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PHYSIOCHEMICAL FACTORS AFFECTING GROWTH OF LACTOBACILLUS SALIVARIUS

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ABSTRACT

Lactobacillus salivarius (*L. salivarius*) is a probiotic bacterial species having a multiple range of therapeutic uses including antibacterial, antifungal and detoxifier. The present study was carried out to explore alternative and more effective medium for its growth. For this purpose, milk products (Skimmed Milk Powder: SMP and Fermented Whey Powder: FWP) supplemented with industrial waste (Corn Steep Liquor: CSL and Yeast Sludge: YS) were studied as alternative media for bacterial growth. Along with media components, physical conditions (Time, Temperature, pH, Agitation, etc.) were also optimized. Among both the milk products, FWP (2%) had shown the highest bacterial growth (11.7 + 0.17 log10 CFU/mL). Bacterial growth was improved by adding 0.3% CSL in both (FWP: 13.8 + 0.7 log10 CFU/mL and SMP: 12.8 + 0.03 log10 CFU/mL) media. FWP medium was comparatively better for growth of *L. salivarius* when 1 % YS was added (13.4 + 0.1 log10 CFU/mL). Optimum bacterial growth was achieved in both the media after 24 hours of incubation at 30 °C temperature, 6.5 pH and 200 rpm agitation. It was concluded that 2% FWP medium supplemented with 0.3% CSL, 1 % YS, 1% Glucose had proven to be better for growth of *L. salivarius*.

Key words: *Lactobacillus salivarius*, optimization, milk products, industrial waste.

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INTRODUCTION

Lactobacilli are Gram's positive rods, non-spore forming, aerotolerant and gives negative result to Catalase test. There are 25 species in clade *L. salivarius* from genus *Lactobacillus* (Salvetti *et al.,* 2018). They have wide range of inhabitants such as gastro intestinal tracts (GIT), fermented food or feed, water and environment, etc. They have multiple beneficiary characteristics such as probiotic, antibacterial, antifungal, food preserving and detoxifier. They are widely used in many commercial products (Parbhurajeshwar and Chandrakanth, 2017).

The *L. salivarius* from gut of animal, human & poultry and milk have shown probiotic characteristics. They act as immune-modulator, health inducer and pathogen inhibitor (Chaves *et al.,* 2017). They have shown antimicrobial activity against pathogens such as *S. mutans* and *C. albicans* (Krzysciak *et al.,* 2017). They have ability to modulate gut microbiota, produce antimicrobial substances (Bacteriocin) and stimulate protective immune response in host. They establish themselves in animal gut and reduce colonization of gut pathogens. Strains of *L. salivarius* have been used to prevent and treat a variety of chronic diseases, including cancer, atopic dermatitis, asthma and halitosis. *L. salivarius* does not pose a health risk to animals or humans when used in a variety of applications (Chaves *et al.,* 2017).

Lactobacilli are fastidious in nature, that's why they need expensive nutrients (carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins) in their medium. Man Rogosa Sharpe (MRS) is standard selective medium, used for *Lactobacillus*. There is a need for finding effective alternative culture media for *Lactobacillus.* Different carbon (glucose, maltose, lactose and whey powder) and nitrogen sources (casein hydro lysate, peptone, yeast extract powder, fish meal, ammonium sulfate and sodium nitrate) are screened for effective growth of *Lactobacillus* media. Di potassium hydrogen phosphate and sodium acetate are positive, and glucose is negative factors for the growth of *Lactobacillus* (Chen *et al.,* 2015). Whey is by product from cheese industry. Fermented whey and skimmed milk are valuable and cheap growth media for *Lactobacillus* (Soenarno *et al.,* 2019). Peptone and Yeast extract are used as milk supplement to enhance the bacterial growth. Skimmed milk supplemented with CSL increased the growth of probiotics such as *Lactobacillus* (Luciana *et al.,* 2013). For growth of *Lactobacillus,* glucose (13.4 g/L), sodium pyruvate (3.4 g/L), meat extract (7.2 g/L), potassium phosphate (2g/L), sodium acetate (5g/L) and ammonium citrate (2g/L) are optimum (Mufidah *et al.,* 2016). Whey medium is proven to be better and coast effective for growth of *Lactobacillus* species (Bovo *et al.,* 2014). Reconstituted whey powder and skimmed milk are optimum for the growth for *Lactobacillus* (Gustaw *et al.,* 2016). The medium containing glucose 2%, yeast extracts 2% and meat peptone 3.5%, is also effective for the bacterial growth. As the physical factors are concerned incubation at 37°C and pH 7.0 along with agitation are optimum for the growth of *L. salivarius* (Malek *et al.,* 2010). This study was designed to prove that milk products (such as SMP and FWP) supplemented with industrial waste (CSL and YS) as complex nitrogen source are ideal candidate for the growth of *L. salivarius*.

MATERIALS AND METHODS

Source of Chemicals: Chemicals such as MRS broth (Oxoid, code # 0041), bacterial agar (Oxoid, code # LP0011), phosphoric acid (Sigma Aldrich, CAS # 7664-38-2), sod. hydroxide (Sigma Aldrich, CAS # 1310-73-2), glucose (Sigma Aldrich, CAS # 50-99-7) and di potassium hydrogen phosphate (Sigma Aldrich, CAS # 7785-11-4) were purchased from local suppliers. CSL was provided by Rafhan maize product company, Faisalabad. YS was obtained from Shakarganj sugar mills, Jhang. SMP and FWP were obtained from local market.

Proximate analysis: Samples of CSL, YS, FWP and SMP were sent to Department of Food Science and Human Nutrition, University of Vet. & Animal Sci., (UVAS), Lahore for proximate analysis.

Source of Bacterial Culture: Identified and characterized broiler isolate, *L. salivarius* (strain CR.4), with accession number RY6: MG938650 was used. It showed 99% similarity with strain KU 163336.1 when NCBI -BLAST was performed. Its glycerol (15% v/v) stock was prepared and stored at -20 °C for future use.

Preparation of Inoculum: Isolate was activated and purified on sterilized MRS agar plate. A tube of 10 mL MRS broth was taken and inoculated with bacterium. It was incubated for 24 hours at 37°C and 10-fold serial dilutions were prepared from its 1 mL. Spread plate method was used to calculate Colony Forming Units (CFU/mL).

Optimization of milk products: On the base of previous study, two milk-based product (SMP, FWP) and two industrial wastes (CSL, YS) as supplements were selected to formulate optimum media for *L. salivarius*. The medium optimizing design, one factor at a time (OFAT) for bacterial growth was followed (Bruns *et al.* 2008). A basal broth was prepared by glucose 1%, di potassium hydrogen phosphate 0.2%, CSL 0.1% and YS 0.1%. This basal broth was used for further modification and statistical optimization.

The broth medium 50 mL was poured into 10 flasks of 250 mL. In this broth SMP and FWP was added (2,4,6,8 and10 %). The pH 6.5 was adjusted by using phosphoric acid and sodium hydroxide dilute solutions. A flask of MRS broth was prepared for comparison of bacterial growth. After autoclaving and inoculation, flasks were incubated at 37°C for 24 h. At the end of fermentation CFU/ mL was calculated.

Optimization of industrial waste: In culture broths (4 % SMP and 2% FWP), CSL (0.01, 0.1, 0.2, 0.3, 0.4, 0.5,0.6, 0.7, 0.8, 0.9, 1%) (Thakur *et al.,* 2019) and YS (0.1, 0.5. 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 %) were added (Mufidah *et al.,* 2016). The pH was adjusted 6.5, after autoclaving and inoculation the flasks were incubated at 37°C for 24 h. At the end of incubation CFU/ mL was calculated.

Optimization of physical factors: Optimized Broths (SMP and FWP) were prepared and optimum physical factors were determined by using method described by Talluri and Lanka, (2017) with minor variation. For this purpose, different ranges of the incubation time (4, 8, 12…48 hours), the temperature (20, 25, 30, 35, 40°C), pH (4, 5, 6, 7, 8) and agitation rate (0, 50, 100, 150, 200 rpm) were determined. At the end of each step CFU/ mL was calculated.

Enumeration of microbial cells: To calculate viable count (CFU/mL), Spread plate method by glass road was used (Thomas *et al.,* 2015). One mL from each sample was serially 10-fold diluted in sterile PBS (pH 7, 0.1 M). The diluted samples (300 µL) were spread on MRS agar plates and incubated at 37°C for 24 h. The colonies on plate were counted by using colony counter. Results were recorded as colony forming units per milliliter (CFU/mL) of *L. salivarius* CR.4.

Statistical Analysis: Results of bacterial growth in media were obtained in form of colony forming units (CFU/ mL). Their Log_{10} CFU/mL were calculated and analyzed by one-way ANOVA using SPSS 16.0 software. Duncan's multiple range test was performed to compare the means. Difference among means was considered significant ($P \leq 0.05$).

RESULTS AND DISCUSSION

The milk-based media when supplemented with nutrients was an ideal choice for *Lactobacillus* growth. Whey is milk serum with high amount of lactose, fats and minerals. Similarly, skimmed milk is high in lactose and minerals. Concentration of lactose was higher in whey powder as compared to skimmed milk powder. Lactose was the main ingredient in them which activates growth of *Lactobacillus.* But they were deficient in proteins (Kaur *et al.,* 2017). The concentration of protein is 36.4% in SMP and 12.2% in FWP (Table. 1). Industrial waste supplements (CSL, YS) can cover up this deficiency, as CSL was rich in protein (39.9%) and YS had high concentration of minerals (40.3%) (Table. 1). CSL and YS are rich source of proteins, amino acids, minerals and vitamins. They are chemically undefined substances, used as milk supplements in industry for bacterial growth (Arakawa *et al.,* 2015).

Table 1: Proximate composition of Yeast sludge, Corn steep liquor, Skimmed milk powder and Fermented whey powder.

Components	Dry Yeast sludge %	Corn steep Liquor %	Skimmed milk powder %	Fermented whey powder %
Energy value (kcal/100 gm)	218.4	225.85	350.08	357.68
Protein	11.36	39.90	36.4	12.2
Lipid	1.6	0.25	0.6	0.8
Minerals	40.3	10.40	8.6	7.5
Crude Fiber	0	0	0	0
Nitrogen free Extract	39.64	16	49.77	75.42
Dry matter	92.90	66.55	95.37	95.92

Table 2: Comparison among media for optimum bacterial growth (*L. salivarius*) under standard conditions.

Sr. #	Media	Composition	Log_{10} CFU/mL
1	Man, Rogosa Sharpe broth	Peptone 1%, meat extract 0.4%, yeast extract 0.4%, glucose 2%, sorbitan mono oleate 0.1%, dipotassium hydrogen phosphate 0.2%, sodium acetate 0.5%, tri ammonium acetate 0.2%, magnesium sulphate 0.02%, manganese sulphate 0.005%, pH 6.2	7.8 ± 0.17
2	Skimmed milk powder medium	Skimmed milk powder 4%, corn steep liquor 0.3%, yeast sludge 0.5%, glucose 1%, Dipotassium hydrogen phosphate 0.2%, pH 6.5	9.36 ± 0.1
3	Fermented Whey powder medium	Fermented whey powder 2%, corn steep liquor 0.3%, yeast sludge 0.5%, glucose 1%, Dipotassium hydrogen phosphate 0.2%, pH 6.5	13.4 ± 0.1

L. salivarius gave highest growth in 4% SMP (11.36 ± 0.11 log_{10} CFU/mL) and 2% FWP (11.6 ± 0.17 log_{10} CFU/mL) (Fig. 1). Both media gave better results (SMP: 12.86 ± 0.03 log_{10} CFU/mL and FWP: 13.86 ± 0.07 log_{10} CFU/mL) when 0.3 % CSL was used (Fig. 2). YS (0.1%) gave the higher growth of *L. salivarius* (9.36 log_{10} CFU/mL) in SMP media but in FWP medium bacterial growth increased up to 13.4 ± 0.1 log_{10} CFU/mL when 1% YS was used (Fig. 3). Milk based media had proven to be better for growth of *Lactobacillus* as compared to standard MRS broth (7.8 ± 0.17 log_{10} CFU/ mL) at same conditions. According to Chen *et al.,* (2015) glucose and whey were ideal carbon source for *Lactobacillus*. Peptone, yeast sludge and CSL were proven to be best nitrogen source for growth of this bacterium. Cheese whey along with skimmed milk and sucrose (1:1) proved to be ideal for bacterial growth (Kusandi *et al.,* 2014). According to Cordeiro *et al.,* (2019), milk supplemented with 30 % whey powder was a better medium for *Lactobacillus. Lactobacillus* have ability to hydrolyze and consume proteins from this medium (Pescuma *et al.,* 2008). Production of *Lactobacillus* biomass increase by increasing concentration of YS in whey (1-10%). But all strains do not show same behavior. For some strains increase in concentration of YS has negative effect on growth of bacteria (Benaissa *et al.,* 2017). CSL is economically cheap source of nitrogen for bacterial media in industry (Tan *et al.,* 2016). But acidic pH of CSL, inhibits bacterial growth when concentration of CSL is increased (Li *et al.,* 2016). Highest growth of *Lactobacillus* was achieved in whey media (6 %) when low concentration of glucose (1.5%) and CSL (1.5%) were added in it. It was even better than standard MRS media used for growth of *Lactobacillus* (Manzoor *et al.,* 2017).

Physical factors such as time of incubation, temperature, pH and agitation rate played crucial role in bacterial growth. Optimum time for bacterial growth was 24 hours, in both media (FWP: 13.4 ± 0.1 $log10_{10}$ CFU/mL, SMP: 9.36 ± 0.1 log_{10} CFU/mL) (Fig. 4). Optimum temperature for *L. salivarius* was 30 ° C (FWP: 12.9 ± 0.03 log_{10} CFU/mL, SMP: 11.9 ± 0.03 log_{10} CFU/mL) (Fig. 5). It was observed in Fig. 6 that on increasing pH from 4-6.5, bacterial growth increased up to 13.7 ± 0.04 log_{10} CFU/mL in FWP medium and 11.6 ± 0.1 log_{10} CFU/mL in SMP medium. Growth of bacterium decreased in both media by increasing pH from 7-8. It was observed in Fig. 7 that in absence of agitation (both anaerobic and aerobic condition) bacterial growth was low (SMP 9.4 ± 0.1 log_{10} CFU/mL, 9.6 ± 0.05 log_{10}

CFU/mL and FWP: 9.9 ± 0.09 log_{10} CFU/mL, 10.5 ± 0.2 log_{10} CFU/mL). By increasing agitation rate (0-200 rpm), bacterial growth increased (SMP: up to 12.5 ± 0.2 log_{10} CFU/mL and FWP:(13.8 ± 0.2 log_{10} CFU/ mL).

Optimum growth time for *Lactobacillus* according to different researches varies. It was 24 hrs according to Kusandi *et al.* (2014), 48 hrs by Bovo *et al*. (2014) and 72 hrs according to Arakawa *et al*. (2015). In MRS media, during first 4 hrs bacterium establish itself in media (lag phase). From 4 to 16 hrs it grew extensively (log_{10} phase), from 16 to 22 hrs its death and division become equal (stationary phase) and after 22 hrs, the death phase starts (Zhang *et al.,* 2013). *L. salivarius* showed maximum growth when incubated at 37 °C, pH 7 and 400 rpm agitation for 24 hrs (Malek *et al.,* 2010). In milk based medium optimum pH for its growth was 6.5-5. Bacterial growth stops when pH drops below 4.5. pH of the media plays important role in the vital functions of cells (enzyme activity, nutrient intake etc.) (Bovo *et al.,* 2014). According to other studies agitation rate has no effect on growth of *Lactobacillus* (Mustafa *et al.,* 2019), which do not support findings of this study.

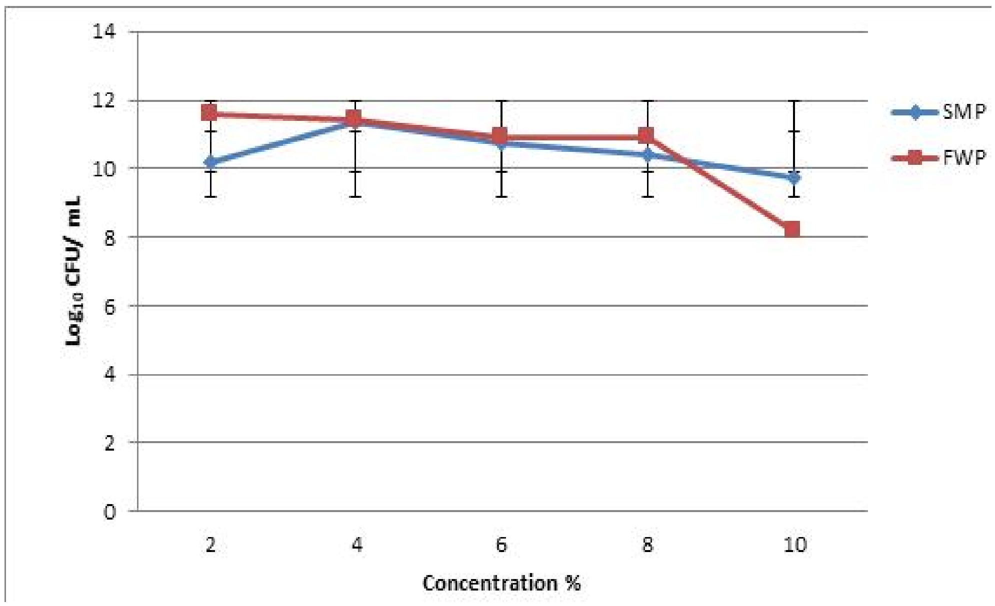

Figure 1: Effect of different concentrations of Skimmed Milk Powder (SMP) and Fermented Whey Powder (FWP) on growth of *L. salivarius* (Log_{10} CFU/mL).

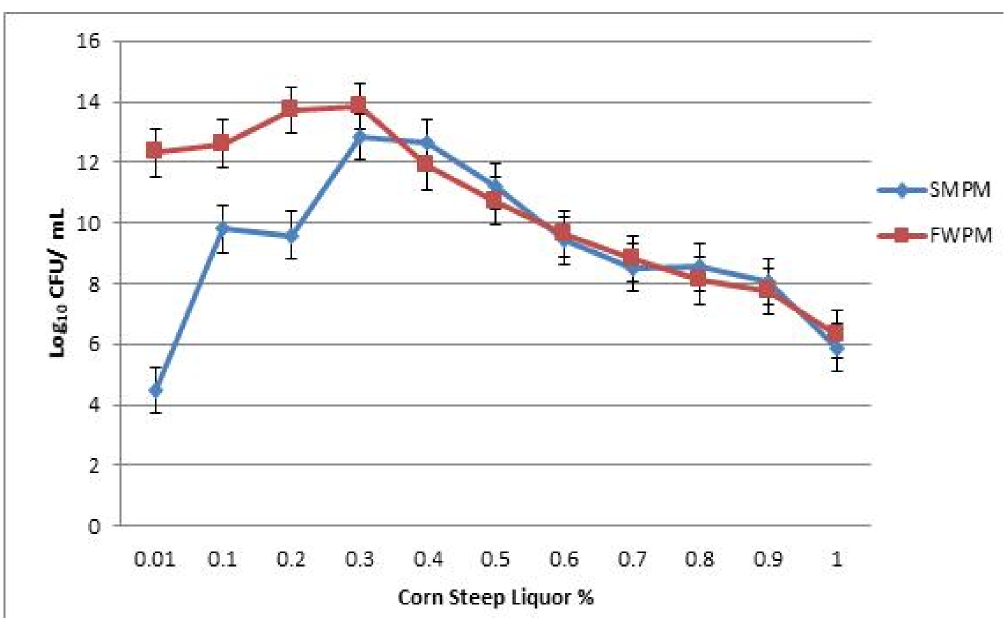

Figure 2: Effect of different concentrations of Corn Steep Liquor on growth of *L. salivarius* (Log_{10} CFU/mL) in skimmed milk powder medium (SMPM) and fermented whey powder medium (FWPM).

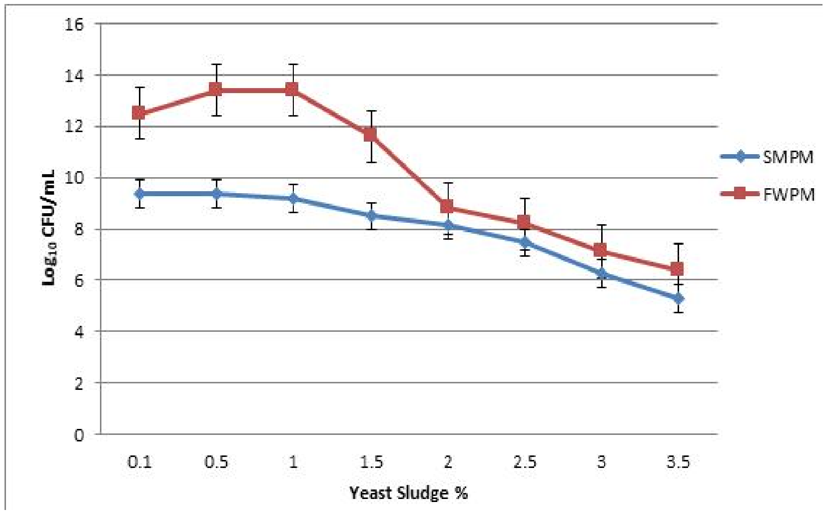

Figure 3: Influence of different concentrations of Yeast Sludge on growth of *L. salivarius* (Log_{10} CFU/mL) in skimmed milk powder medium (SMPM) and fermented whey powder medium (FWPM).

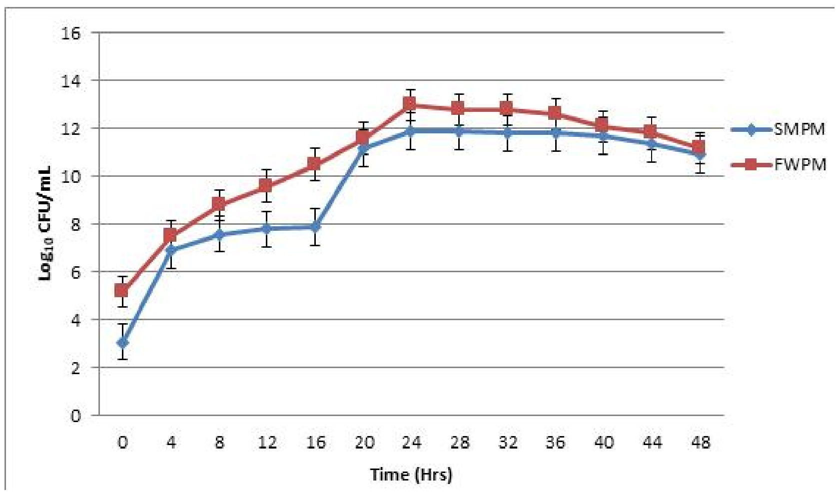

Figure 4: Effect of different time of incubation (Hrs.) on growth of *L. salivarius* (Log_{10} CFU/mL) in skimmed milk powder medium (SMPM) and fermented whey powder medium (FWPM).

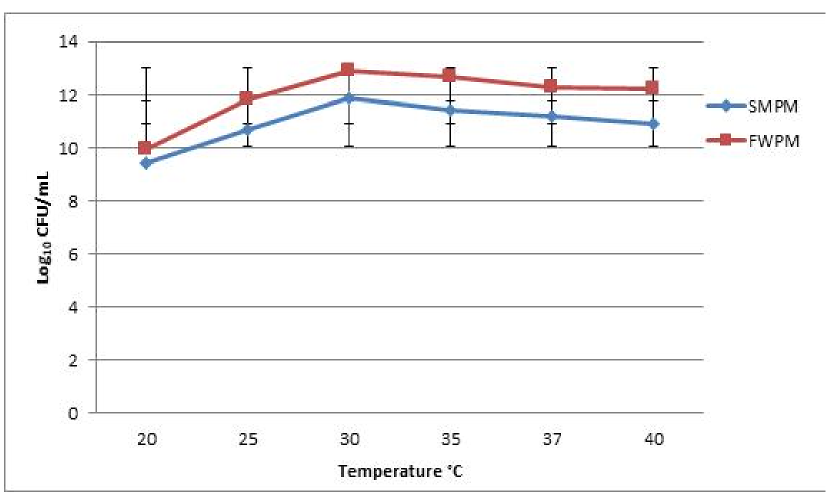

Figure 5: Effect of different temperature (°C) on viable count of *L. salivarius* (Log_{10} CFU/mL) in skimmed milk powder medium (SMPM) and fermented whey powder medium (FWPM).

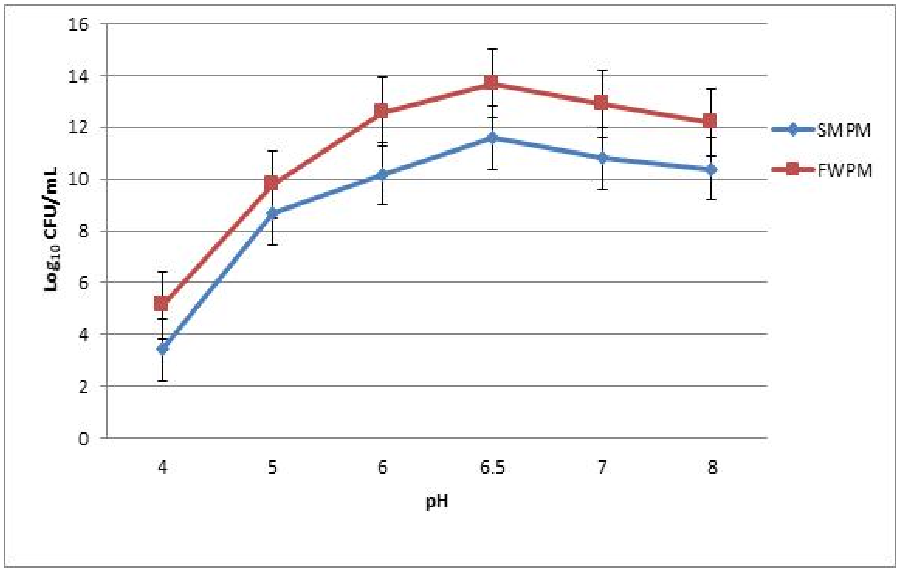

Figure 6: Effect of different pH on viable count of *L. salivarius* (Log_{10} CFU/mL) in skimmed milk powder medium (SMPM) and fermented whey powder medium (FWPM).

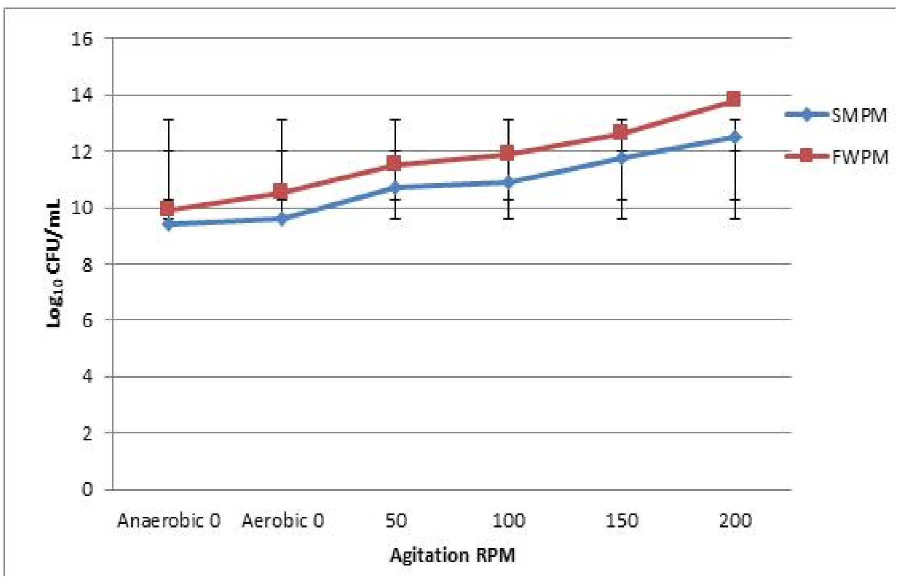

Figure 7: Effect of different agitation rate (RPM) on viable count of *L. salivarius* (Log_{10} CFU/mL) in skimmed milk powder medium (SMPM) and fermented whey powder medium (FWPM).

Conclusion: Fermented whey powder medium supplemented with corn steep liquor, yeast sludge and glucose has proven to be optimum for the growth of *L. salivarius*. Maximum bacterial growth was achieved at 6.5 pH, 30 °C and 200 RPM agitation for 24 hrs. So, it is recommended that experiments should be conducted to test whey based medium for industrial scale production of *L. salivarius*.

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