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Screening of polysaccharides for preparation of α-amylase conjugate to enhance stability and storage life

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ABSTRACT

Nine polysaccharides differing in structure and chemical nature were screened for their ability to conjugate with α-amylase by covalent binding for enhancing the thermal and pH stability of α-amylase. Among these polysaccharides, agar, dextran, pectin and xanthan showed better results but dextran stood out among all the polysaccharide for providing both thermal and pH stability to α-amylase. α-Amylase conjugated with agar, dextran, pectin and xanthan showed antimicrobial property with added preservative (0.2% sodium benzoate) in liquid formulation of α-amylase challenged with *Bacillus subtilis* and *Escherichia coli*. Dextran was the only polysaccharide which showed significant reduction of microbial growth of challenged organisms and aerobic flora without any preservative added. Aerobic flora could flourish well in the liquid α-amylase, but low temperature (4 °C), dextran, and preservative showed synergistic effect in efficiently increasing the storage life of liquid α-amylase.

1. Introduction

Naturally occurring enzymes are often not suitable for biocatalytic processes at industrial level as they get inactivated and/or degraded at extreme temperatures and pH (Dalby, 2007; Iyer & Ananthanarayan, 2008). Many different approaches have been used till date for improvement of enzyme stability in extreme conditions (Fágáin, 2003). Enzyme–polysaccharide interaction is one such approach where the polysaccharides provide rigidity (Klibanov, 1983) and hydration (Srivastava, 1991) to enzymes on conjugation and enhance its stability. Different polysaccharides have been used for many different enzymes to increase their thermal and pH stability. Binding of invertase and cellulase to chitosan (Darias & Villalonga, 2001; Gomez, Ramirez, & Villalonga, 2000) and horseradish peroxidase and glucose oxidase to dextran by non-covalent linkages (Altikatoglu & Kuzu, 2010) have shown improved thermal and pH stability. Covalent binding of amylase to carboxymethyl cellulose (Villalonga, Gomez, Ramirez, & Villalonga, 1999) and dextran (Lenders & Crichton, 1984) has been reported to be successful.

Since each polysaccharide differs in structure and chemical nature, they may bind in a unique and specific way to the same enzyme and thereby provide different levels of thermal and pH stability to the respective enzyme. It is therefore necessary to find the best possible polysaccharide for the enzyme of interest to prepare stable conjugate by covalent binding between the enzyme and polysaccharide. Our previous report on conjugation of α-amylase to dextran showed thermal and pH stability and alteration in the structure (Jadhav & Singhal, 2012). In this work, we screened nine different polysaccharides for conjugation with α-amylase under previously optimized conditions to enhance its stability.

Besides process stability, enzyme–polysaccharide conjugates may also show improved storage stability (Marshall, 1980). Liquid enzymes are more useful over the powder formulations because of ease of preparation and minimal cost of production. For temporary storage, enzymes are generally stored in liquid form. Since liquid enzymes lose 10–20% of its activity during 4–6 months storage, stabilization is a major concern. The general recommendation for storage of enzymes is under refrigeration conditions. Microbial contamination is one of the problems during storage of liquid enzymes during which the contaminating organisms produce wild proteases that can degrade enzyme significantly and utilize them for growth. Moreover water present in liquid enzyme preparations also favours the growth of microorganism. Microbial growth can be inhibited by using some additives (Illanes, 2008). Some additives like glycerol and trehalose protect the enzyme from denaturation by providing protective layer around the enzyme molecules. Since enzyme–enzyme and enzyme–water interactions are mostly

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responsible for microbial contamination in liquid enzymes, it is necessary to reduce these interactions (Chaplin & Bucke, 1990). Enzyme–polysaccharide interactions may alter these unwanted interactions during storage of an enzyme. Reports on antimicrobial activity of polysaccharides are known in scientific literature. Polysaccharides isolated from fungi *Sarcodon imbricatus* are known to possess antibacterial activity (Sulkowska-ziaja et al., 2011). Chitosan based matrices are also reported to show antibacterial activity on oral pathogen (Sarasam, Brown, Khajotia, Dmytryk, & Madihally, 2008). Increased antimicrobial properties against *Escherichia coli* and *Staphylococcus aureus* have been observed after conjugating lysozyme with dextran (Amiri, Ramezani, & Aminlari, 2008).

Crude α-amylase in liquid form favours growth of aerobic flora as well as challenged/added organisms. We did not find any study on microbial growth in liquid formulations of α-amylase. Hence, we undertook a detailed experimental study to observe the effect of enzyme *vis-à-vis* enzyme–polysaccharide conjugates on the growth of microorganisms in the liquid formulations of corresponding α-amylase solutions. In this process, we screened several polysaccharides and focussed on α-amylase–dextran interactions for increasing storage life of α-amylase and suggesting possible mechanisms for the observed effects.

2. Experimental

2.1. Materials

Crude α-amylase from *Bacillus licheniformis* (350 U/mg) was obtained as gift sample from Sigma Chemical, Mumbai, India. It was dialyzed against 20 mM of sodium citrate buffer of pH 5.4 at 4 °C overnight and then used for study. All polysaccharides (agar, carrageenan, carboxymethyl cellulose, dextran, gellan, guar gum, gum Arabic, pectin and xanthan) were purchased from reliable sources. Sodium metaperiodate was obtained from S.D. Fine Chemicals, Mumbai. All other chemicals were of AR grade and procured from reliable sources. *Bacillus subtilis* and *E. coli* were used as model Gram positive and Gram negative organism, respectively, to study storage lifetime of α-amylase.

2.2. Preparation of α-amylase polysaccharide conjugate

Sodium metaperiodate (0.1 M) solution was prepared in 0.1 M sodium acetate buffer of pH 5.0 and used as the oxidizing solution. Polysaccharides *viz*. agar, carrageenan, carboxymethyl cellulose (CMC), dextran, gellan, guar gum, gum Arabic, pectin, and xanthan, 25 mg each was oxidized in 10 ml of oxidizing solution in dark for 90 min, after which the oxidation was stopped by adding 0.3 ml of ethylene glycol, and kept for 1 h in dark. Oxidized polysaccharide solutions were dialyzed against 0.1 M sodium acetate buffer of pH 5.0 at 4 °C overnight. Reducing sites generated on polysaccharide was analysed by 3, 5-dinitrosalicylic acid (DNSA) test (Miller, 1959) to determine the extent of oxidation of the polysaccharide (Lenders & Crichton, 1984). α-Amylase solution (protein concentration of 0.25 mg/ml) was prepared in buffer of pH 5.0 and mixed with equal volume of each oxidized polysaccharide solution and kept for conjugate formation for 20 h at ambient temperature (~28 ± 2 °C) as reported in our previous work (Jadhav & Singhal, 2012). Sodium borohydrate (20 mg) was then added to 10 ml of conjugate mixture to reduce remaining oxidized sites of polysaccharide and kept for 4 h. Finally, all the prepared conjugate solutions were dialyzed against 20 mM of sodium citrate buffer of pH 5.4 at 4 °C overnight (Ahmed, Saleh, & Abdel-Fattah, 2007; Srivastava, 1991; Villalonga et al., 1999). These conjugates were used for analysing the activity and stability of α-amylase.

2.3. α-Amylase activity assay

Enzyme sample (0.5 ml, appropriately diluted) was added in 0.5 ml of 1% starch solution prepared in 0.02 M sodium-citrate buffer of pH 5.4. Mixture was kept at 50 °C for 10 min, DNSA reagent (0.5 ml) added and kept in a boiling water bath for 15 min. Samples were chilled quickly followed by addition of 4.5 ml of distilled water. The absorbance was measured at 540 nm. Enzyme activity was calculated using a standard curve plotted using maltose in the range of 0.0–1.0 mg/ml. One unit of enzyme activity was defined as the micromole of maltose released per minute per ml of enzyme solution at 50 °C.

Protein in the sample was detected using Folin–Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) and it was calculated using bovine serum albumin as a standard in the range of 0.2–1.0 mg/ml.

2.4. Effect of conjugation of α-amylase with different polysaccharides on thermal and pH stability

The free and conjugated α-amylase solutions (2 μg/ml of protein) were evaluated for thermal stability by incubating at 60 °C, 70 °C and 80 °C in 20 mM sodium citrate buffer of pH 5.4 for 15 min followed by quick chilling on ice. The enzyme activity was then assayed and percent residual activity was calculated with respect to their individual original activities Individual original activities were referred to as activities of enzyme and/or enzyme–polysaccharide conjugates under standard assay conditions.

Free and conjugated α-amylase (20 μg/ml of protein) were incubated at ambient temperature in 50 mM citric acid/sodium citrate buffer of pH 3.0–6.0, 50 mM NaH_2PO_4/Na_2HPO_4 buffer of pH 7.0–8.0, 50 mM glycine/NaOH buffer of pH 9.0–10.0. The samples were incubated for 1 h, diluted appropriately in assay buffer and tested for α-amylase activity.

2.5. Effect of conjugation of α-amylase with polysaccharides on its storage life

2.5.1. Effect on the growth of Bacillus subtilis (a model Gram positive organism) and Escherichia coli (model Gram negative organism) in the free and conjugated α-amylase solutions

α-Amylase was conjugated with agar, dextran, pectin and xanthan individually as described in Section 2.2 to prepare four different conjugate solutions. Free and conjugated α-amylase solutions were transferred in sterile 150 ml Erlenmeyer flasks followed by addition of sodium benzoate (0.2%) as a preservative. In order to study the effect of preservative on the growth of organism in the enzyme solutions, similar preparations without any addition of preservative was carried out. All solutions were inoculated with 18 h old culture of *B. subtilis* and incubated at ambient temperature. Growth was observed by measuring absorbance at 600 nm after regular time intervals of 24 h (48–120 h).

Similar experiment was conducted to study growth of *E. coli* in the free and conjugated α-amylase using 18 h old culture of *E. coli.*

2.5.2. Study of growth of aerobic flora in the free and dextran conjugated α-amylase solutions at ambient temperature and 4 °C

Free α-amylase and dextran conjugated α-amylase solutions were prepared as described in Section 2.2, and used with and without preservative (0.2% sodium benzoate) for the study. All the sample solutions were stored at ambient temperature and 4 °C. Samples kept at ambient temperature were analysed for absorbance at 600 nm at 24 h interval, whereas samples at 4 °C were analysed after every 10 days for a period of 60 days.

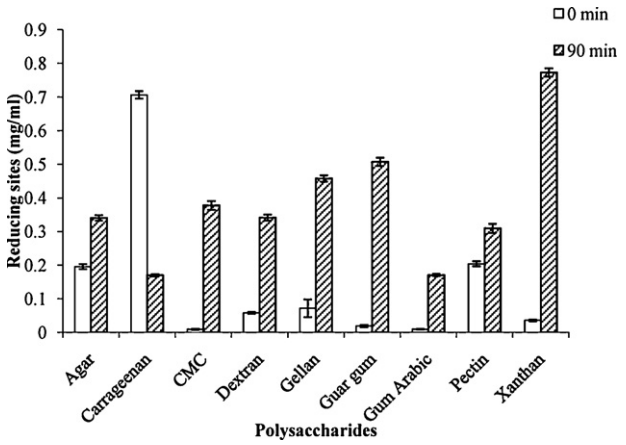

Fig. 1. Reducing sites generated on the polysaccharides after oxidation with 0.1 M sodium metaperiodate for 90 min.

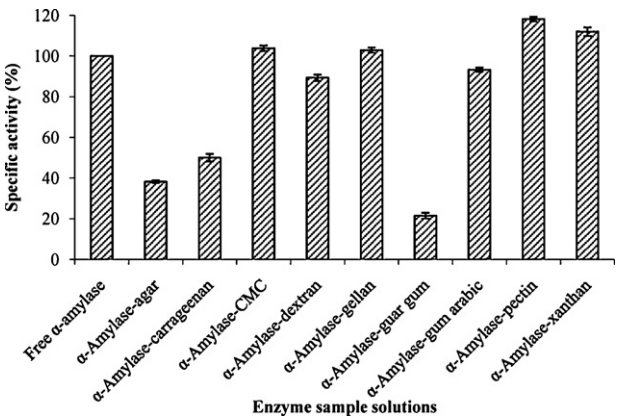

Fig. 2. Percentage of specific activity of α-amylase after conjugating with different polysaccharides.

3. Results and discussion

3.1. Extent of oxidation of different polysaccharides using sodium metaperiodate

Nine different polysaccharides including agar, carrageenan, CMC, dextran, gellan, guar gum, gum Arabic, pectin and xanthan were oxidized using 0.1 M sodium metaperiodate for 90 min. Oxidation of polysaccharide generate aldehydes which are reactive functional groups (Sanderson & Wilson, 1970) and can be analysed using DNSA test. The oxidized form of polysaccharide can be calculated in terms of mg/ml of reducing aldehyde groups present on the polysaccharide. Different levels of oxidation were found for each polysaccharide (Fig. 1). Maximum oxidation and formation of more reducing sites were observed in case of xanthan followed by guar gum, gellan and CMC. Pectin showed minimum level of oxidation which may be due to sufficient number of reducing sites already present on pectin. In case of carrageenan, a reverse trend was observed which may be explained due to possible oxidation of aldehyde groups by sodium periodate to carboxylic acid (Dodd & Hyaric, 1993). The extent of oxidation of each polysaccharide is important because it increases the possibility of binding of enzyme to the respective polysaccharide.

3.2. Residual specific activity of α-amylase after conjugating with different polysaccharides

Specific activity of α-amylase was determined as described in Section 2.3 after binding it with polysaccharides (Fig. 2). α-Amylase bound to CMC and gellan showed 100% retention of original activity whereas that conjugated to pectin and xanthan showed a marginal increase in specific activity. The retention of specific activity was only 21% and 38% after conjugation of α-amylase to guar gum and agar, respectively, while that conjugated with dextran showed 89% retention of original activity, in close agreement with 95% of retained activity we reported in our previous study (Jadhav & Singhal, 2012). Each polysaccharide has a unique combination of monosaccharides and functional groups which is responsible for differences in their structure and also the functional properties. These monosaccharides are linked to each other by specific bonding (α and/or β bonding) to form specific structure of each polysaccharide. Oxidized polysaccharide binds to the enzyme by the formation of carbodiimide bonds between ε-amino group of protein and free aldehyde group of oxidized polysaccharide (Sanderson & Wilson, 1970). Carbodiimide covalent bonding depends on number of free available aldehyde groups and interference by the functional groups as well as structural properties of polysaccharide which may affect the three dimensional structure of enzyme and hence its activity. This may be the reason of different specific activities of enzyme observed after binding with different polysaccharides.

3.3. Effect of conjugation of α-amylase with polysaccharides on thermal and pH stability

The thermal stability of free and polysaccharide conjugated α-amylase was carried out by incubating the enzymes (protein concentration 2 μg/ml) at 60 °C, 70 °C and 80 °C for 15 min. The activity was found to decrease with an increase in temperature for the both free and polysaccharide conjugated α-amylase (Fig. 3). All the polysaccharide conjugated α-amylase preparations were more stable than free α-amylase at each of the temperature used in this study. Agar and guar gum conjugated α-amylase showed highest stability at each temperature among all the polysaccharides used for study but their actual activities were much lower than that of free α-amylase. Xanthan and pectin also showed a large increment in α-amylase stability at each temperature whereas dextran showed almost 10% more stability than free α-amylase at 60 °C and 70 °C, this is in accordance with thermal stability obtained in our previous report (Jadhav & Singhal, 2012).

Similarly, pH stability study showed all the conjugated α-amylase to be more stable towards extreme acidic and alkaline conditions of pH 4 and pH 10, respectively (Table 1). Dextran conjugated α-amylase showed highest pH stability among all the polysaccharides used in the study. The difference of stability

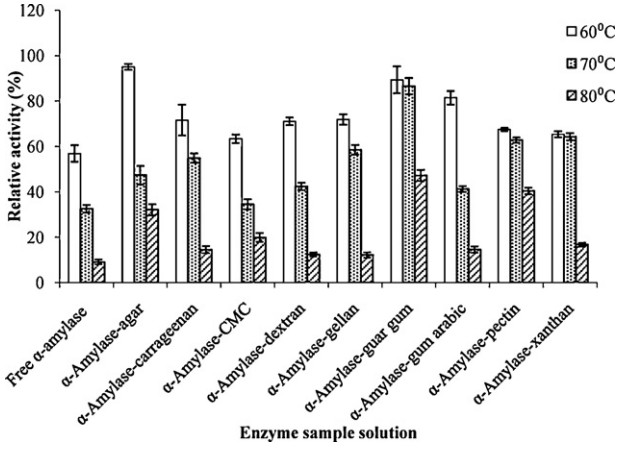

Fig. 3. Profile of free and conjugated α-amylase with respect to thermal stability.

Table 1
Residual activity (%)[a] of free and conjugated α-amylase after incubation in buffer of various pH for 1 h at room temperature.

pH Enzyme sample	3	4	5	6	7	8	9	10
Free α-amylase	2.10	29.89	80.96	75.12	75.88	76.53	70.80	71.62
α-Amylase-agar	10.62	61.49	102.38	77.68	85.44	77.68	97.30	107.13
α-Amylase-carrageenan	8.04	82.53	109.79	112.02	110.24	112.36	138.48	156.72
α-Amylase-CMC	4.41	70.13	88.75	75.17	75.69	76.51	74.82	74.41
α-Amylase-dextran	4.53	62.55	99.39	78.71	85.87	82.06	85.44	76.00
α-Amylase-gellan	2.93	58.25	81.35	75.55	78.36	77.32	75.13	79.82
α-Amylase-guar gum	46.85	123.38	132.73	159.96	99.44	99.75	151.24	237.42
α-Amylase-gum arabic	3.94	58.07	89.49	88.07	88.57	84.26	82.63	76.98
α-Amylase-pectin	3.14	60.62	81.45	78.32	83.10	77.25	78.41	73.32
α-Amylase-xanthan	4.40	64.94	80.79	79.55	83.40	78.67	81.85	73.20

[a] Data are result of triplicates and all the standard deviations are less than ± 5%.

between free and dextran conjugated α-amylase was about 33% at pH 4, while it was 5% at pH 10. Guar gum conjugated α-amylase showed activity more than the original activity after incubating it for 1 h in buffer of pH 4–10 which may be due to release of the enzyme from guar gum binding and masking effects during incubation in various buffer systems. Agar and carregeenan also showed an increased activity at extreme pH values but their actual activities were much lower than free α-amylase.

Stability studies showed dextran conjugated α-amylase to have best thermal and pH stability as compared to all the polysaccharide under study with the better retention of original activity. Among all the polysaccharides evaluated in this work, dextran is a simple polysaccharide of glucose with very short branches and without any attached functional groups. Oxidation produces free aldehyde groups on second and third carbon atoms of glucose in dextran which are relatively free for carbodiimide bond formation (Sanderson & Wilson, 1970). All these properties may favour the strong covalent bonding between simple branched dextran and enzyme without much affect on structure of enzyme. Other polysaccharides like carrageenan, gellan, agar, CMC, pectin and xanthan have varying functional groups and complex structure (Izydorczyk, Cui, & Wang, 2005) which may interfere in the strong covalent binding and affect the structure of the enzyme during covalent binding. Gum Arabic and guar gum are complex and rigid enough to make them unfavourable for binding and maintaining functional properties of enzyme. This may explain higher stability of enzyme–dextran conjugate as compared to other conjugates. Many authors have chosen dextran for conjugation with different enzyme to get higher stability of enzymes (Altikatoglu, Arioz, Basaran, & Kuzu, 2009; Misloviacova, Masarova, Bucko, & Gemciner, 2006; Wongkhalaung, Kashiwagi, Magae, Ohta, & Sasaki, 1985).

3.4. Effect of conjugation of α-amylase with polysaccharides on its storage life

3.4.1. Effect on the growth of Bacillus subtilis (a model Gram positive organism) and Escherichia coli (model Gram negative organism) in the free and conjugated α-amylase solutions

Based on the results obtained from thermal and pH stability, agar, dextran, xanthan and pectin were selected to study storage life of α-amylase with respect to growth of organism in the enzyme solutions. As contaminating organisms grown in the enzyme solution also produces wild amylase, enzyme activity was found to be variable during storage of α-amylase. Growth of the organism was determined using absorbance at 600 nm and also confirmed by spread plate method on agar plate (data not shown). Effect of preservative along with the polysaccharides on the growth of microorganism in the enzyme solution was also studied by maintaining two sets of experiment; one with preservative and another without preservative. Growth of *B. subtilis* (model Gram positive organism) in free and polysaccharide conjugated α-amylase solutions without any preservative was found to increase with increasing time period from 48 h to 120 h (Fig. 4a). Growth of *B. subtilis* in the solution of agar, pectin and xanthan conjugated α-amylase were comparable to growth in the free α-amylase solution but was found to be 40% lower in the dextran conjugated α-amylase solution after 120 h of incubation. This could probably be due to the antimicrobial activity of dextran against *B. subtilis* (Amiri et al., 2008).

E. coli showed about 50% of growth in agar and xanthan conjugated α-amylase as compared to free α-amylase solution whereas pectin did not reduce the growth in enzyme solution after 120 h of incubation (Fig. 4b). Dextran conjugated α-amylase could prevent growth of *E. coli* and it showed 88% reduction in growth as compared to that of in the free α-amylase. Chitosan-dextran based hydrogel had been reported for its antimicrobial properties where

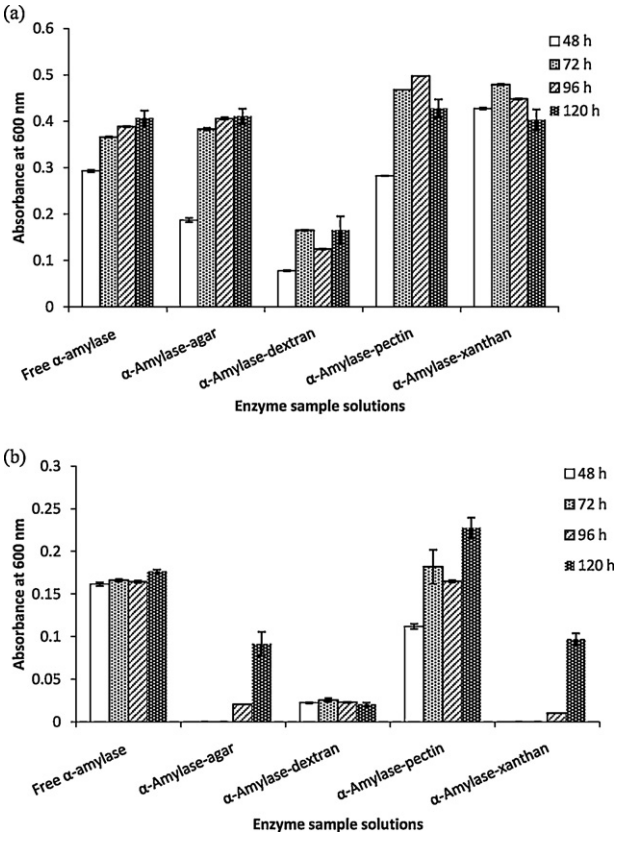

Fig. 4. Study of the growth of model organisms in free and polysaccharide conjugated α-amylase solutions without any preservative added (a) *Bacillus subtilis* (b) *Escherichia coli.*

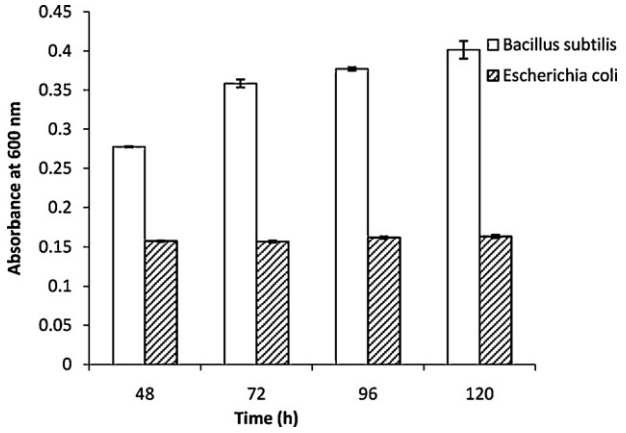

Fig. 5. Study of the growth of model organisms in free α-amylase solution with addition of 0.2% sodium benzoate as a preservative.

dextran aldehyde was found to be antimicrobial component of hydrogel (Aziz, Cabral, Brooks, Moratti, & Hanton, 2012).

Since both of the organisms could grow easily in free α-amylase solutions with added preservatives (Fig. 5), there was no role of only preservative by itself in the protection of α-amylase solution from microbial attack for increasing storage lifetime of α-amylase. Both the model organisms could not grow at all in the polysaccharide conjugated α-amylase with the added preservative, indicating a synergistic effect of preservative and polysaccharide which could completely stop the growth. Though sodium benzoate is well known antifungal agent, scientific literatures has reported its antimicrobial activity against many bacteria including *B. subtilis and E. coli* (Hwang & Beuchat, 1995; Stanojevic, Comic, Stefanovic, & Solujic-sukdolak, 2009; Stanojevic, Comic, Stefanovic, & Solujic-sukdolak, 2010).

Dextran could significantly reduce growth of challenged organism in the α-amylase solution without the help of any preservative and stood out over other polysaccharides.

3.4.2. Study of growth of aerobic flora in the free and dextran conjugated α-amylase solutions at ambient temperature and 4 °C

Growth of aerobic flora was studied in the dextran conjugated α-amylase as compared to free α-amylase solution. Free and dextran conjugated α-amylase preparations with and without preservative was kept at ambient temperature and 4 °C. They were observed for the growth after regular time intervals. The conjugate showed significant reduction of growth of aerobic flora as compared to free α-amylase at ambient temperature after 120 h of incubation (Fig. 6a). While covalently bound dextran showed 60% reduction in growth, the preservative (0.2% sodium benzoate) could reduce it only by 30%. Both the preservative and covalently bound dextran showed a synergistic effect as evident from almost 90% reduction of growth of aerobic flora.

Free α-amylase solution with and without preservative showed the growth of aerobic flora to increase during storage at 4 °C (Fig. 6b). Preservative showed a negative effect on the growth of aerobic flora and reduced the growth as compared to the α-amylase solution without any addition of preservative. At 4 °C, no significant growth was observed in the dextran conjugated α-amylase solution with and without preservative after 60 days of incubation. The synergistic effect of low temperature, dextran and preservative could completely stop the growth of any organism in the enzyme solution and helped in maintaining its activity. Hence dextran may prove to be better option for conjugation of α-amylase to increase its storage life along with increasing stability and maintaining original activity.

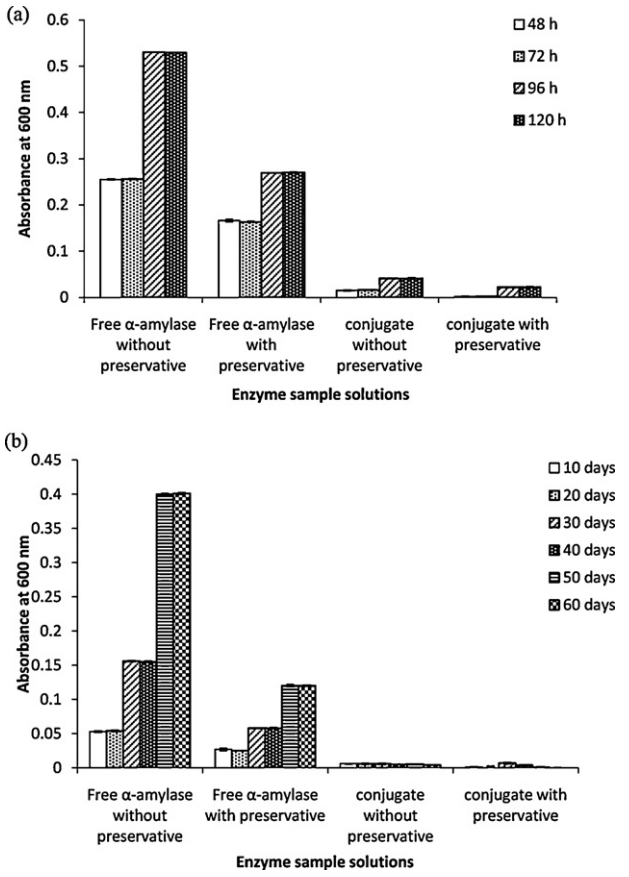

Fig. 6. Study of growth of aerobic flora in free and dextran conjugated amylase solutions with and without preservative stored at (a) ambient temperature (b) 4 °C.

4. Conclusion

Study of screening of polysaccharides for conjugation of α-amylase showed dextran to not only increase thermal and pH stability, but had a powerful antimicrobial action too. A combination of low temperature (4 °C), preservative, and covalently bound dextran can be an effective approach to increase storage life of α-amylase solution. Hence, it can be inferred that dextran conjugated α-amylase can not only be explored for its higher thermal and pH stability, but can also prolong the storage life in liquid formulations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.11.009.

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