *International Journal of Microbiology Research.* 2012, 3(2), 117-123.
[21] D. M. Rothstein, P. E. Delvin, R. L. Cate, *Journal of Bacteriology*. 1986, 168(2), 839-842.
[22] D. Gangadharan, S. Sivaramakrishnan, K. M. Nampoothiri, A. Padey, *Food Technology and Biotechnology.* 2006, 44(2), 269-274.
[23] T. Murugapoopathy, S. Varavinit, V. Arasaratnam, K. Balasubramaniam, *ASEAN Journal of Science and Technology Development.* 1995, 12(1), 43-57.
[24] C. Henderson, *Journal of Agricultural Science.* 1973, 81(1), 107-112.