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Original article

Application of amylase producing bacteria isolated from hot spring water in food industry

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Abstract

Due to the increasing demand for enzymes in various industries, there is enormous interest in research on enzymes suitable for commercial applications and their cost effective production techniques. Thermophilic microorganisms have gained a great deal of attention. Hence, the enzyme from these microorganisms is of special interest because these are not usually denatured by high temperature and are active at elevated temperature. The amylase from thermophilic bacteria of hot springs which are supposed to be unexploited niches may have wide industrial applications. The application of extracted amylase from the bacterial isolates was evaluated for apple and kiwi juice yield and clarification. An application of 0.75 per cent of amylase yielded 58 per cent of apple juice and yielded 54 percent of kiwi juice from J2 isolate. The bacterial amylases were also evaluated for the preparation of bun where the maximum leavening activity of 2.60 ml/h at 0.75 per cent concentration for J2. Further, quality of buns from the selected concentration was also recorded. The loaf volume was recorded to be 177.43 cm^3 and 179.11cm^3 for J2 at the amylase concentration of 0.75 per cent. Hence, amylase yield, stability and the low cost substrate production supported the hypothesis that microbial enzymes have potential in food industries. These natural resources need to be exploited for commercial enzymes.

Key words: Amylase, bacterial isolate, kiwi juice, apple juice, bun making

1. Introduction

Among the starch hydrolyzing enzymes α-amylase (EC3.2.1.1) is a well known endoamylase**.** α- amylase constitute a class of industrial enzymes having approximately a 25% stake in the world enzyme, market. Alpha amylase degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes (Saini *et al.*, 2017). Amylases are used in starch liquefaction process, paper, textile, bakery (Rosell and Dura, 2016), detergent industries (Gupta *et al*., 2008) have potential application in pharmaceutical and fine chemical industries (Singh *et al*., 2016). The microbial production of amylase is more effective than the other sources as the technique is easy, cost effective, consistent and fast which can be modified to obtain enzymes of desired characteristics (Burhan *et al*., 2003). The amylases dominate about 25 per cent of enzyme trade in commercial applications especially for hydrolysis of starch in various industries. Thermostable amylases have been reported from several bacterial strains and have been produced through the use of submerged fermentation (SmF) as well as solid state fermentation (SSF) (Teodoro and Martins, 2000). However, the use of SSF has been found to be more advantageous than SmF and allows the cheaper production of enzymes (Mamo and Gessesse, 1999; Pandey *et al*., 2000). Solid state fermentation is significantly influenced by the nature of solid substrate. The substrate not only supplies necessary nutrients to microorganism but also provide anchorage to the cells. An additional advantage of this technique is that the recovery of products is relatively simple (Subramaniyam and Vimala, 2012). In recent years, the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Abu *et al*., 2005; Pandey *et al*., 2000).

In food industries, amylase enzymes are utilized in baking, brewing, preparation of digestive aids, preparation of cakes, fruits juices and starch syrups (Couto and Sanroman, 2006). Apple and kiwi fruits are two important commercial fruit crops of Himachal Pradesh which are mainly processed for juice making. Apple juice contains considerable amounts of starch, particularly at the beginning of the harvest season. Unripe apples contain as much as 15 per cent starch and up to 1 per cent in the juice after extraction, milling and pressing (Carrin *et al*., 2004).

Starch creates problem during apple juice processing as it complicates filtration and causes post process cloudiness. The enzyme amylase degrades starch into smaller units and contributes to prevent post bottling haze formation. Thus, the amylase can be used to improve the yield and clarification of apple and kiwi juices (Srivastava and Tyagi., 2013). In baking industry, amylase is

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employed to supplement the natural enzymes present in the grains during fermentation by yeast which increases the quality of baking products (Caballero *et al*., 1995).

Keeping in view the current status of knowledge, it is understood that amylase enzyme from thermophilic bacteria of hot water springs which are supposed to be unexploited niches may have wide industrial applications. Therefore, the proposed study was undertaken for isolation and evaluating the application of amylase in juice processing and bakery products.

2. Materials and Methods

2.1 Collection of hot water spring samples

In total 6 water samples were collected from Jeori of Shimla district of Himachal Pradesh in sterilized screw capped tubes and processed for isolation of amylase producing bacteria.

2.2 Isolation and production of amylase producing bacteria

Isolation for amylase producing bacteria from the collected water samples were done by using serial dilution method (Aneja, 2003).

2.3 Screening of amylase producing bacterial isolates

After the isolation bacterial colonies producing clear zones were selected and purified using streak plate technique on the starch medium. The isolates were primarily examined according to their colony morphology.

2.4 Production of amylase

2.4.1 Preparation of seed culture and production of enzyme

The seed culture was prepared by inoculating a loopful of pure culture and incubated for 24 h at 35°C. 2 ml of seed culture was inoculated in 50 ml of production medium (pH 9.0 containing beef extract, 0.25%), peptone (0.15%) and starch (1%). The medium was grown for 48 h at 35°C at 150 rpm in shaking incubator. Production media was centrifuged at 5,000 rpm for 10 min. The supernatant was collected and used further. Uninoculated culture medium was kept as a control.

2.4.2 Amylase assay (Sadasivam and Manickam, 1996)

1 ml of enzyme supernatant was mixed with 1 ml of starch solution with incubation at 27°C for 15 min. 2 ml of DNSA reagent was added to it and the mixture was heated on boiling water bath for 10-15 min. While tubes were warm, 1 ml of potassium sodium tartrate solution was added. After cooling down at room temperature, absorbance of reaction mixture was recorded at 540 nm against reagent blank. The standard curve was made from the stock solution of maltose (10-100 µg/ml). The enzyme activity was expressed in terms of international unit (IU) and specific activity (SA) as one international unit of enzyme activity represents µ moles of glucose released/min/ml of enzyme.

2.5 Applications of amylase in food processing

2.5.1 Effect of amylase on production and clarification of apple and kiwi juice

i. Physico-chemical analysis

Analysis of apple and kiwi fruit juices for various physico-chemical characteristics was conducted by following standard analytical procedures (AOAC, 1984; Ranganna, 1997; Thiamiah, 1997).

ii. Total soluble solids

The total soluble solids (TSS) in fruit pulp was determined with the help of hand refractometer and expressed in degree brix.

iii. Acidity (Ranganna, 1997)

The titratable acidity of kiwi juice and apple juice was determined by titrating the aliquots of known quantity of sample (5 g of juice) against a standardized NaOH (0.1 N) solution to a pink end point using 0.1 per cent phenolphthalein indicator. The values of titratable acidity were expressed as per cent anhydrous citric acid on fresh basis of a given sample.

$$\%\text{Total acid} = \frac{\begin{array}{l}\text{Titre} \times \text{Normality} \times \text{Volume made up} \\ \times \text{equivalent weight of an acid}\end{array}}{\begin{array}{l}\text{Volume of sample taken for estimation} \\ \times \text{aliquot taken for estimation} \times 1000\end{array}} \times 100$$

The fruit after washing, peeling and pulping was treated with amylase at different concentration given below for bacterial isolate in the following manner. Then the beakers with different concentrations were incubated in water bath at 50 ± 2°C without enzyme as a control and with enzyme at different concentrations were varied from 0.5–1.25% (v/v) at interval of 15 min, 30 min, 45 min and 60 min to obtain maximum juice yield and clarity.

With every 15 min of intervals, readings of physico-chemical parameters, *i.e.*, juice yield, absorbance and acid content were noted. Sensory evaluation of kiwi and apple juice was conducted on the basis of color, flavor, taste and overall acceptability on hedonic scale. Taste panel (7-9 members at a time) comprised of faculty members and PG students of Department of Microbiology, Dr. YS Parmar University of Horticulture and Forestry, Nauni-Solan, Himachal Pradesh, India.

2.6 Effect of amylase on bun making

2.6.1 Raw material

Materials used for bun making were collected from local market, *i.e.*, refined flour, yeast, sugar, milk powder, salt, etc.

i. Method

A refined flour of 1.5 kg was taken with 65% of hot water. Water was added in the small amount to mix the flour then 6% of sugar, 45 g of milk powder, 3 % of yeast, 1% oil were also added and mixed properly. The above mixture was treated without enzyme as a control and different concentrations of enzyme varied from 0.5 - 1.25% (w/v). The leavening activity was checked by incubating the mixture for 15 min in hot air oven at 55°C. After 15 min salt was added in small amount and was mixed properly. Further, the above mixture was kept in oven for fermentation for 1.5 h at 35°C, followed by proofing of dough for 1 h at 35°C. Baking was achieved at 175-200°C for 30 min.

2.6.2 Physical characteristics of Bun

i. Loaf volume (Pyler, 1973)

The loaf volume expressed in cubic centimeters was determined by using seed displacement method.

ii. Leavening activity (Caballero *et al.*, 1995)

The leavening activity was estimated in the prepared sample (wheat flour 20 gm, 15 ml water, 1.2 gm sugar, 0.4 gm salt and 0.3 gm of baker's yeast) in 100 ml volumetric cylinders. Maximum leavening rate (ml/h) at 30°C was calculated.

$$\text{Leavening activity (ml/h)} = \frac{\text{Volume of reached at 120 min at 30°C}}{\text{Initial volume of the sample}}$$

iii. Crumb grain (Raganna, 2009)

The crumb grain was collected during cooling and slicing of bread. A total weight of crumb grain was recorded and calculated:

$$\text{Percent crumb grain} = \frac{\text{Weight of crumb grain}}{\text{Weight of sample}} \times 100$$

iv. Crumb colour (Ratika *et al.*, 2015)

A known weight of bread sample was macerated with distilled water and then filtered using whatman filter paper. The filtrate was taken in a cuvett and optical density was measured at 420 nm against the distilled water as blank.

v. Sensory evaluation (Larmond, 1997)

The samples of coded sliced bun were served in cleaned white plate to panelist at room temperature (25°C) for sensory evaluation by hedonic scale where 1= Dislike extremely and 9 = Like extremely. Attributes evaluated in bun were (texture, flavor, colour, taste and overall acceptability), respectively (Amerine *et al.*, 1965).

2.7 Statistical analysis

The data obtained were subjected to analysis of variance technique using (CRD) Completely Randomized Design for laboratory experiment and (RBD) Randomized Block Design for sensory characteristics (Gomez and Gomez, 1976).

3. Results and Discussion

3.1 Isolation, enumeration and screening of amylase producing bacteria

The hot water springs are considered as rich sources of industrially important enzyme producing micro-organisms. The isolation of bacteria was done from the pooled samples collected from hot spring in Jeori of Rampur Bushar of Shimla district by serial plate dilution method and spread plate method on nutrient agar (NA) incubating at 37 ± 2°C. A total of 6 bacterial isolates were isolated from Jeori hot water spring. The selected colonies were grown on the starch nutrient agar medium. The plates were flooded with iodine reagent for the appearance of clear zone post incubation, which indicated the presence of amylase producing strains. In total, 5 isolates (J2, J4, J11, J12, J31 and J32) were found to be the amylase producers, showed clear zones of starch hydrolysis with varying diameters from the pooled samples of water.

The zone size of bacterial colonies ranged between 4.7 to 10.30 mm with enzyme index of 12.80 to 37.00 (Table 1). The isolate J2 from the hot spring exhibited highest zone size of 10.30 mm with enzyme index of 30.60. The isolate J2 have luxuriant growth of isolate when flooded with iodine (Plate1).

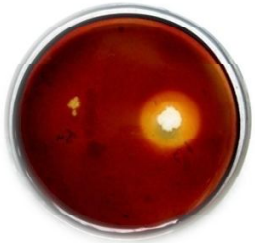


Plate 1: Amylase producing activity of selected bacterial isolates on starch nutrient agar media.

Table 1: Characteristics, amylase activity and viable count of amylase producing bacterial isolates from hot water spring

Isolates	Zone size (mm)	Enzyme index	*Amylase activity (IU)	****Viable count cfu x 10^4
J2	10.30	30.60	52.58	52.6 (7.31)
J3	5.30	13.90	23.34	39.8 (6.38)
J4	9.00	12.80	35.82	41.8 (6.54)
J12	6.38	18.70	21.34	46.8 (6.91)
J32	4.70	19.40	22.57	34.2 (5.93)
$S.E._m$	0.43	0.07	0.87	0.12
CD	1.26	0.20	2.53	0.35

*Amylase activity (IU): µ moles of reducing sugar produced/min/ml
**** viable count: Colony forming unit (Cfu /ml): Number of viable cells in a sample

Different bacterial isolates were studied for the growth and maximum amylase production by the selected bacterial isolates. The results revealed that the growth expressed in terms of viable count as well as amylase activity among isolates from hot springs J2 exhibited highest amylase activity (52.58 IU) (Table1). Based on these observations, isolate J2 was selected and identified as *Bacillus* sp. with accession number [KY990713] and used for further studies.

These studies are in accordance with other research that screened thermophilic amylase producing bacterial strains on starch agar medium (Chauhan *et al.*, 2011), 12 hyper amylase producing strain from the mushroom compost (Vyas and Sharma, 2015), three Iranian hot springs, namelyl; Larijan (67°C, pH 6.5), Mahallat (46°C, pH 7) and Meshkinshashr (82°C, pH 6). They found that Meshkinshahr hot spring was rich in amylase producing bacteria (Fooladi and Sajjadian, 2010) and 12 amylase producing strains from the hot spring of Mukhya kund (61.7°C pH, 7.) and Surya kund (48.0°C pH 7.3) in Unkeshwar district of Maharashtra (Pathak and Rekadwad, 2013).

3.2 Applications of amylase in food industry

The enzyme amylase degrades the starch into smaller units and contributes to prevent post bottling haze formation. Thus, the amylases produced by the selected bacterial isolates were subjected to improve the yield and clarification of apple juice (Figure 1).

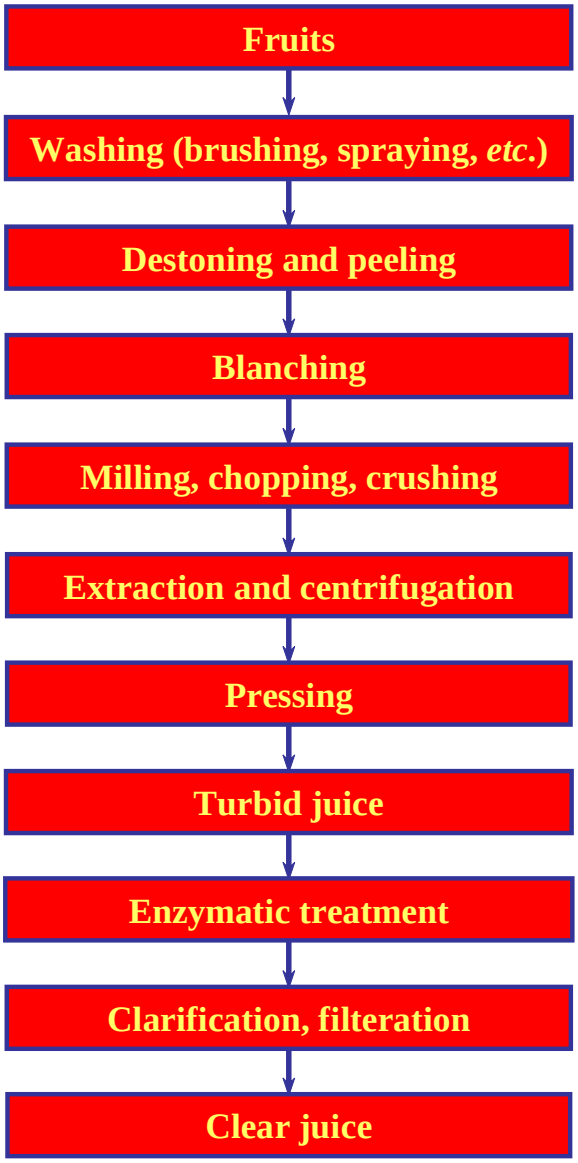


Figure 1: Process for the juice yield and clarification.

3.3 Effect of amylase on apple juice yield and clarification

Application of amylase isolated from hot water spring bacterial isolate was carried out in clarification of juices. The TSS and acidity of raw pulp was observed to be 10.2°C and 1.67%. Table 2 presents the apple juice yield after addition of enzyme at different levels of concentrations at different time intervals. A perusal of data presented in Table 2 revealed that the maximum (60%) juice yield was recorded at 1 per cent enzyme concentration for incubation period of 30 min. The amylase from isolate J2 yielded 58 per cent juice at 0.75 per cent enzyme concentration. The maximum absorbance was observed to be 0.58 at 60 min incubation with 0.75 per cent enzyme concentration of amylase from J2 isolate.

The Figure 2 showed the sensory characteristics of apple juice on 9 Hedonic scale basis**.** The data revealed that the concentration of 0.75 per cent for J2 was adjudged best for taste, colour, flavor and over all acceptability of apple juice.

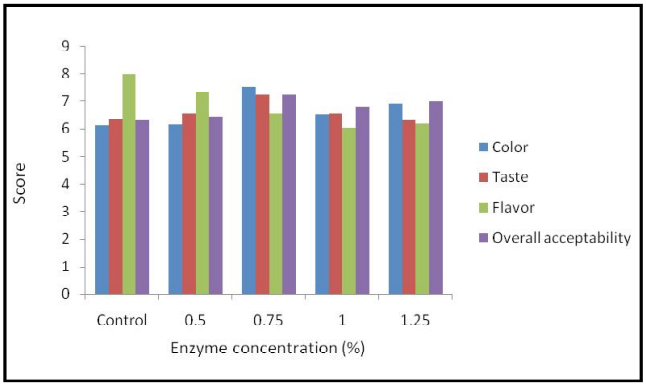


Figure 2: Sensory characteristics of apple juice clarified from selected bacterial amylases.

3.4 Effect of amylases from selected bacterial isolates on the kiwi juice yield and clarification

The amylase produced from the bacterial isolates from hot water spring of Himachal Pradesh was then tested for their application in clarification of kiwi juice. The TSS and acidity of raw pulp was observed to be 14°C and 1.74%. The data presented in Table 2 showed that kiwifruit juices yield was found to be maximum at 1.25 per cent (52 per cent with 0.49 absorbance) concentration with incubation period of 45 min. The Figure 3 showed the sensory characteristics of kiwi juice (9 Hedonic scales)**.** The data revealed that the amylase concentration of 1.25 per cent for J2 was evaluated best for taste, colour, and flavor and over all acceptability of apple juice.

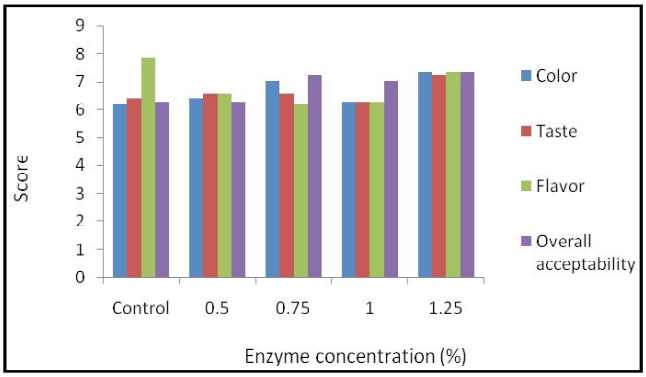


Figure 3: Sensory characteristics of kiwi juice clarified from selected bacterial isolate.

The possible reason for juice yield and clarification of juices with amylase enzymatic treatment may be due to degradation polysaccharides. Enzymatically clarified juice results in viscosity reduction and cluster formation, which facilitates the separation through centrifugation or filtration. Increase in amylase concentration increased the rate of clarification by exposing part of the positively charged protein beneath, thus reducing electrostatic repulsion between cloud particles which cause these particles to aggregate to larger particles and eventually settle out. As a result, the juice becomes more concentrated in respect of flavor and colour (Abdullah *et al*., 2007). In one more researcher, also reported that the juice yield and clarification are the function of enzyme hydrolysis which assesses

the variables of enzymatic treatment of fruit pulp particularly temperature, time, pH and amylase concentration (Teodoro and Martins, 2000). In order to minimize the turbidity in the mixed juices of apple, banana and sapodilla fruit, the enzyme preparations containing a combination of pectinase and amylase in the ratio of 2:1 reduced the viscosity (Vinjamuri and Bhavikatti, 2015).

Table 2: Effect of amylases from selected bacterial isolates on the apple juice yield and clarification

Time (min)	Concentration of enzyme (%)	Apple juice		Kiwi juice	
		(%) yield	OD (nm)	(%) yield	OD (nm)
15	Control	16	0.25		
	0.5	48	0.34	40	0.22
	0.75	50	0.38	36	0.23
	1.0	46	0.2	44	0.21
	1.25	50	0.28	48	0.2
30	0.5	40	0.25	40	0.28
	0.75	50	0.39	44	0.30
	1.0	48	0.42	40	0.35
	1.25	48	0.31	44	0.33
45	0.5	42	0.32	44	0.32
	0.75	44	0.45	44	0.37
	1.0	48	0.3	48	0.43
	1.25	46	0.39	52	0.49
60	0.5	42	0.37	44	0.36
	0.75	58	0.58	48	0.39
	1.0	50	0.31	44	0.43
	1.25	48	0.38	40	0.45
$CD_{(0.05)}$		2.13	0.025	2.12	0.022

3.5 Effect of amylases on the softening of dough for buns making

The prime function of amylase is to liquefy and hydrolyze starch. The action of amylase in the saccharification which provides fermentable sugars to yeast (Figure 4). The Table 3 showed the testing concentration of enzyme from selected isolates for checking the leavening activity.

Table 3: Effect of amylase from selected bacterial isolates on leavening activity of dough

Amylase concentration (%)	Leavening activity of dough (ml/h) J2
0.5	2.00
0.75	2.60
1.0	2.30
1.25	2.20
$CD_{(0.05)}$	0.21
SE_m	0.07

The data revealed that the best leavening activity was observed to be 2.60 ml/h at 0.75 per cent enzyme concentration for J2. The loaf volume was recorded to be 179.11 for J2 at the concentration of 0.75 percent (Table 4). The similar results were obtained for the sensory quality characteristics, *viz.*, taste, colour and overall acceptability of buns (Figure 5).

Table 4: Effect of amylase from selected bacterial isolates on bun quality characteristics

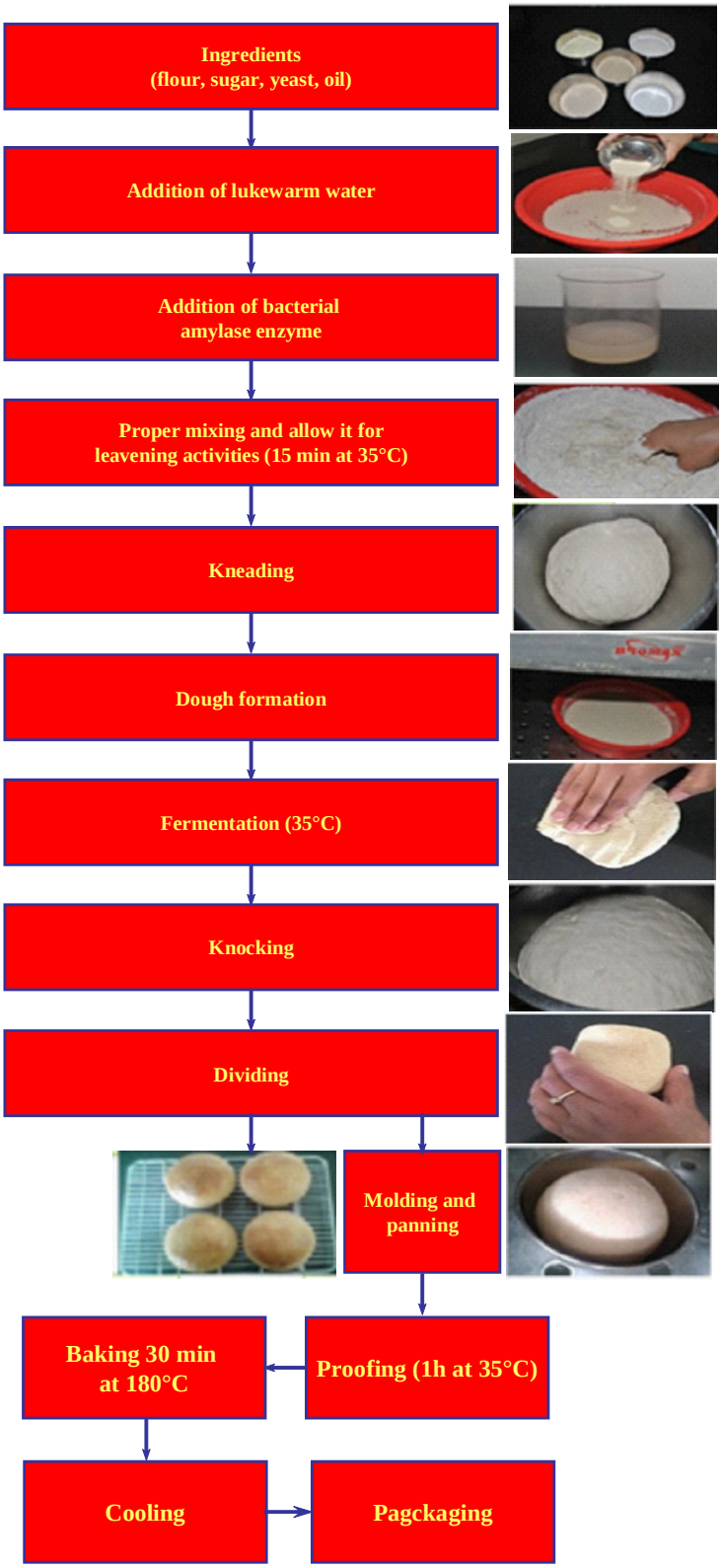
Amylase Concentrations (%)	Loaf volume (cm^3)	Crumb grain (%)	Crumb color
	J2		
Control	154.60	0.008	0.55
0.5	160.33	0.112	0.62
0.75	179.11	0.171	0.72
1.0	171.80	0.115	0.54
1.25	170.00	0.114	0.63
$CD_{(0.05)}$	2.15	0.002	0.02
SE_m	0.72	0.001	0.008

The effect of α-amylase on the bakery products was evaluated with bran. The inclusion of higher amount of wheat bran in formulations provided a significant decrease ($p < 0.05$) in the total hydrolysable starch amount of bread. They reported that the use of α-amylase in bread-making processes could provide technological advantages improving quality of breads without markedly changes in their glycaemic index (Sanz-Penella *et al*., 2014). In one more observation, it was reported that small dose (10 g/100 kg) of β-amylase showed the improvement in quality of dough for bread making. However, high dose of amylase leads to reduction in dough elasticity and over production of dextrin that causes a sticky content of dough making it very hard to handle for bread making. They further suggested that amylase acts on the starch resulting in increase in the fermentable sugars and change the rheological characteristics of dough which improves the bread quality and nutritive value of bread (David *et al*., 2014).

The influence of different climatic conditions was evaluated on the activity of alpha-amylase in wheat samples and bread quality parameters. They reported that decrease in Mixo lab parameter torque C3 and specific bread loaf volume, as well as increase in the breakdown torque (C3-C4) of samples harvested in 2013 were also observed, which could be attributed to rainy weather influencing increase in alpha-amylase activity (Sadasivam and Manickam, 1996).

4. Conclusion

It can be concluded from the present investigations that the isolate *viz*., J2 from hot water spring of Jeori (Shimla), Himachal Pradesh was found to be potential sources for amylase production. The 1 per cent concentration of amylase was found to increase the yield and clarification of apple and kiwi juices. The bacterial amylase was evaluated for preparation of bun and 0.75 per cent of J2 isolate improved the quality of buns considerably. Hence, amylase yield, stability and the low cost substrate production supported the hypothesis that microbial enzymes have potential in food industries. These natural resources need to be exploited for commercial enzymes.



Process of the development of burns

Figure 4: Process of making buns.

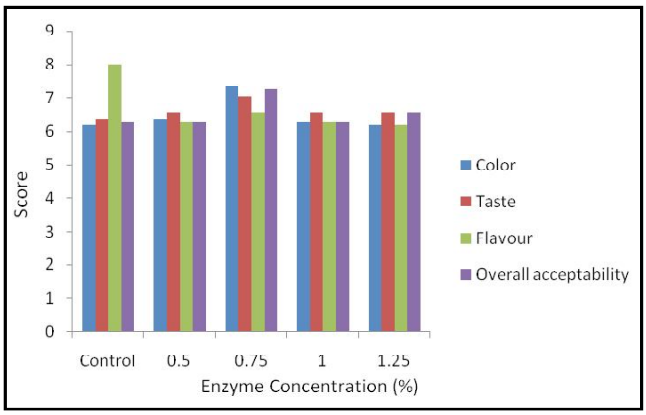


Figure 5: Sensory characteristics for bun (9 Hedonic scale).

Conflict of interest

We declare that we have no conflict of interest.

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