PRODUCTION OF BACTERIOCIN FROM *PEDIOCOCCUS PENTOSACEUS* ISOLATED FROM *KURDAI*

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Abstract
Kurdai is a traditional fermented whole wheat snack food, native to Maharashtra and parts of Gujarat. The microbes responsible for kurdai fermentation have been successfully isolated and identified as Leuconostoc lactis, Pedicoccus pentosaceus, Enterococcus faecium, Staphylococcus hominis, Klebsiella pneumonia and Lactobacillus plantarum. Pedicoccus pentosaceus one of microbes responsible for kurdai fermentation were identified. In the present work, Pedicoccus pentosaceus was explored for the production of pediocin which is a Class II bacteriocin produced by lactic acid bacteria, because they are heat stable and non-modified lantibiotics. Bacteriocins have been drawing the attention of the food industry because of their increased specificity and effectiveness at low concentrations in order to prevent the development of pathogens. The activity of pediocin was evaluated using various commercial strains as indicators such as Escherichia coli MTCC 40, Bacillus cereus MTCC 1272, Leuconostoc mesenteroides NCIM 2198, Lactobacillus fermentum NCIM 2165 and Lactobacillus helveticus NCIM 2733. It was shown that the highest pediocin activity was expressed against Lactobacillus helveticus NCIM 2733 with 4.89 ± 0.13 Activity Units (AU)/mL being produced. Pediocin production was then optimized by controlling the incubation time, temperature seed age, inoculum density and initial pH of the growth media.

Keywords: Bacteriocin, *Pedicoccus pentosaceus*, *Kurdai*, Food preservation

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1.INTRODUCTION

Fermentation is the scientific process of breakdown of complex sugars to simpler substances by microbial action. Consumption of fermented foods has been a tradition throughout history mainly because they were produced as a method to prevent spoilage. There has been little scientific awareness on indigenous traditional fermented foods (Davidson and Ziavonic, 2003). Since fermented foods are a rich hub of natural microflora, systematic studies of indigenous fermented foods would be of great benefit in addressing the rising issue of food security.

Kurdai is Indian traditional wheat fermented food, native to Maharashtra and parts of Gujarat. It is prepared by soaking whole wheat in water for 3 days, during which the batter undergoes fermentation. The batter is then finely ground to obtain a milky white extract, which is cooked with water forming soft and stiff dough. The dough is then passed through a press to get *Kurdai*, which is then dried and stored. It is locally popular as a snack food after being deep fried for consumption. Its nutrition profile and fermentation biochemistry have never been studied. The significance of the present study lies in studying the microbial aspects of *Kurdai* as a novel ecological niche, and exploring the biotechnological potential of the isolates.

The major target of food processing such as fermentation is preservation. With microbes developing resistance to antimicrobial compounds, and increased public concern towards the use of synthetic approaches, natural methods of food preservation have been the focus of many research groups. Natural antimicrobials from microbial sources have the

potential to be included in food systems as edible components (Davidson and Ziavonic, 2003). *Pedicoccus pentosaceus* appears to be of great interest for food preservation, due to the production of antimicrobial peptide pediocin, a potent bacteriocin (Daeschel and Klaenhammer, 1985). *Pedicoccus pentosaceus* has applications in brewery industry and sausage fermentations, apart from applications as probiotic bacteria (Raccach, 1987).

Pediocins, along with other bacteriocins produced by *Pediococcus spp*. fall under the Class II bacteriocins produced by LAB- heat stable, non-modified lantibiotics. These bacteriocins have been drawing the attention of the food industry because of their increased specificity and effectiveness at low concentrations in order to prevent the growth of certain foodborne pathogens and have the ability to neutralize bacterial endotoxic activity. Considering the fact that pediocins have been assigned a GRAS status since 1988 (Federal Register, 1988), Pediocin producing strains could be applied as freeze dried cultures in food products and in antimicrobial packaging in order to control the growth of food borne pathogens (Benmechernene et al, 2013; Espitia et al, 2013; Huang et al, 2009). Studies on characterization of indigenous strains will provide new insights into optimizing their bacteriocin production and increase their potential use in a large number of food products.

In the present work, *Pediococcus pentosaceus* previously isolated from *Kurdai* were screened for their pediocin activity against commercial indicator strains. In addition, One Factor At a Time (OFAT) optimization of physical parameters responsible for pediocin production have also been studied, namely- production media, seed age, inoculum density, incubation temperature, production time and initial pH of media.

2. MATERIALS AND METHODS

Materials

All the chemicals used while carrying out the present work were of analytical grade and were from Himedia Laboratories, Mumbai, India. The commercial strains employed in the study were obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India and Microbial Type Collection Centre (MTCC), Chandigarh, India.

Methods

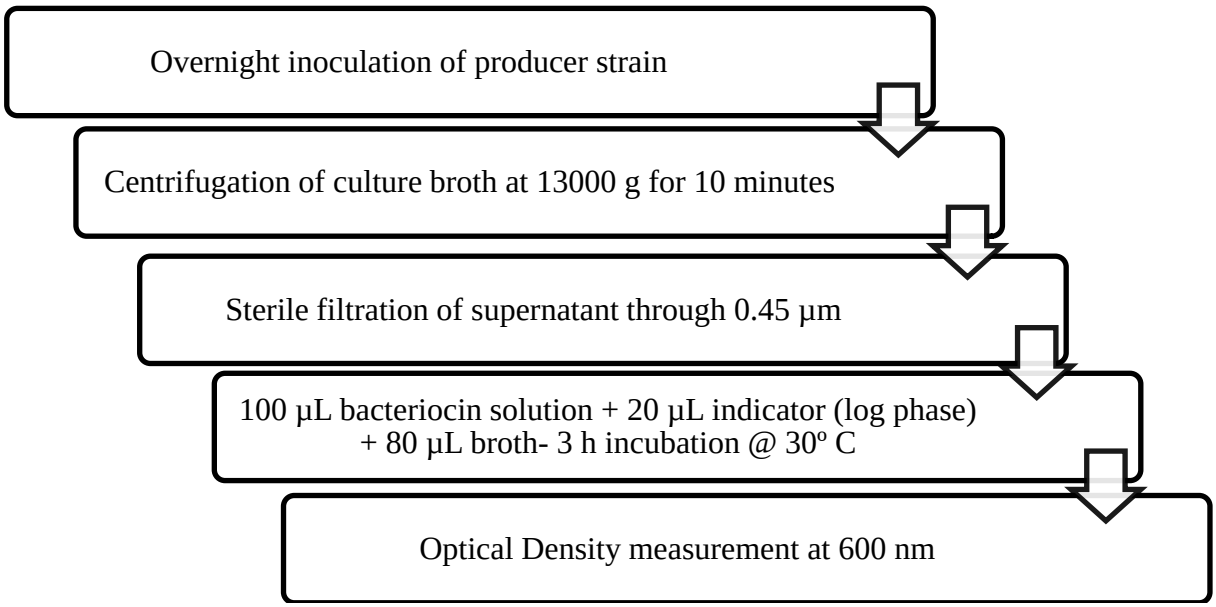
Kurdai preparation and identification of microbes

200 g of whole wheat was soaked in 250.0 mL of tap water for 72 h. Soaked wheat was ground to a coarse consistency. The ground slurry was then filtered through muslin cloth and filtered slurry was further subjected to fermentation for overnight at 30℃ to get fermented *Kurdai* batter. Then microbes were isolates from *kurdai* batter. Then isolates were sequenced at National Centre for Cell Science, Pune, India and Chromous Biotech, Bangalore, India. Analysis of the sequence was done using Seq scanner v1.0 (Applied biosystems). Species identification was done using NCBI nucleotide BLAST.

Screening of commercial indicators for maximum pediocin activity

Pediococcus pentosaceus strains were isolated from *Kurdai* and studied for their ability of production pediocin using commercial strains as indicators- *Escherichia coli* MTCC 40, *Bacillus cereus* MTCC 1272, *Leuconostoc mesenteroides* NCIM 2198, *Lactobacillus fermentum* NCIM 2165 and *Lactobacillus helveticus* NCIM 2733. de Man et al (1960) MRS medium was used as the growth as well as the production medium for initial studies. It has been postulated that activity of pediocin depends on incubation temperature of indicator strain (Bauer et al., 2005). In the present study, to avoid ambiguity, all indicator strains were studied for the activity assay at their logarithmic phase of growth; it was also ensured that they were cultured at the same temperature (37^{o}C). Pediocin activity assay was performed as per the spectrophotometric method reported (Toba et al., 1991). From these results, the strain that showed the highest bacteriocidal activity was employed for optimization studies.

Spectrophotometric method employed for assay:



Screening of production media and study of physical parameters responsible for pediocin activity:

Production media reported previously in literature for pediocin production were employed and compared against the growth media (MRS) for pediocin activity (Nel et al., 2001; Vijay Simha et al., 2012). Nel H A *et al.* (2001) and Vijay Simha B *et al.* (2012) have reported an improvement in bacteriocin activity upon supplementation with components as bacteriological peptone, tween 80, manganese sulphate and glucose. The selected *Pediococcus pentosaceus* isolate was grown in the above mentioned production media and the bacteriocidal activity was compared. The details of MRS medium composition (de Man *et al.*, 1960) were Peptone - 10 (g/ L), Meat extract – 8 (g/ L), Yeast extract – 4 (g/ L), D(+)- Glucose – 20 (g/ L), Dipotassium hydrogen phosphate – 2 (g/ L), Tween 80 – 1 (g/ L), Diammonium hydrogen citrate – 2 (g/ L), Sodium acetate – 5 (g/ L), Magnesium sulphate -2 (g/ L) and Manganese sulphate - 0.04 (g/ L). This compared with production medium 1 composition (Nel, H. A. *et al*., 2001) were MRS medium - 55.15 (g/ L), Bacteriological peptone – 17 (g/ L), Manganese sulphate - 0.14 (g/ L), Tween 80 – 30 (g/ L) and also production medium 2 (Vijay Simha, B. *et al.*, 2012) such as MRS medium - 55.15 (g/ L) and Glucose 20 (g/ L).Physical parameters responsible for pediocin production such as seed age (6 to 36 hours), inoculum density (10^{-1} to 10^{-5}), incubation temperature (25°C to 39°C), production time (14 to 28 hours) and initial pH of media (5 to 9) were studied in order to optimize bacteriocin production.

Statistical analysis

All tests were performed in triplicates, and the p value was determined using Microsoft Excel program in order to establish statistical significance of the data.

3. RESULTS AND DISCUSSION

Identification of microbes in *Kurdai* batter

The sequences obtained were checked for sequence homology using NCBI BLAST and the obtained results were summarized in Table1.

Table 1: Identified microbes in *Kurdai* batter

Isolate ID	Accession No.	Description	Sequence homology
A	AB681816.1	*Leuconostoc lactis*	99%
B	JN128742.1	*Pediococcus pentaceus*	99%
C	JN128742.1	*Pediococcus pentaceus*	99%
D	NR042254.1	*Lactobacillus plantarum*	99%
E	AB481104.1	*Enterococcus faceium*	98%
G	-	Seq failure	-
Y1	GQ417066.1	*Klebsiella pneumoniae*	99%
Y2	NR036956.1	*Staphylococcus hominis*	99%
Y3	GQ417066.1	*Klebsiella pneumoniae*	99%
Y4	-	Seq failure	-

Pediocin activity of commercial strains

The growth curve of *Pediococcus pentosaceus* was derived. MRS broth was inoculated with an overnight culture of *Pediococcus pentosaceus.* The Optical Density (OD) was measured at 600 nm against uninoculated MRS broth as the blank. Measurements were taken at regular intervals of time and the growth curve was obtained as shown in Fig 1.

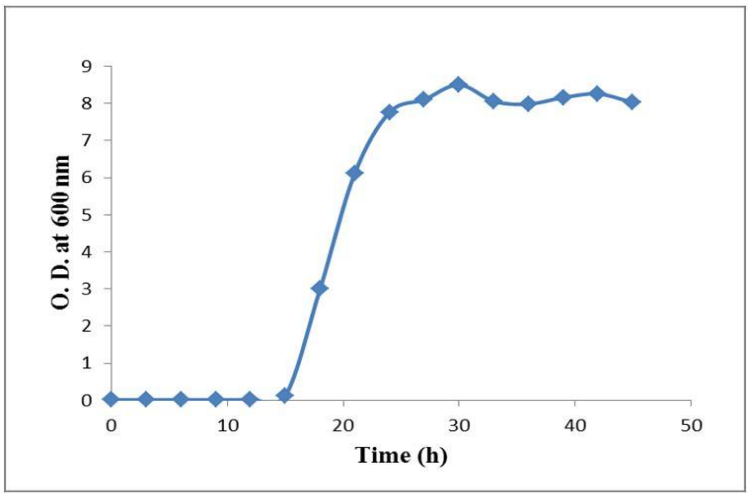


Fig. 1--Growth curve of *Pediococcus pentosaceus* isolated from *Kurdai*

The preliminary studies (Fig. 2) showed that the indigenous isolate showed activity against all 5 indicator strains. The highest pediocin activity was observed against strain *Lactobacillus helveticus* NCIM 2733 i.e. 4.89 ± 0.25 Activity Units (AU)/ mL, among the selected commercial strains. *Escherichia coli* MTCC 40 followed in sequence at 3.55 ± 0.12 AU/ mL. The least activity was expressed against *Lactobacillus fermentum* NCIM 2165 (1.34 ± 0.04 AU/ mL). A narrow spectrum of activity for class II bacteriocins has been

previously reported (Drider *et al*, 2006), and it has been postulated that activity of pediocin depends on incubation temperature of indicator strain (*Bauer et al*, 2005). The authors suggest that fluidity of cell membrane, coupled with its protein and lipid content determine its resistance to the formation of pores on itself by the antimicrobial peptide. Pore formation is the crucial step to bacteriocin attack on the target strain's cell. It is not presently possible to comment on spectrum of activity of our strain, since there is a need to do further studies. In the present study, to avoid ambiguity, all indicator strains were studied for the activity assay at their logarithmic phase of growth; it was also ensured that they were cultured at the same temperature (37^{o}C). Yet, further studies on the target cells and their growth kinetics are required for a complete understanding of the mechanism due to which *Lactobacillus helveticus* NCIM 2733 shows to be the less sensitive towards pediocin action by the indigenous isolate. Because of these results, *Lactobacillus helveticus* NCIM 2733 was chosen as the indicator strain for further studies.

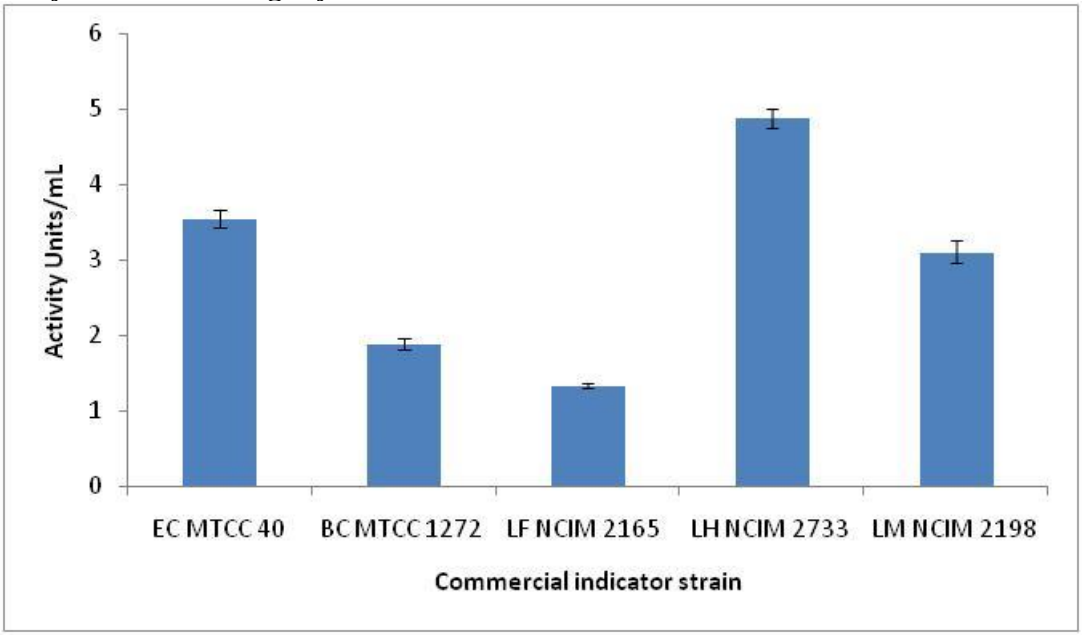


Fig. 2-- Pediocin activity as expressed against different indicators (n=3)

Optimization of physical parameters

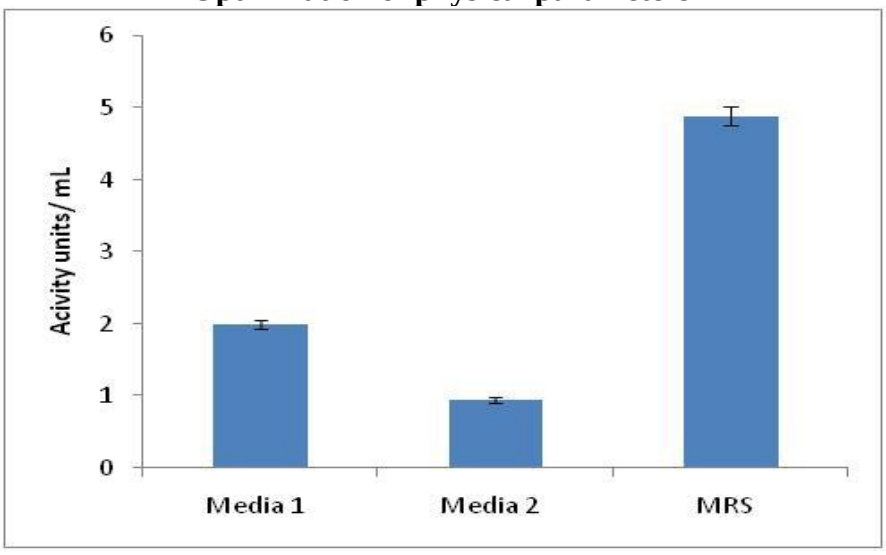


Fig. 3-- Screening of different production media (Media 1, Media 2) for pediocin (n= 3)

The indigenous isolate expressed considerably activity of pediocin shown in Fig. 3. For subsequent studies, MRS medium without supplementation, which showed an activity of 4.89 ± 0.25 AU/ mL, was used as production medium. It could be a possibility that supplemented components reduced the bioavailability of the original components of the MRS medium. Since MRS medium contains 20g/L glucose, supplementation could have possibly led to substrate inhibition. It has been reported that higher levels of carbon sources and an imbalance in C/N ratio reduce bacteriocin production (Guerra and Pastrana, 2002). Incorporation of manganese sulphate had an inhibitory effect on pediocin production; contrary to reports (Anastasiadou *et al*, 2008). Further studies are required to establish as to which among the supplementary components to MRS media caused the activity to reduce.

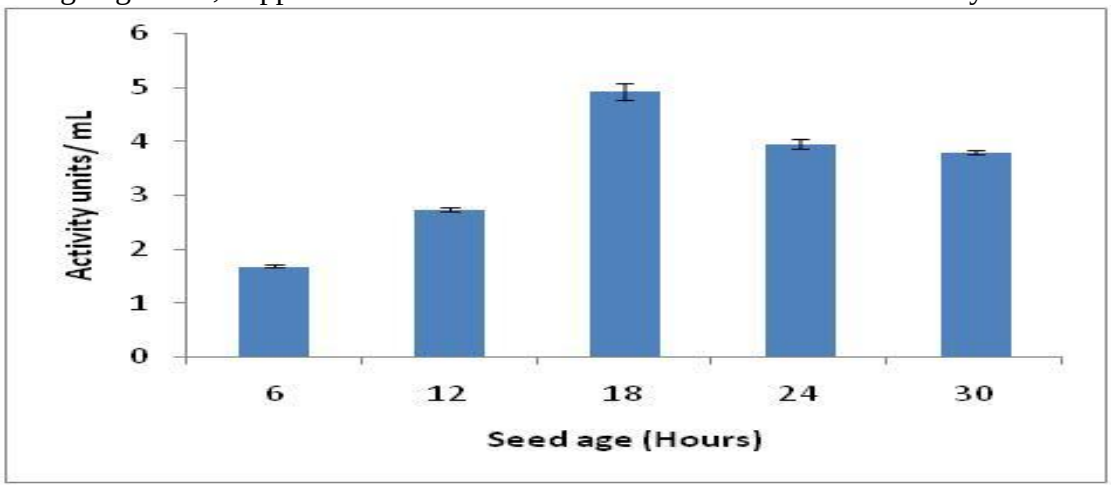


Fig. 4-- Effect of seed age on pediocin activity (n=3)

As observed, a seed age of 18 hours enabled highest pediocin activity, at 4.93 ± 0.31 AU/ mL (Fig. 4). The growth curve of the isolated strain shows the exponential phase between 14 and 28 hours (Fig 1), in a total life cycle of 48 hours. It can be hypothesised that at 18 hours of growth, the cells attain synchronization, within a few hours of the beginning of log phase, and hence show improved activity when inoculated at a seed age of 18 hours

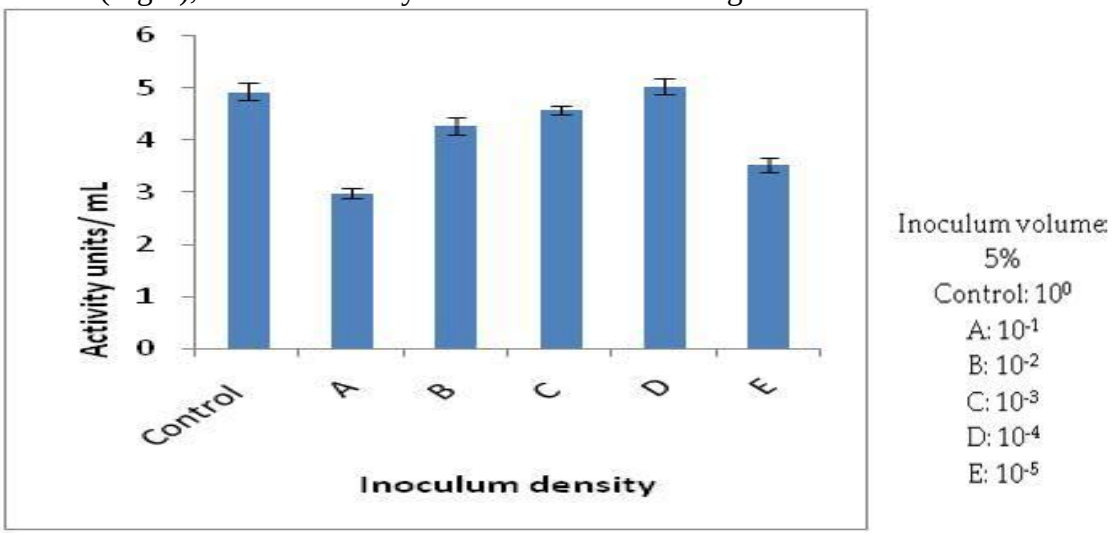


Fig 5-- Effect of inoculum density on pediocin activity (n=3)

To study the effect of inoculum density, cells were serially diluted and inoculated at different dilutions and examined for pediocin production. Pediocin activity was found to

initially increase with dilution of inoculum (Fig. 5) until a factor of 10^{-4} (4.93 ± 0.31 AU/ mL), beyond which the activity tends to drop. Yet, the increase in pediocin activity at a factor of 10^{-4}, as opposed to the control inoculum without any dilution, is insignificant ($p>0.05$). Hence inoculum density is not a significant factor for the strain under study for the production of pediocin.

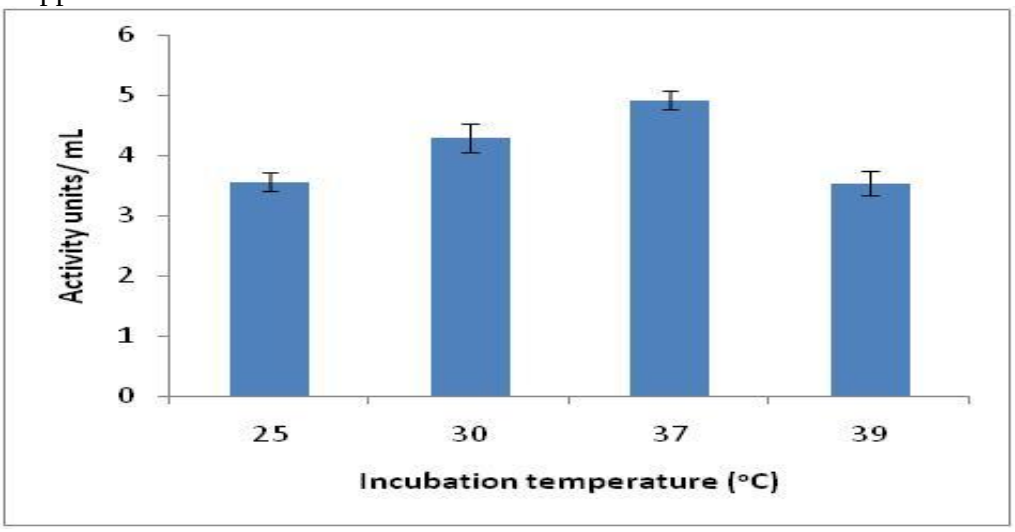


Fig 6-- Effect of incubation temperature on pediocin activity (n=3)

The optimum production temperature (Fig. 6) and pH (Fig. 7) were observed to be at 37°C and 6 units respectively (4.93 ± 0.31 AU/ mL). These values correspond to the optimum growth conditions of temperature and pH of the strain, making bacteriocin production a growth related trait as described previously (Ray, 1995).

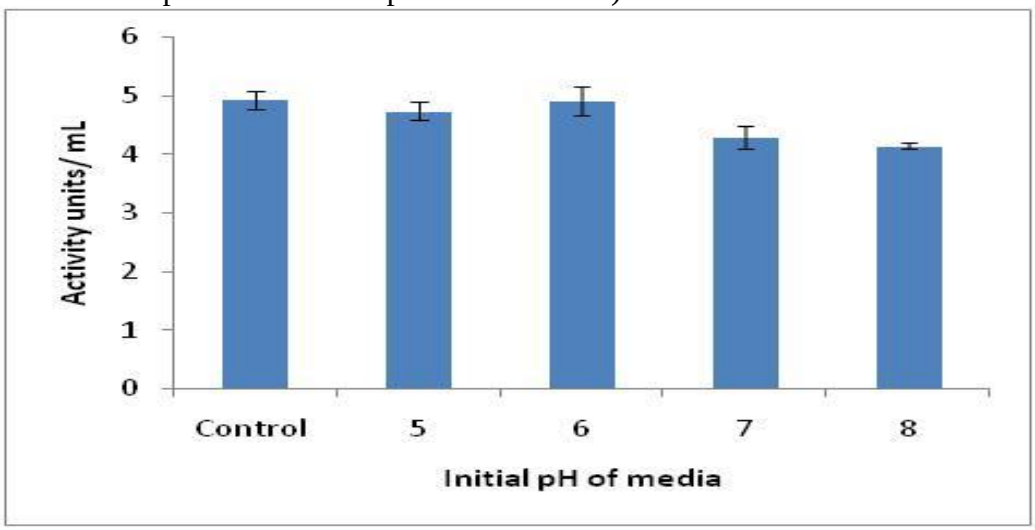


Fig. 7-- Effect of initial media pH on pediocin activity (n=3), Control: pH 6.3

The highest activity was observed at an incubation time of 21 hours (Fig. 8) which is approximately the mid log phase of growth for the strain (Fig1). The cells lost their pediocin production capability beyond mid log phase, although to a lower extent. Since pediocin production is growth related (Ray, 1995), as the cells slowly reach the stationary phase, there is a drop in the pediocin activity.

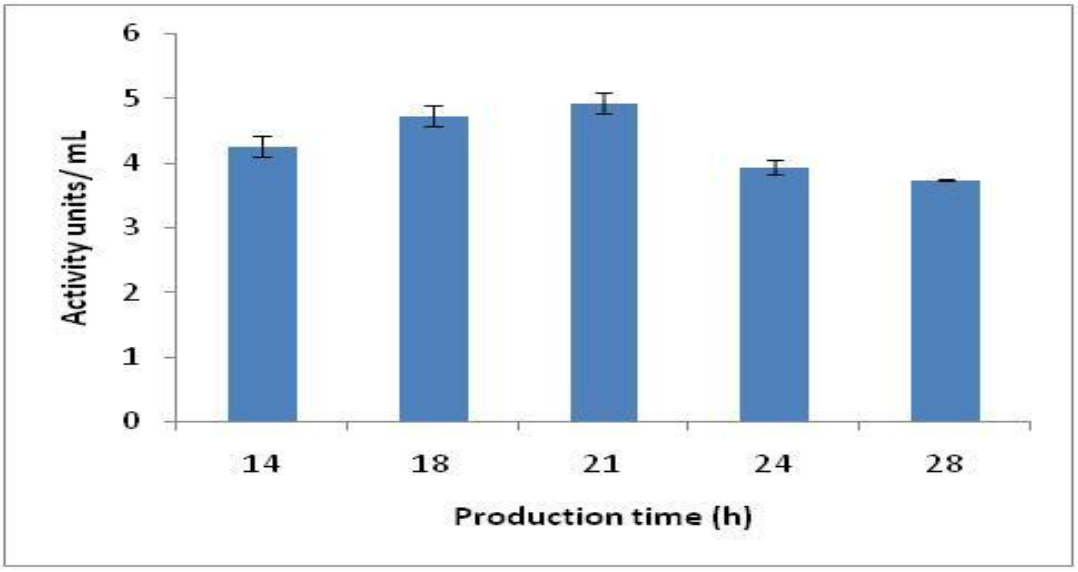


Fig. 8-- Effect of incubation time on pediocin activity (n=3)

4. CONCLUSION

Attempts to optimize the physical parameters for pediocin production by *Kurdai* isolate *Pediococcus pentosaceus* showed little or no improvement in pediocin activity. The scope of the work lies in studying the chemical parameters responsible for pediocin production followed by further attempts at optimization.

Acknowledgement

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