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Alpha-Glucosidase Inhibitory Functional Fermented Milk Products (Product A & Product B) Developed Using Proteolytic Lactobacilli Cultures with Supplementation of Whey Protein Powder S(WPC-70)

Samlesh Kumari[1]*, Anil Kumar Puniya[2] and Sudhir Kumar Tomar[2]

[1]Centre of Excellence on Soybean Processing and Utilization, ICAR-Central Institute of Agricultural Engineering, Bhopal-462038, Madhya Pradesh, India
[2]Department of Dairy Microbiology, ICAR-National Dairy Research Institute, Karnal 132001, Haryana, India

**Corresponding author*

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A B S T R A C T

Alpha-glucosidase inhibitors regulate hyperglycemia by impeding the rate of carbohydrate digestion in the small intestine and thereby hampering the diet associated acute glucose excursion. In the present investigation it was observed that peptide extracted from fermented milk were inhibitory against α-glucosidase11-26% inhibition respectively. Lactobacilli fermented milk products were developed based on the best AGI inhibitory potential of milk peptides released during fermentation by selected lactobacillus cultures (*Lb. rhamnosus* DH2, *Lb. salivarius* 695 and *Lb. salivarius* 696. Two products (Product A and Product B) were developed using response surface methodology with independent variables: (incubation period, inoculum level and WPC (70) ;) and dependent variables (alpha glucosidase inhibition, pH and lactic acid). Among two optimized products (A and B), Product A (25.23%), showing higher AGI activity as compared to Product B (12.7%). The present study has culminated into development of fermented milk product with AGI potential using non alpha glucosidase producing well characterised proteolytic lactobacilli as starter. There is a great scope for exploring more number of specific proteolytic strains of lactobacilli capable of producing anti-hyperglycemic peptides/bioactive factors through milk fermentation.

Introduction

Today, while most of the diseases prevalent in the 19th century have been eradicated, yet the present generation is paradoxically plagued with a fresh set of diseases creatively called "lifestyle diseases". These diseases are the result of the fast-paced lifestyle that inevitably accompanies the developments (http://www.drhealth.md/life-style-diseases/). Lifestyle diseases such as obesity, cancer, cardiovascular diseases (CVD), type-2 diabetes (T2D) and hypertension have become an epidemic in our modern society.

Among these, diabetes mellitus is a looming epidemic of the 21st century poses major threat to global health, affecting almost all major sections of society the prevalence of T2D continues to increase worldwide.

The fundamental mechanism underlying hyperglycemia in diabetes mellitus involves the over production of glucose (excessive hepatic glycogenolysis and gluconeogenesis) and or decreased utilization of glucose by the tissues (Latner, 1958). Complication related to hyperglycemia is the most common cause of mortality in almost 50% of diabetic patients (Ceriello *et al.,* 2004; Vasudevan and Ballantyne, 2005). Therefore, treatment remedies used for hyperglycemia are a cause of concern as most of the anti hyperglycemic drugs available in market carry certain socioeconomic burden and side effects as well. The natural therapeutic approach to hyperglycemia is use of digestive enzyme inhibitors. Inhibitors of enzyme responsible for carbohydrate digestion i.e. α-glucosidase and α-amylase are used to achieve greater control over hyperglycemia in T2D. Agents with α-glucosidase and amylase inhibitory action delay carbohydrate digestion in the small intestinal tract and thereby reduce meal-induced rises in blood glucose and plasma insulin (Clissold and Edwards, 1988; Toeller, 1994). Although clinical inhibitors of αglucosidase are widely available, alternative α-glucosidase inhibitors are continually sought in the hope of minimising side-effects and reducing drug costs. There is also the potential for functional foods containing inhibitory activity, and many natural, plant and food-based sources have been screened (Yamada *et al.,* 2007; Al-Zuhair *et al.,* 2010; Tundis *et al.,* 2010; Mohamed *et al.,* 2012; Nair *et al.,* 2013; Katekhaye *et al.,* 2013). During recent years, there has been an increase in the interest in understanding the relationship between food and health all over the world. Most important among all these is diet management and nutritional therapies such as nutraceuticals, functional and fermented dairy products. Functional dairy products containing nutraceuticals and fermented milks produced with incorporation of these ingredients with specific health benefits are of potential interest. Now a days, many functional dairy products available in market contains lactobacillus culture. Lacticacid bacteria (LAB), especially Lactobacilli are frequently used in products for human consumption, cultured milks, and various pharmaceutical preparations (Maha, 2013). Fermentation of milk with lactobacillus species getting more attention globally so, it can be said that probiotics dairy products became integral part of our diet in modern era life. Functional peptides from egg white protein hydrolysates displayed the potential α-glucosidase inhibitory activity. Among the eight synthetic peptides, two peptides, Arg–Val–Pro–Ser– Leu–Met and Thr–pro–Ser–Pro–Arg, demonstrate higher α-glucosidase inhibitory activity with an IC 50 values at 23.07 and 40.02 µmol/L, respectively. These results showed that the potential of bioactive peptides from the egg white protein exhibiting the α-glucosidase inhibitory activity could be considered as an ingredient for functional food product with the antidiabetic activity (Yu *et al.,* 2011). Peptides released in fermented dairy products possesses functional attributes such as anti-oxidative, anti-pathogenic etc. (Power *et al.,* 2013; Kumari and Vij 2015).

Proteolytic activity is very important characteristic of LAB. They produce therapeutic benefits and also increase physiological activity of cultured dairy products by liberating a number of biologically active peptides. The main aim of this study was to determine the therapeutic properties of bioactive peptides released from prepared fermented (by selected Lactobacillus species) milk proteins.

Careful control of the blood glucose level delays or protects against the development of severe complications and therefore, development of fermented milk products containing potential α-glucosidase inhibitors can be beneficial for the prevention or improvement of diabetic complications. Fermented milk with lactobacillus species continue to play an important role in the nutrition and also linked to the treatment of various diseases due to multiple beneficiary compositions having no side effect and lactobacillus fermented milk foods can be used as an alternative approaches to treat diabetes. Therefore, inconclusive knowledge is available about the bioactive factor present in these functional dairy foods responsible for inhibiting the digestive enzymes i.e. α-glucosidase. To explore the potential of dairy-based lactobacilli fermented milk product in management of hyperglycemia and other factors which contribute to this complicated metabolic syndrome diabetes mellitus current work is designed and two fermented milk products were developed with potential proteolytic lactobacillus cultures.

Materials and Methods

All the chemicals/reagents used in this study were of analytical grade purchased from himedia and sigma Aldrich. Whole milk and plastic cups for Lactobacilli fermented milk preparation were collected from Experimental Dairy, ICAR-NDRI, Karnal. Skimmed milk powder and whey protein powder (WPC-70) were procured from Modern Dairies, Karnal.

Proteolytic activity on skim milk agar

Lactobacillus cultures collected from collected from NCDC, ICAR-NDRI, Karnal. *Lactobacillus* cultures were screened for proteolytic activity on skim milk agar. Skim milk agar was prepared by adding autoclaved skim milk @10 % in nutrient agar media and was poured in sterilised Petri plates. Activated *Lactobacillus* cultures were spotted on skim milk agar plates and were incubated for 24h at 37°C. Differentiation of microorganisms was based on the coagulation and proteolysis of casein. Proteolytic activity was demonstrated by a clearing zone in the medium surrounding the bacterial growth.

Screening of non-α glucosidase producing lactobacillus cultures

For this, cultures were grown in MRS broth for 18 h and cell pellets were harvested after centrifuging 5.0 mL grown cultures at 500 g/5 min. Harvested cell pellets were washed twice with sterile phosphate buffer (pH 6.8) in order to remove residual broth particles. The resultant pellets were resuspended in 5.0 mL of phosphate buffer and 500 µL of α-glucosidase specific substrate i.e. p-Nitrophenylα-D-glucopyranoside (pNPG; 20 mM) was added, vortexed and incubated at 37°C for 24 h. Phosphate buffer with pNPG (without culture) was kept as control. After incubation, enzyme production potential was assessed with α-glucosidase assay kit (Sigma)and absorbance of p-nitrophenol released was taken at 405 nm in SpectroStar nano plate reader BMG Labtech.

Experimental plan and design for optimization of different variables for developing anti- hyperglycaemic lactobacilli fermented milk

To describe the nature of the response surface in the optimum region, a two factor (3 levels at each level) second order central composite rotatable design (CCRD) was adopted.

The independent factors viz. percent of inoculums (x1), incubation period (x2) and percent of WPC/ SMP (x3) was considered for the optimization of processing variables for titratable acidity, total viable count and α-

glucosidase inhibitory potential. The selective range for the variables was 1- 2.5% for inocula, 8-18h for incubation period and 1-3 % for WPC/ SMP.

Preparation of fermented milk with NAGP lactobacillus cultures

Milk was procured from Experimental Dairy, NDRI, Karnal. This milk was fermented at 37°C/ 12 h using the selected Lactobacillus cultures. Fresh whole and skim milk powder were used for preparation of standardized milk by using Pearson square method to adjust the fat at 2.5%, SNF 11% and TS 13.5%. (Fig.2)

Physicochemical and microbiological analysis of developed products

The pH of the product was determined electrochemically with a pH meter by the method described in IS: SP 18 (Part XI, 1981). The pH meter was first calibrated using standard buffers of pH 4.0 and 9.2 and standardized using pH buffer of 7.0 at 20±0.1°C. 3.8.2. Titratable acidity was determined by the procedure described in IS: 1479, Part I, ISI (1960).

Ten grams of the sample was taken in a beaker and titrated against 0.1 NaOH using phenophthalein as indicator till the appearance of light pink tinge, which persisted for 30 seconds.

The titratable acidity was expressed as percent lactic acid. Acidity (% Lactic acid) = 9 VN/X N = Normality of NaOH, V = Volume of NaOH (ml), X = Amount of sample taken (gm

Total lactic acid bacteria count was done by plating the appropriate dilution of the developed product in MRS (de Mann Rogosa and Sarpe) agar at 30°C for 24h. Coliform count of the developed product was done by pour plating with violet red bile agar and incubation at 37°C for 24-48h. Yeast and mold count was done by plating in appropriate dilution in Potato Dextrose Agar (PDA) at 22-25°C for 72h.

Anti- hyperglycaemic functional peptides extraction from fermented milk products

Water-soluble extracts of the milks were obtained by centrifugation at 10000 × g for 30min at 5°C and filtration through a Whatman No 42 filter and subjected to step wise filtration Further the collected water extract was filtered with 0.45µm syringe filters and the obtained filtrate was passed through 0.22µm syringe filters.

Finally, for getting supernatant 0.22µm filtrate was centrifuged at 10,000g for 10min. The supernatant was subjected to ultrafiltration using 30kDa, 10kDa and 3kDa MWCO. Bioactive peptides rich fractions were obtained by passing supernatant through vivaspin 10kDa, and 3kDa MWCO membranes and these fractions were analyzed for α- glucosidase inhibitory potential.

Statistical analysis

Excel was used for feeding and analysis raw data obtained from different experiments and further Graph pad prism was used to represent data. Data were analyzed by using completely randomized factorial design. Analysis of variance was conducted; when significant effect was detected, the means were separated by Fisher Least Square Analysis.

Optimization study data were analyzed by Completely Randomized Design as per the methods described by Steel and Torrie (1980). Storage study data were examined using Factorial CRD. The values for microbial counts were log transformed before analysis.

Results and Discussion

Proteolytic activity on skim milk agar

All the NAGP cultures have given zone of proteolysis on skim milk agar nearly 20mm, which proves that these cultures possess good proteolytic potential. Zone of precipitation given by lactobacilli cultures viz; DH2, NCDC-695, NCDC-696 and their combinations (DH2+695 and DH2 + 696) are shown in Fig.3. It is very clear from the results obtained on skim milk agar that the selected Lactobacillus cultures were highly proteolytic as diameter of zones ranged between 19.25± 0.52 to 21.25±0.28mm. These patterns of proteolysis corresponded with the growth patterns of these microorganisms. Several researchers have reported wide variations in the proteolytic abilities of LAB (Hickey *et al.,* 1983; Oberg *et al.,* 1991). For the production of α-glucosidase inhibitor bioactive peptides, lactobacilli cultures DH2 alone and in combination with NCDC 695 and 696 were found highly proteolytic and it was necessary. Proteolytic potential of these microorganisms are important to ensure their growth in milk.

Development of α-glucosidase inhibitory lactobacillus fermented milk using response surface methodology

Water soluble 30kDa extracts of Lactobacillus fermented milk showed appreciable AGI activity. Protein/peptide content of water extract of was lactobacilli fermented milk was analysed showed that proteins are having potential to inhibit α-glucosidase enzyme. Further for optimization of lactobacilli fermented milk, MWCO membrane milk protein extracts were considered as the bioactive factor responsible for α-glucosidase inhibition. For optimization of product three responses were optimized and among these one of the response was α-glucosidase inhibitory potential exhibited by the peptide extracted from lactobacilli fermented milks prepared with the suggested conditions or formulation by software.

As there was scanty information on the development of anti-hyperglycemic fermented milk, the Central Composite Rotatory Design package (Design Expert 7.0.0) of Response surface methodology technique was applied to the optimize the levels of various unknown variables e.g. percent of inoculums level, time of incubation and percent of WPC-70 for the development of product. The various ranges of these variables were selected during preliminary trials, viz., time of incubation (6 to 11h), inoculum level of lactobacilli (1-2.5 %) and % of WPC-70 (1-3%) for the development of lactobacilli fermented milk. The computation suggested about 20 formulations as delineated of the product in Table.1.

For the development of Lactobacilli fermented milk with anti-hyperglycemic potential, the responsible bioactive peptides extracted from milk should show considerable α-glucosidase (digestive enzyme) inhibition. In such developed novel fermented milk technological factors such as pH and titratable acidity are per se to play an important roles in sensory acceptability of the product. These variables were optimized in such a way that the lactobacilli fermented contain WPC-70 at appropriate level. In case of both the lactobacilli fermented milk products (Product A and Product B) responses affected by variable factors have been discussed here.

Table.1 Experimental variables for anti- hyperglycaemic lactobacilli fermented milk products (coded and actual values)

Independent Variables	Coded Values	Coded Level					
		Low Actual	High Actual	Low Coded	High Coded	Mean	Standard Deviation
Incubation period (h)	A	6.00	11.00	-1.000	-1.000	8.500	2.066
Inoculum level (%)	B	1.00	2.50	-1.000	-1.000	1.750	0.620
WPC-70 (%)	C	1.00	3.00	-1.000	-1.000	2.000	0.826

Table.2 Central composite design matrix with the experimental data on responses of product-A and B for response surface analysis [α-glucosidase inhibitory *Lactobacillus* fermented milk products]

Exp. No.	Run	Factors			Responses					
		Inb. period (%)	Inl. level (%)	WPC-70 (%)	AGI activity (%)		pH		Lactic acid (%)	
					Pdt. A	Pdt. B	Pdt. A	Pdt. B	Pdt.. A	Pdt. B
1	20	6	1	1	7	4	6.24	6.22	0.2	0.2
2	13	11	2.5	1	38	16	4.33	4.33	0.89	0.89
3	5	6	2.5	1	7	3	6.34	6.14	0.2	0.2
4	15	11	2.5	1	22	11	4.58	4.58	0.74	0.74
5	19	6	1	3	8	4	6.44	6.44	0.19	0.19
6	8	11	1	3	26	14	4.52	4.52	0.78	0.78
7	2	6	2.5	3	10	2	6.11	6.11	0.28	0.28
8	10	11	2.5	3	28	17	4.82	4.82	0.71	0.71
9	1	4.3	1.75	2	7	4	6.45	6.45	0.19	0.19
10	11	12	1.75	2	28	10	5.31	5.31	0.68	0.68
11	12	8.5	0.49	2	14	6	5.77	5.77	0.23	0.23
11	9	8.5	3.01	2	14	9	4.62	4.62	0.49	0.49
12	6	8.5	1.75	0.32	11	8	4.82	4.82	0.53	0.53
13	7	8.5	1.75	3.68	12	8	4.84	4.84	0.47	0.47
14	18	8.5	1.75	2	15	11	4.53	4.53	0.62	0.62
15	17	8.5	1.75	2	15	11	4.53	4.53	0.62	0.62
16	14	8.5	1.75	2	15	11	4.53	4.53	0.62	0.62
17	4	8.5	1.75	2	15	11	4.53	4.53	0.62	0.62
18	3	8.5	1.75	2	15	11	4.53	4.53	0.62	0.62
19	16	8.5	1.75	2	15	11	4.53	4.53	0.62	0.62
20	20	6	1	2	15	11	4.53	4.53	0.62	0.62

Fig.1 Flow diagram of lactobacillus fermented milk product A and product B

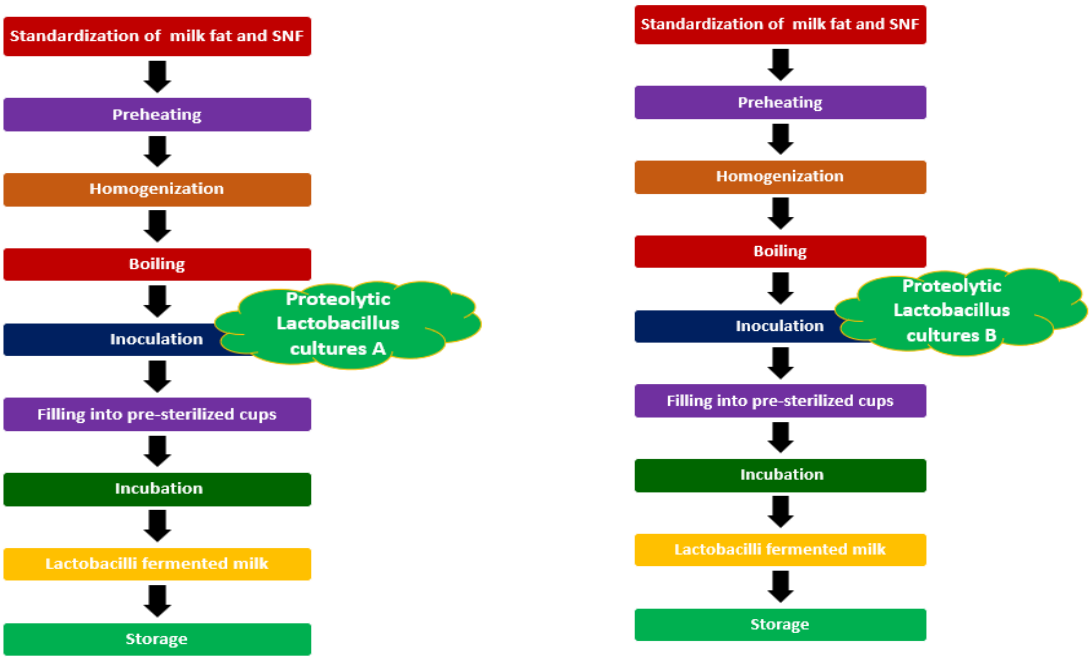


Table.3 Comparison of actual and predicted values of responses

Attributes	Predicted values*		Actual values**	
	Product A	Product B	Product A	Product B
AGI (%)	24.9	13.6	25.23	12.7
pH	4.37	4.34	4.29	4.31
Lactic acid (%)	0.75	0.77	0.833	0.81

*Predicted values given by Design Expert Version 7.0.0
**Actual values (average of 3 trails) of the opitimised product

Table.4 Chemical and microbiological analysis of the products

Parameters	Control	Product A	Product B
pH	4.33± 0.08	4.09±0.02	4.32 ± 0.41
Titratable acidity (% LA)	0.82± 0.04	0.91±0.02	0.89 ± 0.37
Protein content (%)	2.87±0.05	2.55±0.09	2.57±0.03
Fat (%)	2.5± 0.08	2.5±0.00	2.5± 0.01
SNF (%)	9.3±0.07	11.00± 0.08	11.00±0.06
Total solids (%)	11.8±0.06	13.5±0.12	13.7±0.16
Lactobacilli count (CFU/gm)	8.98±0.52	9.12±0.70	8.80±0.61
Coliform	-	-	-
Yeast and mold	-	-	-

Final Equation in Terms of Actual Factors

(Product A)

AGI= - 4.05553 +1.62734 * Incubation period -1.76752 * Inoculum Level + 2.93499E-003 * WPC (70) -1.06667 * Incubation period * Inoculum Level -0.50000 * Incubation period * WPC (70) +3.33333 * Inoculum Level * WPC (70) + 0.2750 * Incubation period2 +0.85598 * Inoculum Level2 -0.40239 * WPC (70)2

(Product B)

AGI= -33.20050+6.91833 * Incubation Time +6.54299* Inoculum level +0.96327 * WPC -70-0.2666 * Incubation Time * Inoculum level +0.10000* Incubation Time * WPC -70+0.66667* Inoculum level * WPC -70-0.28305* Incubation Time2-1.57360* Inoculum level 2-0.70837* WPC -702

It can be observed from the Table.1 that AGI activity of product A and B ranged between 7 to 38 % and 4 to 17 % respectively. In case of product A, the formulation that showed highest AGI activity value contained 10.48 incubation period, 1.48 inoculum level and 1.25 WPC-70, and product B 10.73 incubation period, 1.65 inoculum level and 1.87 WPC-70. At interaction level, it has been interpreted by ANOVA that the combined effect of incubation period and inoculum level (AB), incubation period and WPC-70 (AC) and incubation period (A2) had a positive effect ($P<0.05$) on AGI activity of the products.

However, incubation period WPC-70 (AC) as well as inoculum level and WPC-70 (BC) had non-significant effect ($P<0.05$) on AGI activity of the products (Fig.3a-c and Fig.4a-c). The response surface equations derived from prediction of the effect of 3 factors on AGI activity of the products as shown in the response plots.

The main aim of optimization was to determine the best possible combination of the three factors that would result in most acceptable products with respect to maximum AGI activity as well as physical attributes (pH and acidity).

It was done through a criterion (Table.2) used for the optimization of the product in which range of all the three variables and responses obtained were fed into the software to obtain the level of importance.

Fig.2 Zone of proteolysis on skim milk agar produce by selected lactobacilli cultures (Data are presented as means ± SD (n=3)

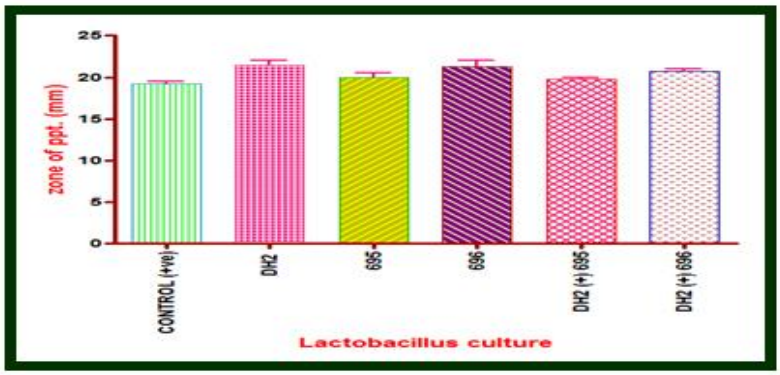
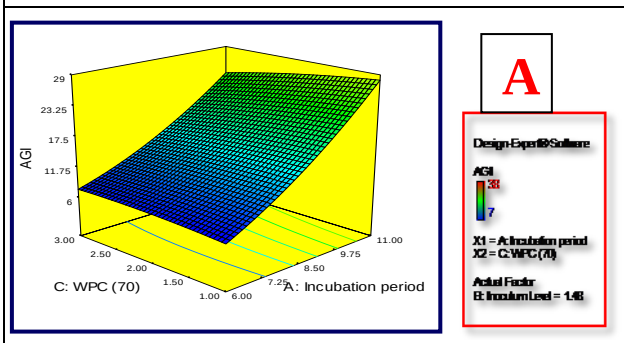
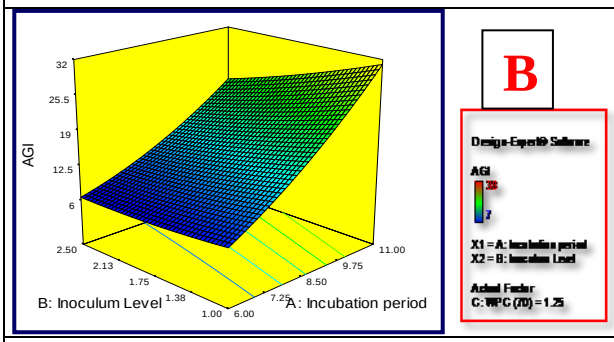


Fig.3 Response surfaces showing effect of incubation period, inoculum level and WPC-70 on AGI Activity for Product-A





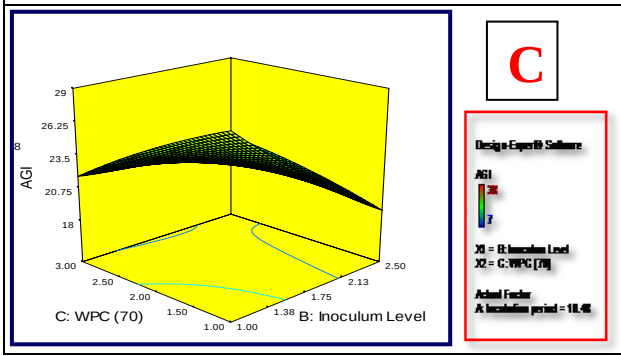
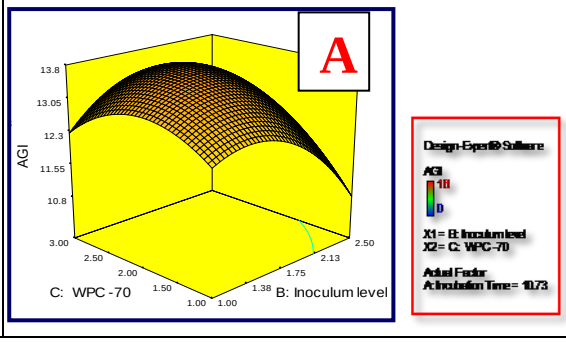
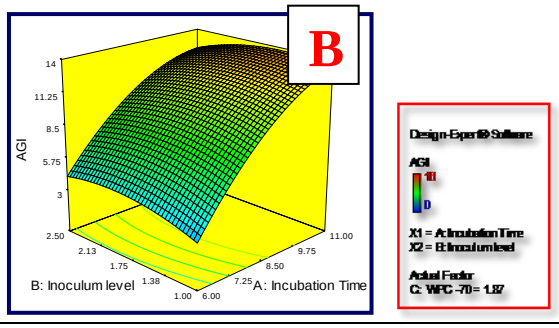
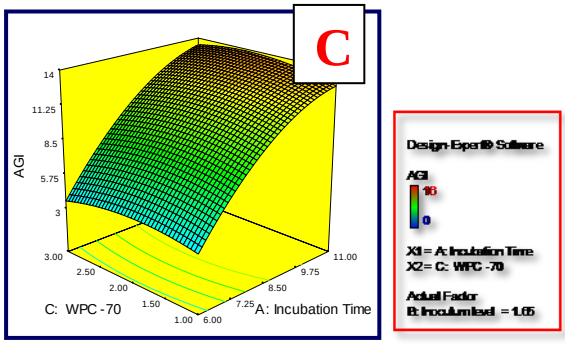


Fig.4 Response surfaces showing effect of incubation period, inoculum level and WPC-70 on AGI Activity for Product-B







Based on this analysis of the fermented products formulated using different levels of inoculum level, incubation period and WPC-70, a combination of the factors was obtained with very good desirability for further evaluation (Table.4).

The predicted values obtained through Design Expert package (7.0.0), were also compared with the actual values of AGI activity, pH and acidity for both the products (Table.2).

Evaluation of physicochemical and microbiological properties of the product

The gross composition of the final product is listed in Table.3. The influence of peptides presents in WPC-70 and fermented milk was hydrolyzed by the proteolytic enzymes released by lactobacilli culture combination. Furthermore, the incorporation of micronutrients to the milk, such as peptides and amino acids, may be useful to reduce fermentation time. It is accepted that food

proteins may act as precursors of biologically active peptides with different physiological effects (Hernández-Ledesma *et al.,* 2005). The firmness of fermented milk is highly dependent on total solids content (Tamime and Deeth, 1980; Penna *et al.,* 1997), on the protein content of the product (Tamime *et al.*, 1984; Dave and Shah, 1998b; Trachoo and Mistry, 1998), and on the type of protein (Tamime *et al.*, 1984; Cho *et al.*, 1999). In the present study, total solids content for fermented milks was 13.5%. WPC, which is an excellent substrates for production of health beverages as it is cheap and widely available in large volumes. It can also be used as a substrate for production of different microorganism or their metabolites (Mrvcic *et al.*, 2008).

Future Prospects

Fermented milk products were prepared with the combination of these non α-glucosidase producing Lactobacillus cultures and these were showing good fermentation attributes with considerable sensory attributes score. These culture combination were taken for further research work. Protein/ peptide obtained from fermented milks prepared with the culture combination; DH2+695 and DH2+696 showed highest AGI percentage and accordingly, these cultures combinations were selected for further product optimization. Lactobacilli fermented milk. were optimized with the help of response surface methodology; using three independent variables viz: incubation period, inoculum level and level of WPC-70 % on the basis of AGI activity. Two fermented milk products were developed (product A; incubation period-18h, inoculum level-1.5 and WPC-70 %- 1.25 and B; incubation period-18h, inoculum level-1.65 and WPC70 %- 1.65). On optimization, peptide extracted form product A showed higher AGI potential as than to product B peptides.

Fermented milk derived peptides possesses potential AGI activity and these peptides are generated in the naturally prepared fermented milk without any artificial/synthetic ingredient. This was probably the first study, about identification of bioactive factor responsible for digestive enzyme inhibition through milk fermented milk by non-α-glucosidase inhibitory lactobacilli strains. Moreover, preliminary beneficial effects of milk derived bioactive peptides on target diseases should be considered carefully before it can be formulated as chemotherapeutical agents or may try to use them directly in their viable condition.

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