

THERMAL STABILITY OF CELL AND α -AMYLASE PRODUCED BY *BREVIBACILLUS BORSTELSENSIS* R1 IN DIFFERENT IMMOBILIZATION MEDIA

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ABSTRACT

Oceans contain diverse microbial habitat. Bacteria have been regarded as treasure of many useful enzymes viz., amylases, proteases, lipases, hydrolases and reductases. Among them amylolytic enzymes have great biotechnological applications and economic exploitations. Extension of the use of immobilized enzymes to other practical processes requires new methodologies and a better understanding of current techniques. By cell/enzyme immobilization on carrageenan and sodium alginate there was an increase in α -amylase production by more than 100% at 60°C, the property of which is useful in food processing industries. The α -amylase was found to be useful in bakery, food, fodder for poultry, automation dishwashing and laundry industries. The α -amylase was also useful in solving the problem of

water pollution of industrial effluents and sewage water by hydrolyzing the substrates.

KEY WORDS: α -amylase, *Brevibacillus borostelensis* R1, agar-agar, agarose, acrylamide, sodium alginate and carrageenan.

INTRODUCTION

Cell/enzyme immobilization is defined as confining the enzyme molecules to a distinct phase from the one in which the substrates and the products are present; this is achieved by fixing the enzyme molecules to or within some suitable material ^[1]. The characteristics of the matrix are of paramount importance in determining the performance of the immobilized enzyme system. Ideal support properties include physical resistance to compression, hydrophilicity,

inertness towards enzymes, ease of derivatization, biocompatibility, resistance to microbial attack, and availability at low cost ^[2]. There are four principal methods available for immobilizing cells/enzymes: adsorption, covalent binding, entrapment and membrane confinement.

In adsorption method the enzyme molecules adhere to the surface of the carrier matrix due to combination of hydrophobic effects and the formation of several salt links per enzyme molecules. The binding of enzyme molecules to the carrier matrix is usually very strong, but it may be weakened during the maintenance of many factors like addition of substrate, pH and ionic strength. Loading of about 1gm enzyme/gm matrix is possible.

In covalent binding method the enzyme molecules are attached to the carrier matrix by the formation of covalent bonds. As a result, the strength of binding is very strong and there is no enzyme loss during use. The covalent bond formation occurs with the side chains of amino acids of the enzyme, their degree of reactivity being dependent on their charged status. Enzyme loading is quite low 0.02-0.3gm/gm of matrix. Matrix used is agarose, cellulose, sepharose and other polysaccharides.

In entrapment method enzyme molecules are entrapped within suitable gels or fibers and there may or may not be covalent bond formation between the enzyme molecules and the matrix. Acrylamide and bisacrylamide are mixed to form a gel containing the entrapped enzyme which may be used to form small beads or a film on a solid support. Entrapment in calcium alginate is the most widely used method. Enzyme loading is very high 1gm/gm of gel or fiber. The entrapment method is based on the occlusion of an enzyme within a polymeric network that allows the substrate and products to pass through but retains the enzyme ^[4].

In membrane confinement, the enzyme molecules are usually in an aqueous solution may be confined within a semipermeable membrane which, ideally, allows a free movement in either direction to the substrate and products but does not permit the enzyme molecules to escape. Immobilized enzymes typically have greater thermal and operational stability than the soluble form of the enzyme, used in non-aqueous systems, support continuous production system, reduce effluent handling problems during recovery and enzyme is used at much higher concentration. The entrapment technique reported to improve productivity ^[4]. Many applications of immobilized cells/enzymes from bacteria in different matrices by various

immobilization methods have been reported by Betancor *et al.* ^[5]. Immobilization of amylase and cells were reported in agar and agarose ^[6], sodium alginate ^[7] and carrageenan ^[8].

MATERIALS AND METHODS

Collection of the marine water samples

Marine water samples were collected from Rushikonda, coastal area of Visakhapatnam, Andhra Pradesh, India. The water samples were collected in sterile BOD bottles (Borosil) and brought to the lab, stored in the refrigerator until it was used.

Primary screening of α -amylase producing Bacteria

The collected marine water samples were diluted by serial dilution technique. The diluted samples of 10^{-4} to 10^{-6} (0.1ml) were spread with L-shaped glass rod by spread plate technique on the starch agar plates. After incubation at 37°C for 24hours, the plates were flooded with Lugol solution (1% iodine in 2% potassium iodide w/v) ^[9]. The zone of hydrolysis measuring more than 11mm were selected for further screening of amylase activity. The potent bacteria showing more than 11 mm were selected and studied in detail for identification by referring the standard description of Bergey's manual of determinative bacteriology ^[10].

Estimation of amylase by DNS method

The selected amylase producing bacteria cultured in nutrient broth (NB) were further screened by estimation of α -amylase activity according to 3, 5 dinitro salicylic acid (DNS) method ^[11]. One unit of enzyme activity is defined as the amount of enzyme that releases 1.0 mmol of reducing sugar (maltose) per minute under the assay conditions.

Cell and enzyme immobilization

The cells of *Brevibacillus borstelensis* R1 and α -amylase enzyme were immobilized with different agents. Then the immobilized cells of *Brevibacillus borstelensis* R1 and α -amylase were incubated in orbital shaking incubator (120rpm) for 24hrs at different temperatures under optimized conditions- Pikovskaya's medium (Himedia) at pH 7.0, 1% salinity, 5% jaggary, 1% beef extract (Himedia) and 0.8% calcium chloride (Merck).The cell/enzyme immobilization was studied to understand the thermal stability and increase of α -amylase production by *Brevibacillus borstelensis* R1.

Immobilizing Media Used And Methods Of Immobilization

Agar-Agar

Hundred ml of sterile 3% agar solution was prepared and allowed to cool upto 45⁰C. In cell immobilization 10 loopful of *Brevibacillus borstelensis* R1 culture was added to 10ml of agar solution, mixed thoroughly for homogeneous distribution of cells, then poured in a sterilized petri plate and allowed to solidify. After solidification, the agar was cut with sterile knife into 1cm³ blocks. Ten grams of cell immobilized blocks were added to 100ml of SmF production medium in a sterilized erlenmeyer flask and incubated. In enzyme immobilization instead of culture, 2.0 ml of crude supernatant (α -amylase) of *Brevibacillus borstelensis* R1 was mixed thoroughly with 10.0ml (3%) agar solution and the same procedure was followed.

Agarose

Agarose is a polysaccharide which contains alternating 1, 3 linked D Galactopyranose and 1, 4- linked 3, 6-anhydro-L- Galactopyranose (Linear polymer). The above procedure was followed for immobilization of cell/enzyme.

Acrylamide

Polyacrylamide gel-aqueous solutions of acrylamide, N'-N'-ethylene bisacrylamide were polymerized in presence of suitable initiator and accelerator. Cell/enzyme was entrapped within lattice of 10ml of 10% polyacrylamide gel [3.3ml acrylamide-bis-acrylamide stock solution (30:0.8), 2.5ml resolving gel buffer stock solution (1.5M Tris pH 8.8), 4.0ml H₂O, 0.004ml TEMED and 0.1ml 10% ammonium persulfate (freshly prepared)]. Ten loopful of *Brevibacillus borstelensis* R1 culture in 10ml of the polyacrylamide solution was added in a beaker and mixed thoroughly for homogeneous distribution and poured in a petriplate. The gel was allowed to stand for 30min for polymerization. The acrylamide gel was cut into 1cm³ blocks. Ten grams of cell immobilized blocks were added to 100ml of SmF production medium in an erlenmeyer flask and incubated at different temperatures in orbital shaking incubator. In enzyme immobilization, 2.0ml of crude supernatant (α -amylase) of *Brevibacillus borstelensis* R1 was added to 10.0ml of the polyacrylamide solution in a beaker and mixed thoroughly for homogeneous distribution and the above procedure was followed.

Sodium Alginate

Bacteria get entrapped in a polymeric network formed by ionic cross-links in alginate. Alginate is a hydrophilic polysaccharide. It has an interrupted structure of stretches of alpha - 1, 4 linked alpha di-gluco pyranosyluronic acid residues and stretches of beta-1, 4 linked

manno pyranosyluronic acid residues stretches where both uronic acids occur in alternate sequences. Alginate solution produces gel in presence of calcium ion (sensitive to PO_4^{2-}). Addition of Ca^{2+} ions induces interactions between the polysaccharide chains of alginate forming a network.

Hundred ml of 3% alginate solution was prepared and sterilized. The alginate was boiled in a water bath until the slurry was formed and it was cooled. Five ml of sterile slurry was taken in a 25ml beaker. In cell immobilization 5 loopfuls of pure culture of strain was added and thoroughly mixed for homogeneous distribution of cells. Five milliliters sterile syringe was used to drop alginate drop by drop in 50ml CaCl_2 (0.1M) solution in a beaker for the formation of beads. The beads (Calcium alginate) were allowed to stay for 30minutes for stabilization. Calcium chloride solution was discarded and the beads were rinsed in distilled water thrice. Extra beads can be stored in normal saline (0.85% NaCl) solution. In enzyme immobilization, 2.0ml of crude supernatant (α -amylase) of *Brevibacillus borstelensis* R1 was added to 10ml of alginate and thoroughly mixed for homogeneous distribution in slurry instead of culture and the above procedure was followed. Ten grams of cell/enzyme immobilized beads (about 15 beads weigh one gram) were added to 100ml SmF production medium in an erlenmeyer flask and incubated.

Carrageenan

Carrageenan is a linear sulphated polysaccharide containing D-galactose, 3, 6-anhydro-D-galactose and their sulphate ester derivatives. Among carrageenans, K^+ -carrageenan is insoluble in cold water. The procedure for cell and enzyme immobilization was carried out as above for alginate. The production medium of cell immobilized beads was incubated in orbital shaking incubator (120rpm) at different temperatures (4°C , 25°C , 37°C , 50°C and 60°C) for 24hrs. After incubation, the beads were separated by filtration and the medium was subjected to centrifugation at 5,000 rpm for 15 minutes at room temperature. The supernatant was collected in sterile test tube and the pellet was discarded. The supernatant (0.5 ml) was used for the amylase assay by DNS method. Thermal stability of immobilized enzyme activity at different temperatures was studied by taking triplicate sample assay. The enzyme immobilized beads were incubated in orbital shaking incubator at 120rpm with starch as substrate at different temperatures (4°C , 25°C , 37°C , 50°C and 60°C) for 15minutes. The amylase activity was determined by DNS method.

RESULTS

Cell Immobilization

Five cell immobilization media, agar-agar, agarose, acrylamide, sodium alginate and carrageenan were used at different temperatures; 4°C, 25°C, 37°C, 50°C and 60°C to study the increase in α -amylase activity by the immobilized cells of *Brevibacillus borostelensis* R1 (Figures 1 a-f). By cell immobilization the α -amylase activity was found to be increased in agar-agar by 19.8% and 16.5% at 4°C and 37°C, respectively. The enzyme activity in agarose enhanced by 5.5% and 1.7% at 4°C and 37°C respectively. In acrylamide it was increased by 35.6%, 8.4%, 10.6%, 75.2% and 97% at 4°C, 25°C, 37°C, 50°C and 60°C respectively. In sodium alginate the enzyme activity enhanced by 10.1%, 8.3%, 7.9%, 97.5% and 107.9% at 4°C, 25°C, 37°C, 50°C and 60°C respectively. In carrageenan the enzyme activity was increased by 20.4%, 10.6%, 88.3% and 103.13% at 4°C, 37°C, 50°C and 60°C respectively. The highest α -amylase activity obtained is shown in table 1.

Table- 1. Immobilization of *Brevibacillus borostelensis* R1 at optimal temperatures in different media

Immobilization media	Temperature(°C)	Amylase activity (U/ml) in control	Amylase activity (U/ml) in test	% of increase in enzyme activity
Sodium alginate	60	2041±32	4245±43	107.9
Carrageenan	60	2041±32	4146.±32	103.13
Acrylamide	60	2041±32	4021±16	97
Agar-agar	4	1220±35	1462±36	19.8
Agarose	4	1220±35	1265±9	5.5

Values presented in the table are means of triplicates±SD.

Table -2. Enzyme activity of *Brevibacillus borostelensis* R1 at optimal temperatures in different immobilization media

Immobilization media	Temperature (°C)	Amylase activity (U/ml) in control	Amylase activity (U/ml) in test	% of increase in enzyme activity
Acrylamide	60	2141±32	4818±32	125
Sodium alginate	50	2019±32	4128±28	104.4
Carrageenan	50	2019±32	3627±23	79.6
Agar-Agar	50	2019±32	3019±32	49.5
Agarose	50	2019±32	2073±12	2.6

Values presented in the table are means of triplicates±SD.

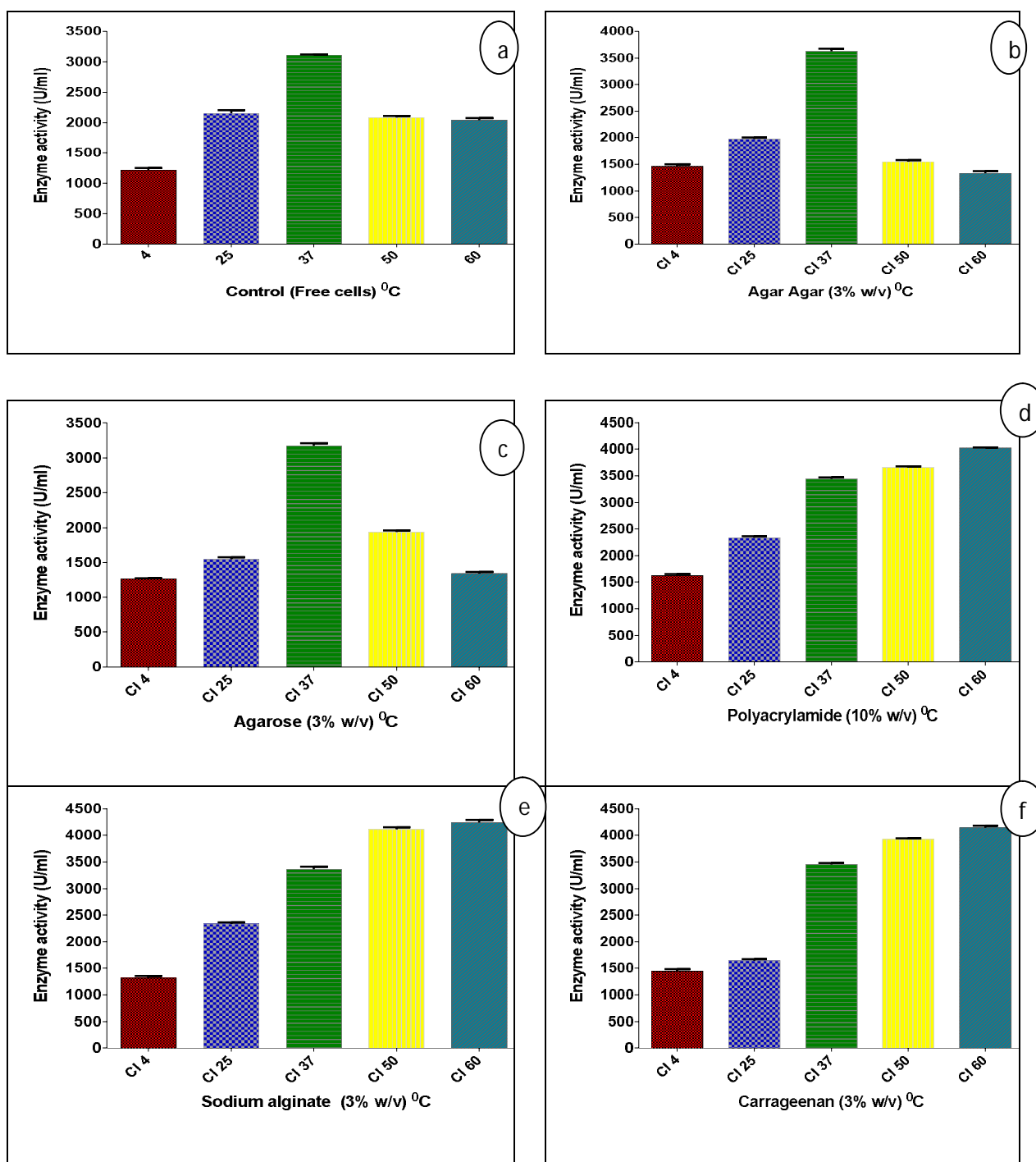


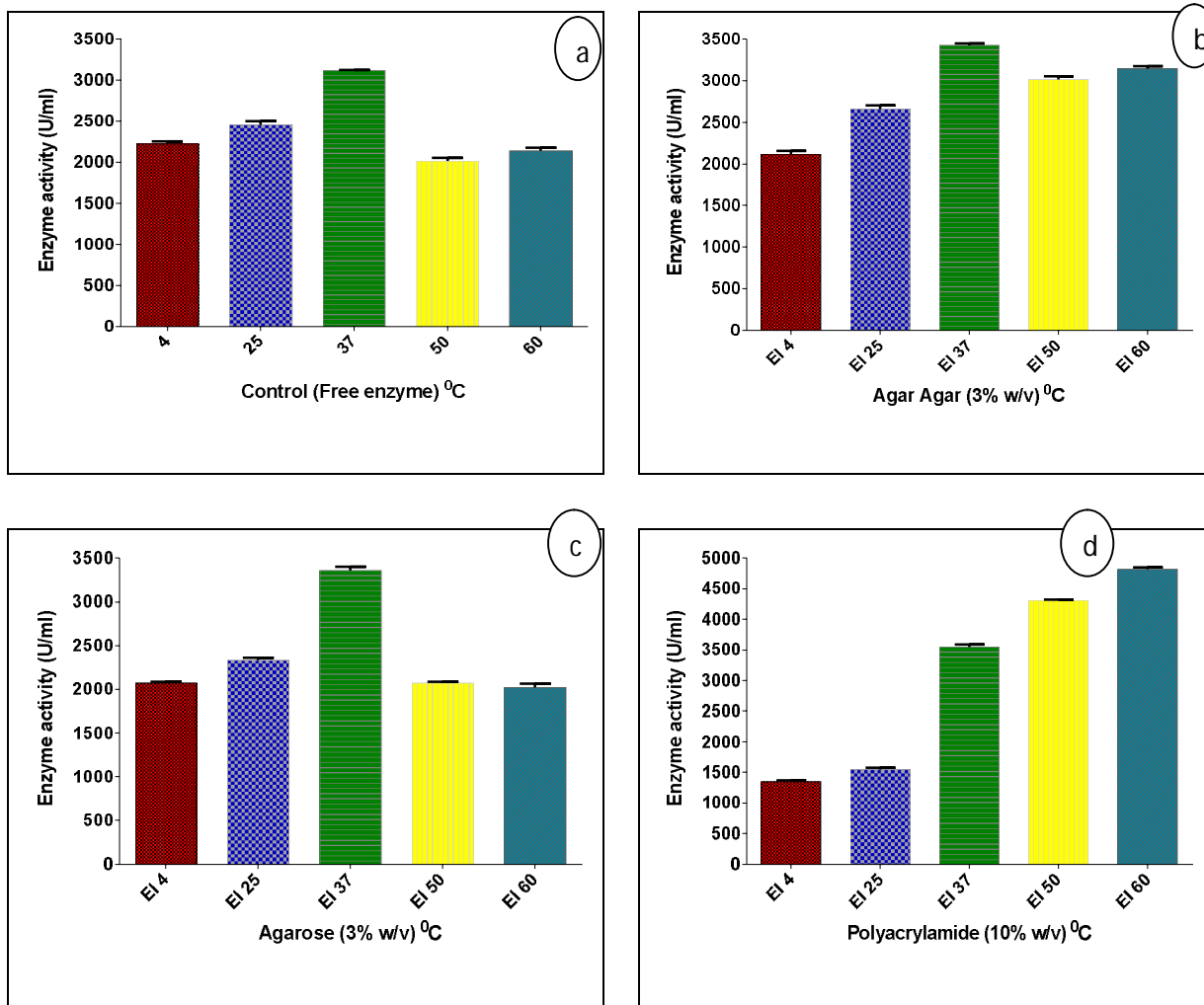
Fig: 1 Optimization of α -amylase production by Free (F) and Cells Immobilized (CI) of *B. borstelensis* R1 in different immobilized media at different temperatures. (a) Control (b) Agar-Agar (c) Agarose (d) Acrylamide (e) Sodium alginate and (f) Carrageenan.

Y bars indicate the standard deviation of mean value.

**** $P < 0.0001$ Values differ significantly at $p < 0.05$.

Enzyme Immobilization

Five enzyme immobilization media, agar-agar, agarose, acrylamide, sodium alginate and carrageenan were used at different temperatures; 4°C, 25°C, 37°C, 50°C and 60°C to study the increase in α -amylase activity by *Brevibacillus borostelensis* R1 (Figures 2 a-f). By enzyme immobilization the α -amylase activity was found to be increased in agar-agar by 8.14%, 10%, 49.5% and 46.7% at 25°C, 37°C, 50°C and 60°C respectively. The enzyme activity in agarose was enhanced by 7.8% and 2.6% at 37°C and 50°C respectively. In acrylamide it was increased by 3.8%, 113.7% and 125% at 37°C, 50°C and 60°C respectively. In sodium alginate the enzyme activity enhanced was by 8.6%, 6.6%, 13.7%, 104.4% and 102% at 4°C, 25°C, 37°C, 50°C and 60°C respectively. In carrageenan the enzyme activity was increased by 11.6%, 4.5%, 79.6% and 9.3% at 25°C, 37°C, 50°C and 60°C respectively. The highest amylase activity obtained is shown in table 2.



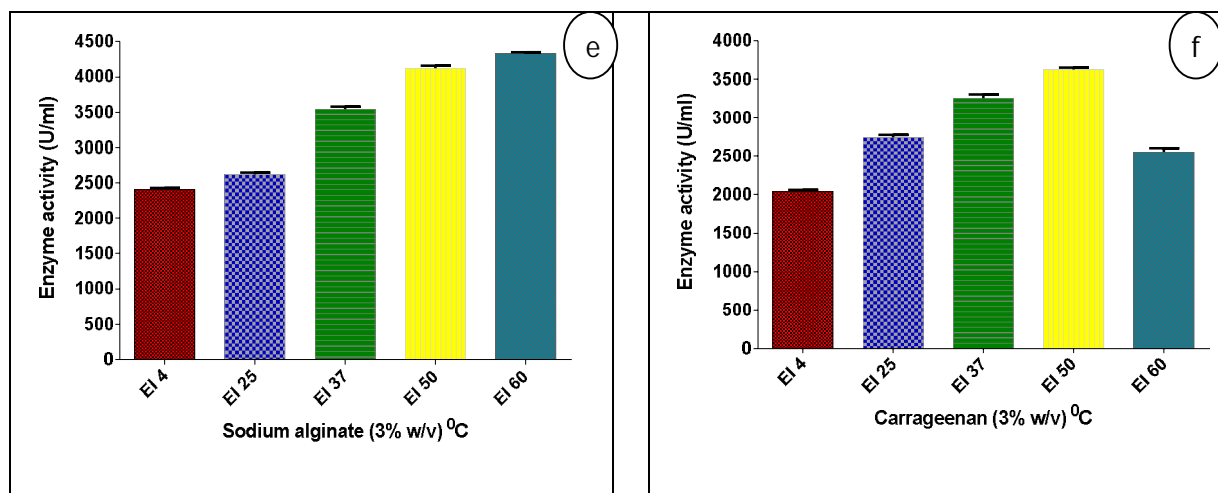


Fig: 2 Free (F) enzyme (α -amylase) and Enzyme Immobilized (EI) of *B. borstelensis* R1 in different immobilized media at different temperatures. (a) Control (b) Agar-Agar (c) Agarose (d) Acrylamide (e) Sodium alginate and (f) Carrageenan.

Y bars indicate the standard deviation of mean value.

**** P < 0.0001 Values differ significantly at p<0.05.

DISCUSSION

Sodium alginate is found effective in increasing thermal stability of immobilized *Brevibacillus borostelensis* R1. Cell immobilization in alginate was reported in *Bacillus licheniformis* [12] and in *Bacillus circulans* GRS 313 [13]. Jamuna & Ramakrishna [14] reported maximal enzyme yield (90.0 U/ml) at 4% (w/v) alginate concentration. Goksungur & Zorlu [15] studied that *Bacillus subtilis* had produced thermostable α -amylase by entrapping in calcium alginate gel capsules. Lamas *et al.* [16] reported that *Bacillus sp.* when immobilized in 1% sodium alginate gave highest enzyme yield. Konsoula & Liakopoulou-Kyriakides [17] reported the increase of thermal stability of cells immobilized in carrageenan by using entrapment technique. Alpha-amylase immobilized at 3% concentration of acrylamide (10%) media have produced increased production of amylase with increased optimum temperature to 60°C. Mahajan *et al.* [18] checked the suitability of amylase entrapped in agarose beads for use in pharmaceutical industry. Raviyan *et al.* [19] reported the thermal stability of *Bacillus licheniformis* in agar medium. Abou-Elela *et al.* [20] studied thermal stability of α -amylase from *Aspergillus oryzae* entrapped in polyacrylamide gel at 50-70 °C. The thermal stability of amylase was increased when immobilized in sodium alginate to 50°C [21] and 60°C [22] in *Bacillus species*.

CONCLUSION

The α -amylase activity in different immobilization media was studied. By cell immobilization, the α -amylase activity was found to be increased by 5.5%, 19.8%, 97%, 103.13%, 107.9% in agarose, agar-agar, acrylamide, carrageenan, sodium alginate, at 4⁰C, 4⁰C, 60⁰C, 60⁰C and 60⁰C respectively. By enzyme immobilization, the α -amylase activity was found to be increased by 2.6%, 49.5%, 79.6%, 104.4%, 125% in agarose, agar-agar, carrageenan, sodium alginate, acrylamide at 50⁰C, 50⁰C, 50⁰C, 50⁰C and 60⁰C respectively.

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