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Production of Halophilic α-Amylase by Immobilized Cells of Moderately Halophilic *Bacillus* sp. Strain TSCVKK

Kanthi K. Kiran[1*], Podili Koteswaraiah[1] and T. S. Chandra[2]

[1]Biochemistry Laboratory, Department of Chemistry, IIT Madras, Chennai, Tamil Nadu, India.
[2]Department of Biotechnology, IIT Madras, Chennai, Tamil Nadu, India.

Author's contributions

This work was carried out in collaboration between all authors. KK performed the literature search, designed and executed the experiments and wrote the manuscript. PK helped in performing literature search; enzyme assays and in writing the manuscript. TSC supervised the study. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To investigate the effect of cell immobilization on amylase production by the moderately halophilic bacterium, *Bacillus* sp. strain TSCVKK and to compare the properties of the amylase produced under immobilized conditions with the enzyme produced by the free cells.
Study Design: Cell immobilization.
Place and Duration of Study: Department of Chemistry, Biochemistry Lab, Indian Institute of Technology (IIT Madras), Chennai, Tamil Nadu, between Jan 2009 and March 2009.
Methodology: *Bacillus* sp. strain TSCVKK was immobilized in alginate, agar, polyacrylamide and gelatin. Production of amylase was determined using 3, 5-dinitrosalicylic acid (DNS). Effect of NaCl, pH, temperature on the activity of amylase was determined and compared with the amylase produced by the free cells.
Results: Maximum production of 832 mU/ml was achieved with an initial cell load of 1.2% (w/v; wet weight) of 24 h grown cells immobilized in 2% agar of 4 mm^3 block size using GSL-2 medium containing 10% NaCl and 1.5% dextrin at pH 8.0 at 30ºC after 36 h of growth. Amylase production was lower when the cells were immobilized in alginate (211 mU/ml) or with the free cells of same biomass concentration as used for immobilization

**Corresponding author: Email: Kanthi_kiranin@yahoo.com;*

(333 mU/ml). Amylase was not produced when gelatin or polyacrylamide was used as the immobilization matrix. The immobilized cells in 2% agar could be used up to 5 cycles without much reduction in amylase production. Amylase produced through cell immobilization retained all the properties that were shown by amylase produced under submerged fermentation.

Conclusion: Agar was the suitable matrix to immobilize *Bacillus* sp. strain TSCVKK for amylase production. Amylase produced under immobilization conditions retained its temperature, salt and pH requirements. Immobilized cells were used for 5 cycles without much decrease in production.

Keywords: Moderate halophile; Bacillus sp. strain TSCVKK; α-amylase; cell immobilization; agar; alginate; repeated batch fermentation.

1. INTRODUCTION

Alpha amylases are a class of industrial enzymes that randomly cleave α-1, 4 linkages in amylose chain generating glucose, maltose, and maltotriose units. They constitute about 25% of enzyme market for sugar, textile, paper, brewing and food processing industries (Rao et al., 1998; Sivaramakrishnan et al., 2006). Novel enzymes are in demand for biocatalysis at extremes of pH, temperature, salinity and organic solvents. In this context, extracellular hydrolytic enzymes from halophiles have drawn attention owing to their function under low water activity conditions (Mohapatra et al., 1998).

Cell immobilization is a well documented method employed for enhanced production of biocatalysts using matrices like agar, alginate and carrageenan. The advantages being repeated or prolonged use of cells, easier downstream processing, reduced risk of contamination, continuous fermentation in less sophisticated reactors (Mamo and Gesseasse, 1997), higher yields of enzyme activity and operational stability; greater resistance to environmental perturbations and lower effective enzyme cost (Kierstan and Coughlan, 1985). However, disadvantages include low activity, diffusional restriction and the leaching of cellular components (Babu and Panda, 1991; Prabhune et al., 1992). Though halophilic bacteria are known to produce halophilic amylase in submerged state but only one report exist till to date on amylase production by cell immobilization of *Halobacterium salinarum* (Bagai and Madamwar, 1997) and there are no such reports on a moderate halophile. We reported previously the production of detergent and surfactant stable amylase which is, halophilic and alkali-tolerant by a newly isolated moderately halophilic, alkali-tolerant *Bacillus* sp. strain TSCVKK (Kiran and Chandra, 2008). Here we report for the first time an amylase production by the agar immobilized cells of this strain.

2. MATERIALS AND METHODS

2.1 Bacterial Strain and Inoculum Preparation

Bacillus sp. strain TSCVKK (MTCC 8373, NCIM 5249, DSM 19277 and LMG 24087 and Gen Bank accession number EF419834) was grown for 24 h in Great salt lake-2 (GSL-2) medium containing (gl^{-1}): soluble starch, 10; citric acid, 0.5; tryptone, 2; yeast extract, 2; NaCl, 100; $MgSO_4.7H_2O$, 10; KCl, 5; NH_4Cl, 2; $NaHCO_3$, 1; KH_2PO_4, 0.5; trace metal solution, 2ml l^{-1}; metal chloride solution, 5ml l^{-1} (Kiran and Chandra, 2008). Initial pH was adjusted to 7.5 with

0.1N NaOH prior to autoclaving. Trace metals and metal chloride solutions were autoclaved separately and added after cooling. One ml of this culture broth was inoculated into 50 ml of GSL-2 medium, initial pH 8.0, containing 0.4% yeast extract, 1% dextrin and 0.2% $CaCl_2$ and was grown for 12, 24 or 36 h with agitation at 180 rpm at 30ºC.

2.2 Immobilization of Strain TSCVKK

After 24 h of growth the broth was centrifuged at 10000 rpm for 15 min at 3ºC and the cell pellet was washed and suspended in 5 ml of 100 mM Tris buffer, pH 7.5 containing 1.8% NaCl, and was stored at 4ºC. This was used for immobilization in alginate, gelatin, polyacrylamide and agar. Five ml of cell suspension corresponds to 0.6 g wet weight of 24 h grown cells in log phase which was 1.2% (w/v) of initial cell load (ICL) in 50 ml of the production medium.

2.3 Alginate Immobilization

Cell suspension prepared as above was mixed with 5 ml of 4% alginate solution to give a final concentration of 2% alginate (w/v), 0.9% NaCl, cell loading of 0.6 g wet cells per 10 ml gel. The slurry was extruded through a 10 ml syringe fitted with 21G needle into 100 ml of 100 mM calcium chloride solution to form the beads. The beads were cured in 100 mM Tris buffer, pH 7.5, containing 10% NaCl and 100 mM calcium chloride solution for 24 h at 4ºC and were washed in sterile 10% NaCl solution.

2.4 Gelatin Immobilization

Cell suspension prepared as above was mixed with 5 ml of 20% sterile gelatin to get a final concentration of 10% gelatin, 0.9% NaCl, cell loading of 0.6 g wet cells per 10 ml gel that was maintained at 45ºC, and poured into a sterile petri dish. Also gelatin was solidified using 10 ml of 5% glutaraldehyde for cross linking at 30ºC (Veelken and Pape, 1982). Solidified and cross-linked gelatin was cut into small cubes (4 mm^3), washed thoroughly with sterile 100 mM Tris buffer containing 10% NaCl (pH 7.5) for the complete removal of excess glutaraldehyde and was treated with sterile buffer for 1 h at 4ºC.

2.5 Polyacrylamide Immobilization

Cell suspension was mixed with 5 ml of sterile 15% acrylamide monomer (14.25 g of acrylamide and 0.75 g of bis-acrylamide) and 10 mg of ammonium persulphate followed by 10 µl of tetramethylethylenediamine (TEMED) were added. The contents were mixed and poured into a sterile plate and were allowed to polymerize. The solidified matrix was cut into cubes of 4 mm^3 and was treated with sterile buffer for 1 h at 4ºC (Veelken and Pape, 1982).

2.6 Agar Immobilization

Cell suspension was mixed with 5 ml of warm 4% agar solution at 45ºC to get a final concentration of 2% agar, cell loading of 0.6 g wet cells per 10 ml gel. The contents were mixed and poured onto a sterile plate and cut into cubes of 4 mm^3 (Kunamneni et al., 2005). The cubes were stored at 4ºC in sterile 100 mM Tris buffer, pH 7.5, containing 10% NaCl for 1 h.

2.7 Conditions for Immobilization in Agar Blocks

Different parameters like initial cell load (ICL) (0.6, 1.2, 1.8 and 2.4%), dextrin concentration (0.5, 1.0, 1.5 and 2.0%), and size of agar cubes (2, 4 and 8 mm^3) and agar concentration (1, 2, 3 and 4%, w/v) were evaluated for their effect on amylase production by cell immobilization.

2.8 Production of Alpha-Amylase by Immobilized Cells of *Bacillus* sp. Strain TSCVKK

Immobilized cells (beads or blocks) were transferred to 50 ml of GSL-2 broth and incubated at 30ºC at 120 rpm for 72 h. Parallel experiments with an equal amount of free cells (0.6 g of wet cells) served as a control. Growth was monitored by measuring the optical density of the culture broth at 600 nm in a spectrophotometer and amylase production for every 12 h in the cell free supernatants.

2.9 Amylase Assay

Amylase was assayed as described by Bernfeld (1955). One ml of the reaction mixture contained 500 µl of 1% soluble starch (prepared in 100 mM Tris buffer, pH 7.5, 10% NaCl and 5 mM $CaCl_2$), 425 µl of buffer and 75 µl of culture supernatant and was incubated for 15 min at 55ºC. The amount of reducing sugars released was quantified by 3, 5-dinitrosalicylic acid (DNS) using maltose as standard. Enzyme activity was expressed as milli International Unit per ml (mIU/ml or mU/ml), which is one thousandth of an IU that was defined as the amount of enzyme releasing 1 micromole of maltose equivalent per minute. An enzyme blank with DNS added prior to enzyme served as control. The effect of NaCl (0, 5, 10, 15 and 20%), pH (6.5-9.5) and temperature (40-75ºC) on crude amylase from the culture broth were tested as described earlier (Kiran and Chandra, 2008). Relative amylase activity was expressed as percentage of maximum amylase activity detected in the assay.

2.10 Microscopic Examinations

Scanning electron microscopy (SEM) was carried out to determine the distribution and possible morphological alterations of cells during immobilization. Agar cube with immobilized cells was finely cut with a sterile knife. Cut end facing upwards and finely crushed agar block were fixed with 2.5% glutaraldehyde on separate cover slips and gradually dehydrated with ethanol (10 to 90% with 10% increments). The cover slip was mounted on an ion sputtering device (JEOL-JFC-1100E, JEOL Japan) and a 10 mA current was passed for 5 min. About 300 Aº thickness of gold coated sample was mounted and scanned using FEI Quanta 200 Scanning Electron Microscope, Hillsboro, USA.

2.11 Repeated Batch Fermentation

After each fermentation cycle of 36 h, the spent medium was decanted and the agar blocks were carefully collected and washed with sterile buffer (100 mM Tris containing 10% NaCl; pH 7.5) and transferred aseptically to a fresh GSL-2 broth to continue the fermentation. This process was repeated up to 5 cycles (total 180 h). Growth was measured by determining the optical density of the culture broth at 600 nm. One absorbance unit was equivalent to 0.35mg/ml dry weight of cells. All the experiments were performed in triplicates and data presented as mean ± SE.

3. RESULTS

3.1 Selection of Suitable Matrix

Among various matrices tested for cell immobilization, agar was found to be the suitable matrix for amylase production by strain TSCVKK (Fig. 1). Enzyme production under immobilized condition started at 12 h and reached a maximum level (722 mU/ml) by 36 h and then gradually decreased. In contrast the alginate immobilized cells and the control (free cells) yielded only 211 and 333 mU/ml respectively. The amount of cells leaked was more in agar immobilized cells (4.9 mg/ml of dry cells) compared to alginate immobilized cells (1.6 mg/ml of dry cells). Amylase was not produced when gelatin or polyacrylamide was used as immobilizing matrix.

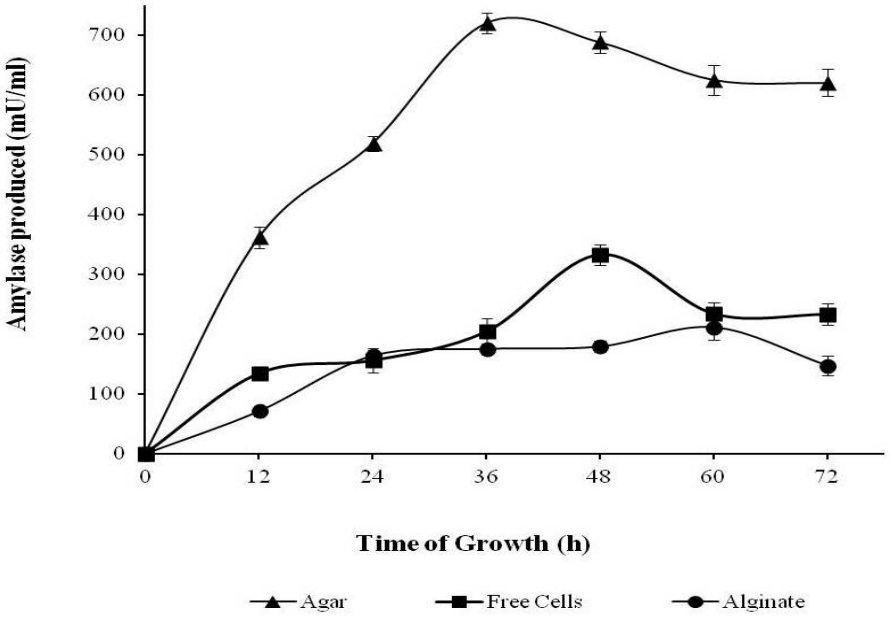

Fig. 1. Effect of different matrices on amylase production by immobilized cells of *Bacillus* sp. strain TSCVKK. -▲-, agar immobilized cells; -■-, free cells, and -●-, alginate immobilized cells

3.2 Conditions for Immobilization in Agar

Different parameters were analyzed for maximum amylase production during immobilization in agar. The age of strain TSCVKK cells used for immobilization showed a strong influence on amylase production. Amylase production of 722 mU/ml was obtained when cells in the logarithmic growth phase (24 h) were used for immobilization, whereas cells at early log phase (12 h) or at late log phase (36 h) could induce only 404 and 460 mU/ml respectively. The efficiency of immobilized system is dependent on initial cell load (ICL). ICL of 1.2% (w/v) of 24 h-grown-cells of strain TSCVKK gave highest amylase production of 722 mU/ml after 36 h of growth and it decreased at other concentrations (Fig. 2).

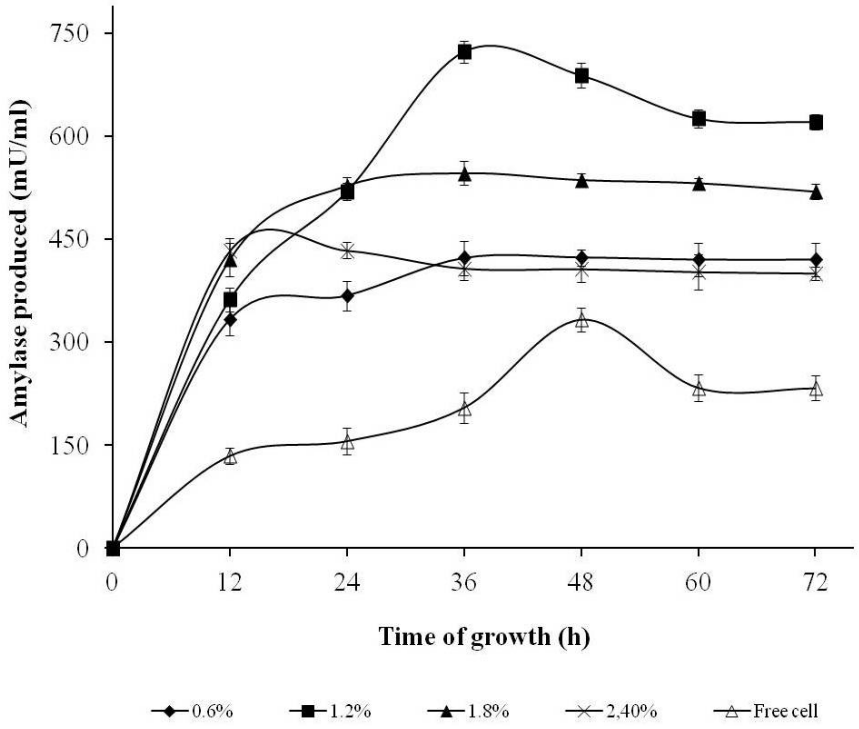

Fig. 2. Effect of different initial cell loading on amylase production by agar immobilized cells of *Bacillus* sp. strain TSCVKK (-♦-, 0.6%; -■-, 1.2%; -▲-, 1.8%; -x-, 2.4% and -Δ-, free cells)

Substrate concentration influenced amylase production by the immobilized cells of strain TSCVKK. Maximum amylase production of 832 mU/ml was obtained with 1.5% (w/v) of dextrin in the production medium. With 0.5, 1.0, and 2.0% (w/v) of dextrin, production was decreased to 369, 722, and 631 mU/ml. Agar concentration and size of agar cube influenced amylase production by the immobilized cells of strain TSCVKK. Amylase production was highest (832 mU/ml) with 2% (w/v) agar, while with 1, 3 and 4% agar it reduced to 225, 421 and 293 mU/ml respectively. Maximum amylase production (832 mU/ml) was achieved with cubes of size 4 mm^3 and was decreased to 685 and 742 mU/ml with cubes of 2 or 8 mm^3 respectively. The optimum concentration of matrix material and size of the cubes may differ with respect to organism and product of interest.

SEM images of the immobilized cells of strain TSCVKK was similar to that of free cell with no alterations in the morphology with more density of cells near the surface of agar cube (Fig. 3a, 3b & 3c).

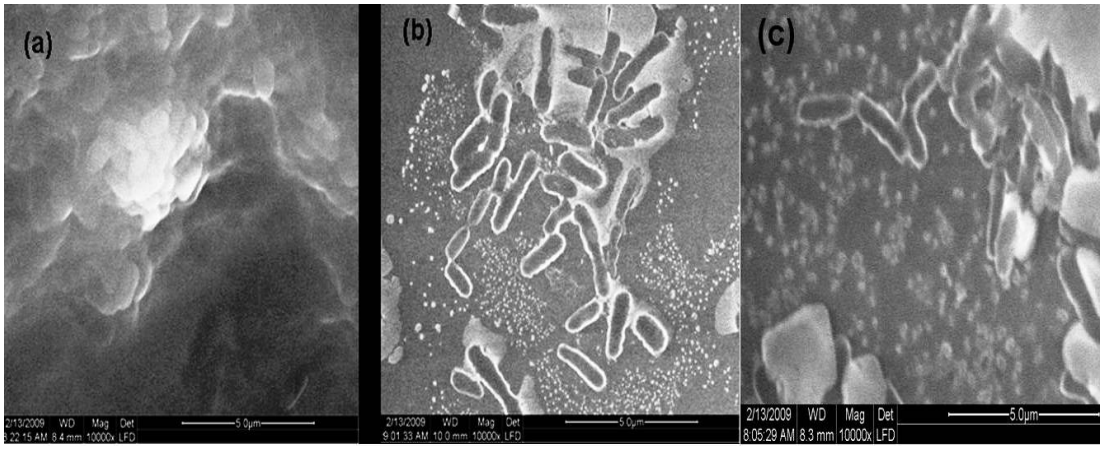

Fig. 3. Scanning electron microscopic images of *Bacillus* sp. strain *TSCVKK* after agar entrapment [Cross section of the cube (a); crushed agar cube (b) and free cells (c)

3.3 Repeated Fermentation with the Immobilized Cells

Amylase production continued to be 832 mU/ml for three cycles and gradually decreased in the 4th and 5th cycles. Cell leakage from agar cubes also increased (Fig. 4). Amount of cells leaked from agar cubes was 5 mg dry weight of cells /ml in first 3 cycles and increased to 6 mg dry weight of cells /ml by the end of 5th cycle.

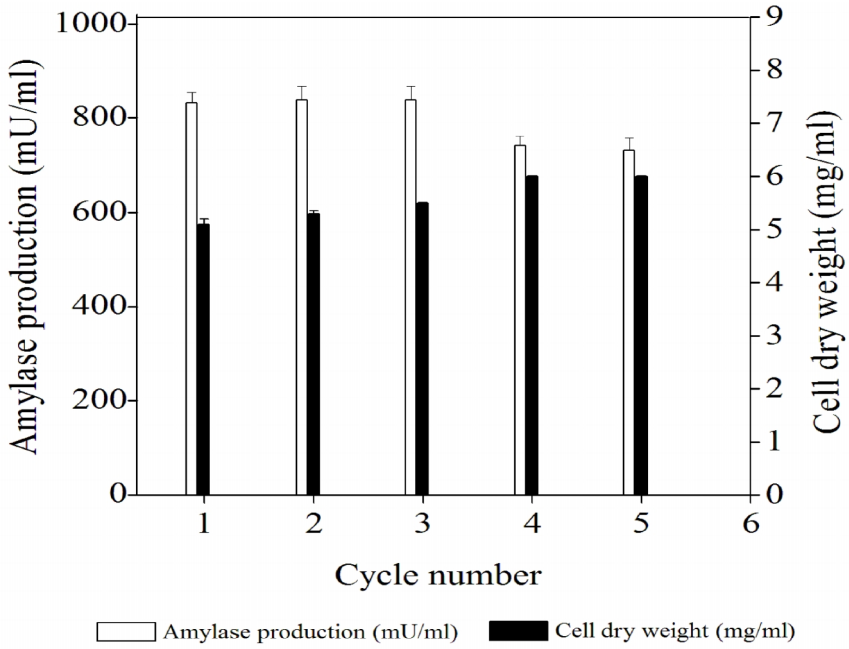

Fig. 4. Repeated batch fermentation with agar immobilized cells of *Bacillus* sp. strain TSCVKK. -□-, amylase production (mU/ml) and -■-, Cell leakage (mg/ml)

3.4 Properties of Amylase in Culture Supernatant

In order to determine the influence of immobilization on the properties of amylase secreted by the immobilized cells, properties of amylase obtained by the immobilized cells was compared with the amylase produced by free cells with respect to NaCl, pH and temperature.

3.4.1 Effect of NaCl

Amylase obtained from the immobilized cells showed no activity when the assay was carried out in absence of NaCl in the reaction mixture. Activity increased with increase in NaCl from 2 to 10%. At 10% NaCl, enzyme activity was 100% (832 mU/ml) and 98% of the activity was retained at 15% NaCl (Fig. 5a).

3.4.2 Effect of pH

Amylase obtained from immobilized cells showed 100% activity (832 mU/ml) at pH 7.5 (Fig. 5b). It retained 60% of activity at pH 9.0 suggesting alkali-tolerant nature. At pH of 6.5, 71% of activity was retained but when pH further reduced to 6.0 and 5.5 there was a drastic reduction.

3.4.3 Effect of temperature

Amylase obtained from immobilized and free cells showed 100% activity at 55ºC (832 mU/ml). More than 74% of activity was retained at 45 to 60ºC (Fig. 5c).

The above results were similar to the results obtained with the enzyme produced by free cells of strain TSCVKK.

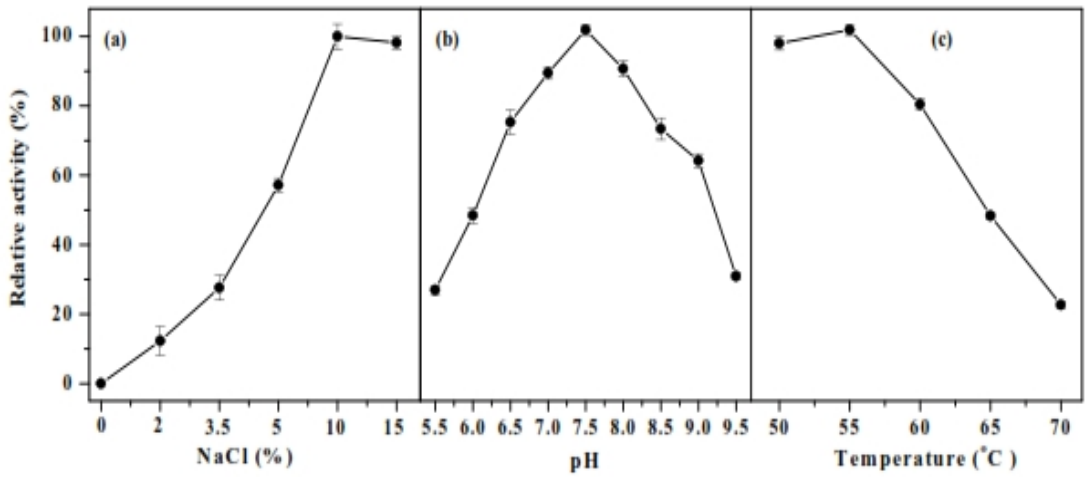

Fig. 5. Properties of alpha amylase produced by the agar immobilized cells of *Bacillus* sp. strain TSCVKK. (a) NaCl; (b) pH and (c) Temperature. Strain TSCVKK was immobilized in agar and grown in GSL-2 broth with 1.5% dextrin; 10% NaCl and 0.2% $CaCl_2$. Relative activity, 100%, is equal to 832 mU/ml

4. DISCUSSION

Selection of an appropriate matrix for cell immobilization (CI) is critical for obtaining an enhanced enzyme production (Ramakrishna and Prakasham, 1999). Alginate was shown to be the best matrix for CI for amylase, protease, xylanase and tannase production (Konsoula and Liakopoulou-Kyriakides, 2006; Kunamneni et al., 2005; Amani et al., 2007; Mohapatra et al., 2007) but use of agar entrapped cells is rare (Tonkova et al., 1994). Amylase was produced using agar beads for a thermophilic strain of *B. licheniformis* 44MB82-A (Dobreva et al., 1996). In the present study agar beads could not be used owing to an adverse effect of temperature on cells when molten agar of 50-55ºC was used for bead formation.

CI may enhance or decrease or unalter the enzyme productivity. Enhanced amylase production was shown in agar immobilized cells of *B. licheniformis* 44MB82-A (Dobreva et al., 1996). In contrast immobilized *Bifidobacterium bifidum* No.1, 791 showed lower production compared to free cells (Ryed, 2007). Agar immobilized cells of strain TSCVKK showed increased amylase production in comparison with alginate where production was decreased. This could be attributed to high substrate mass transfer rates during cell immobilization (Jamuna and Ramakrishna, 1992). Cross linking agents like bis-acrylamide monomer or glutaraldehyde used for CI with polyacrylamide or gelatin might have altered the cell physiology that hinders amylase production (Ramakrishna and Prakasham, 1999).

Age of biomass used for CI showed strong influence on amylase production. Cells of strain TSCVKK in logarithmic growth phase produced higher amylase than in early or late logarithmic growth phase. This is mainly due to cells in logarithmic growth phase are metabolically active.

CI of strain TSCVKK with higher biomass decreased the amylase production, might be due to competition between the cells that lead to substrate limitation. Immobilized cells of *Streptomyces griseoloalbus* showed decreased α-galactosidase production at higher biomass concentration (Anisha and Prema, 2008). Concentration of major carbon source influenced amylase production by the immobilized cells of strain TSCVKK. Maximum amylase was produced with 1.5% dextrin in production broth by the immobilized cells, higher than required by submerged cells that produced with 1% dextrin (Kiran and Chandra, 2008). Lower substrate concentration decreased the amylase production due to substrate limitation. High substrate concentrations are required to maintain high cell densities in immobilized systems for higher enzyme production. Increase in substrate concentration beyond 1.5% lead to a decrease in amylase production due to catabolite repression in strain TSCVKK (Kiran and Chandra, 2008) or by the acid end products accumulated during fermentation. Immobilized cells of *B. licheniformis* KBR6 produced maximum tannase with 2% tannin in the growth medium than free cells that produced maximum tannase with 1.5% tannin (Mohapatra et al., 2007).

Concentration of matrix material determines the efficiency of CI as it influences diffusion properties and strength of the matrix material. Higher agar concentration lead to decreased amylase production due to decreased diffusion of nutrients in and out of the matrix material as noticed in *S. griesioloalbus* (Anisha and Prema, 2008). In *B. licheniformis* NCIM-2042, increase in matrix concentration decreased protease production (Potumarthi et al., 2008) whereas in *B. licheniformis* 44MB82-A, amylase production was highest with 4% agar (Dobreva et al., 1996). SEM examination of agar entrapped cells of strain TSCVKK showed a random distribution of rods and the cells were densely distributed (Fig. 3a). Cells in agar blocks were clustered in the areas of growth in furrows of agar block as reported by other

researchers (Kocher and Mishra, 2009). Therefore agar immobilization provided a suitable micro-environment to strain TSCVKK for its growth without changing cell morphology (Fig. 3b and 3c). To the best of our knowledge there was no report on SEM of halophilic amylase producing agar entrapped cells although vast literature exists on alginate entrapped non-halophilic cells. Immobilized cells of strain TSCVKK could be able to produce amylase continuously for 5 cycles without much decrease in the production. In a similar study with immobilized *B*. *licheniformis* 44MB82-A amylase was repeatedly produced for 5 cycles (Dobreva et al., 1996). Maximum amylase was produced under immobilized conditions by strain TSCVKK at the end of 36 h, which was 12 h less than the time taken by cells in submerged fermentation (48 h) (Kiran and Chandra, 2008). This is significant because 12 h could be saved in each fermentation cycle. In *S*. *griseoloalbus* the time taken for highest α-galactosidase production was reduced by 24 h on immobilization (Anisha and Prema, 2008). Difference in the course of amylase biosynthesis between immobilized cells and free cells could be due to altered physiology and metabolism of cells under immobilized conditions (Ramakrishna and Prakasham, 1999).

Immobilization of strain TSCVKK in agar did not alter the properties of amylase. The properties were similar to that produced by free cells suggesting the production of a stable biocatalyst by the immobilized cells.

5. CONCLUSION

In summary, under immobilized conditions in 2% agar of cube size $4mm^3$, with 1.5% dextrin, 10% NaCl, pH 8.0 at 30ºC, *Bacillus* sp. strain TSCVKK produced 832 mU/ml of halophilic; alkali-tolerant, detergent and surfactant stable alpha amylase. This was 1.4 fold higher in comparison to our earlier report on amylase production using free cells in submerged fermentation (592 mU/ml) (Kiran and Chandra, 2008). This is the first report on amylase production by immobilized *Bacillus* sp. strain TSCVKK and only the second report on halophilic amylase production by any halophilic bacterium through cell immobilization, the first one being amylase production by an extreme halophile, *H. salinarium* (Bagai and Madamwar, 1997). This amylase can be useful in harsh industrial processes where low water activity would inhibit enzymatic conversions and also in detergent, pharmaceutical industries and in food industry for the fermentation of salted foods.

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COMPETING INTEREST

Authors have declared that no competing interests exist.

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