サンザイム
Sanzyme Biologics

PrOme™ - BS
SAN 144 BS

In-Vitro Studies

SANZYME BIOLOGICS (P) LTD
Regd. Office : Plot No.13. Sagar Society, Road No.2,
Banjara Hills, Hyderabad-500 034.,Telangana, India
Phone : +91 40-4858 9999 | Fax : +91 40-4858 9913 |
Website : www.sanzymebiologics.com |
Email : info@sanzymebiologics.com
CIN : U24110TG2016PTC112002

Factory Unit I: Door No: 7-4-115, Sy. No: 258 & 259, Gaganpahad, Bangalore Road, Hyderabad – 500052.,Telangana, India

Factory Unit II: Plot Nos. 19 to 22, Sy. No's. 321/1,11,12,13 & 276 & 277, Biotech Park, Phase - III, Genome Valley, Karkapatla (V), Markook (Mdl.), Siddipet (Dist.), Pin Code : 502 281,Telangana, India

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QUALITY CONTROL DEPERTMENT

Acid Tolerance Studies

Product: PROME-BS

Strain: *Bacillus subtilis* SAN 144BS (Encapsulated)

Objective: To determine Acid tolerance assay of PROME-BS

Initial Potency for Study: 3000 CFU/ml

- **Acid Tolerance & Recovery Assay Protocol:**
    1) Prepare appropriate buffers as glycine HCl (Gly-HCl, pH 2.2 and 3.0) and Phosphate Buffered Saline (PBS, pH 7.0)
    2) Use overnight grown *Bacillus subtilis* SAN 144BS (~12 hour) culture, wash it thrice with PBS
    3) This washed and diluted culture is diluted at 1:10 dilution in appropriate buffer at pH 2.0, 3.0 and 7. 0
    4) Measure Optical Density at 600 nm in a spectrophotometer at every hour for 6 hours starting from '0' hour
    5) At the same time, for each hour sample is subjected for assay by taking 500 µl of sample, pour plate with Nutrient agar medium in triplicates, and incubate at 37 ^{0}C for 24 hours.
    6) Keep the culture sample prepared in normal saline without acid treatment as control.
    7) Below assay protocol can be adopted for pour plating.
    8) Plating:
        a) Set out sterile petri plates and add 1 ml of control and heat treated final dilution into each petri plate and then pour 15-20 ml molten Nutrient Agar (NA) medium into each of the petri plate and mix thoroughly (temperature around 45 °C)
        b) Incubate the solidified plates in an inverted position at 37 °C for 24 hours.
    9) Counting:
        a) Count the number of CFU in each petri plate and calculate by taking the average number of CFU.

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10) Calculation:

$$\text{Viable Count} = \frac{\text{Average No. of CFU x Dilution factor}}{\text{Weight of sample taken x } 10^9} = \text{Billion CFU /g}$$

*Note: Perform all the dilutions in the vicinity of Uni directional Air Flow Unit
*Note: Vortex all the serial dilutions, while performing the assay.

- Media Composition:
  - Nutrient Agar : Hi media Code: M001
- Preparation of sterile phosphate buffer (pH 6.8)
  - Weigh accurately 6.8 g of Potassium dihydrogen phosphate and dissolve in 100 ml distilled water, then add 1.0 g Sodium hydroxide and make up the final volume to 1000 ml with distilled water. The solution shall be sterilized with steam at 121 °C (or 15 lbs pressure) for 20 min and then cool.

Result:

Time (Hours)	pH 2.0 (CFU/ml)	% of Recovery	pH 3.0 (CFU/ml)	% of Recovery	pH 7.0 (CFU/ml)	% of Recovery
0	3000	100.00	3000	100.00	3000	100.00
1	2955	98.49	2979	99.29	2963	98.77
2	2876	95.85	2946	98.21	2954	98.46
3	2842	94.72	2904	96.79	2945	98.15
4	2740	91.32	2871	95.71	2861	95.38
5	2660	88.68	2796	93.21	2861	95.38
6	2558	85.28	2775	92.50	2852	95.08

Conclusion:

- PROME-BS was recovered 85.28% (2558 CFU/ml) at pH 2 and 6 Hours Incubation
- PROME-BS was recovered 92.50% (2775 CFU/ml) at pH 3 and 6 Hours Incubation
- PROME-BS was recovered 95.08% (2852 CFU/ml) at pH 7 and 6 Hours Incubation

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QUALITY CONTROL DEPERTMENT

Bile Tolerance Studies

Product: PROME-BS

Strain: *Bacillus subtilis* SAN 144BS (Encapsulated)

Objective: To determine Bile tolerance assay of PROME-BS

Initial Potency for Study: 3000 CFU/ml

- **Bile Tolerance & Recovery Assay Protocol:**
  1. Prepare appropriate bile solutions at concentrations 0.15%, 0.3% and 0.6%
  2. Use overnight grown *Bacillus subtilis* SAN 144BS (~12 hour) culture, wash it thrice with PBS
  3. This washed and diluted culture is further diluted at 1:10 dilution in appropriate bile solution at 0.15%, 0.3% and 0.6% concentration
  4. Measure Optical Density at 600 nm in a spectrophotometer at every hour for 6 hours starting from '0' hour
  5. At the same time, for each hour sample is subjected for assay by taking 500 µl of sample and pour plate with Nutrient agar medium in triplicates and incubate at 37 ^{0}C for 24 hours.
  6. Keep the culture sample prepared in normal saline without bile solution treatment as control.
  7. Below assay protocol can be adopted for pour plating.
  8. Plating:
     a) Set out sterile petri plates and add 1 ml of control and heat treated final dilution into each petri plate and then pour 15-20 ml molten Nutrient Agar (NA) medium into each of the petri plate and mix thoroughly (temperature around 45 °C)
     b) Incubate the solidified plates in an inverted position at 37 °C for 24 hours.
  9. Counting:
     a) Count the number of CFU in each petri plate and calculate by taking the average number of CFU.
  10. Calculation:

$$\text{Viable Count} = \frac{\text{Average No. of CFU x Dilution factor}}{\text{Weight of sample taken x } 10^{9}} = \text{Billion CFU /g}$$

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*Note: Perform all the dilutions in the vicinity of Uni directional Air Flow Unit
*Note: Vortex all the serial dilutions, while performing the assay.

- Media Composition:
  - Nutrient Agar : Hi media Code: M001
- Preparation of sterile phosphate buffer (pH 6.8)
  - Weigh accurately 6.8 g of Potassium dihydrogen phosphate and dissolve in 100 ml distilled water, then add 1.0 g Sodium hydroxide and make up the final volume to 1000 ml with distilled water. The solution shall be sterilized with steam at 121 °C (or 15 lbs pressure) for 20 min and then cool.

Result:

Time (Hours)	Bile 0.15 % (CFU/ml)	% of Recovery	Bile 0.3 % (CFU/ml)	% of Recovery	Bile 0.6 % (CFU/ml)	% of Recovery
0	3000	100.00	3000	100.00	3000	100.00
1	2986	99.52	2969	98.96	2953	98.44
2	2964	98.81	2938	97.92	2888	96.25
3	2943	98.10	2891	96.36	2822	94.06
4	2893	96.43	2844	94.81	2728	90.94
5	2822	94.05	2774	92.47	2691	89.69
6	2764	92.14	2688	89.61	2569	85.63

Conclusion:

- PROME-BS was recovered 92.14% (2764 CFU/ml) at 0.15% Bile concentration and 6 Hours Incubation
- PROME-BS was recovered 89.61% (2688 CFU/ml) at 0.3% Bile concentration and 6 Hours Incubation
- PROME-BS was recovered 85.63% (2569 CFU/ml) at 0.6% Bile concentration and 6 Hours Incubation

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QUALITY CONTROL DEPERTMENT

Thermostability Studies

Product: PROME-BS

Strain: *Bacillus subtilis* SAN 144BS (Encapsulated)

Objective: To determine the Thermostability activity of PROME-BS

Initial Potency for Study: 10 Billion CFU/g

Temperatures Exposed: 120 ºC and 130 ºC

Time: Each sample was exposed to the said temperatures for 10 and 20 minutes

Heat Treatment & Recovery Assay Protocol:

1) Sample Preparation:
    a. Take 1.0 g of sample and transfer to 100 ml sterile standard volumetric flask.
    b. Add approximately 60 ml of sterile phosphate buffer (pH 6.8) and shake vigorously.
    c. Transfer 1 ml of this stock solution into 9.0 ml of phosphate buffer at pH 6.8 in a sterile test tube and mix thoroughly.
    d. Continue serial dilution until the desired dilution series is obtained.
2) Heat Shock:
    a. Allow the final dilution to stand in the water bath at 120 °C & 130 °C for 10 & 20 minutes respectively
    b. Cool the test tubes to room temperature.
    c. Use final dilution without heat shock as control sample.
3) Plating:
    a. Set out sterile petri plates and add 1 ml of control and heat treated final dilution into each petri plate and then pour 15-20 ml molten Nutrient Agar (NA) medium into each of the petri plate and mix thoroughly (temperature around 45 °C)
    b. Incubate the solidified plates in an inverted position at 37°C for 24 hours.
4) Counting:
    a. Count the number of CFU in each petri plate and calculate by taking the average number of CFU.

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5) Calculation:

$$\text{Viable Count} = \frac{\text{Average No. of CFU x Dilution factor}}{\text{Weight of sample taken x } 10^9} = \text{Billion CFU /g}$$

*Note: Perform all the dilutions in the vicinity of Uni directional Air Flow Unit
*Note: Vortex all the serial dilutions, while performing the assay.

- Media Composition:
  - Nutrient Agar : Hi media Code: M001
- Preparation of sterile phosphate buffer (pH 6.8)
  - Weigh accurately 6.8 g of Potassium dihydrogen phosphate and dissolve in 100 ml distilled water then add 1.0 g Sodium hydroxide and make up the final volume to 1000 ml with distilled water. The solution shall be sterilized with steam at 121°C (or 15 lbs pressure) for 20 min and then cool.

Results:

Sample No.	Initial Potency	Temp. Exposed	Time	Recovery Potency	
	Bn CFU/g	ºC	min.	Bn CFU/g	%
1	10.0	120	10	10.0	100%
2	10.0	120	20	9.5	95%
3	10.0	130	10	9.0	90%
4	10.0	130	20	8.0	80%

*Bn ➔ Billion
*Temp. ➔ Temperature

Conclusions:

- PROME-BS was recovered at100 % (10.0 Billion CFU/g) when treated at 120 ºC for 10 minutes
- PROME-BS was recovered at 95 % (9.5 Billion CFU/g) when treated at 120 ºC for 20 minutes
- PROME-BS was recovered at 90 % (9.0 Billion CFU/g) when treated at 130 ºC for 10 minutes
- PROME-BS was recovered at 80 % (8.0 Billion CFU/g) when treated at 130 ºC for 20 minutes

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ร้านฉลาดคิด® by Siriraj
งานพัฒนาและสร้างประโยชน์จากนวัตกรรมและเทคโนโลยี
ฝ่ายวิจัย สำนักงานคณบดี คณะแพทยศาสตร์ศิริราชพยาบาล
มหาวิทยาลัยมหิดล โทร.0-24192995-7 โทรสาร.0-24183307
เลขประจำตัวผู้เสียภาษี 0994000158378

Room 542, Research Laboratory Unit, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wanglang Rd., Siriraj, Bangkoknoi, Bangkok 10700, Thailand

Antimicrobial Resistant Gene Detection Report

Date: 04.02.2020

Name & Address of customer		Sanzyme Biologics Plot No. 13, Sugar Society, Road No.2, Banjara Hills, Hyderabad-500 034	
Sample ID / type		*Bacillus subtilis* San144BS	
Date of sample receipt	22-01-2020	Method of test	Real-time PCR with specific probes
Date of sample testing	23-01-2020 to 03-02-2020	Condition of sample when receive	O.K.

Results

	Antimicrobial classes	Target gene	Results
1	β-lactams	CTX-M1	NF
2	(penicillin,	CTX-M2-M74	NF
3	amoxicillin,	CTX-M8-M25	NF
4	cephalosporin)	CTX-M9	NF
5		PER	NF
6		VEB	NF
7		CMY1-MOX	NF
8		CMY2-LAT	NF
9		DHA	NF
10		FOX	NF
11		ACT-MIR	NF
12		OXA-1	NF
13		OXA-9	NF
14	Carbapenems	KPC	NF
15		GES	NF
16		NDM	NF
17		VIM	NF
18		IMP	NF
19		OXA-48	NF
20	Folate pathway	*sul1*	NF
21	inhibitors	*sul2*	NF
22		*sul3*	NF
23		*dfrA1*	NF
24		*dfrA5-14*	NF
25		*dfrA12*	NF
26		*dfrA17*	NF

	Antimicrobial classes	Target gene	Results
27	Polymyxins	*mcr-1*	NF
28		*mcr-2*	NF
29	Tetracyclines	*tetA*	NF
30		*tetB*	NF
31	Phenicols	*cmlA*	NF
32		*floR*	NF
33		*catA1*	NF
34		*catB3*	NF
35	Aminoglycosides	*aacC1*	NF
36		*aacC2*	NF
37		*aacC4*	NF
38		*aphA1*	NF
39		*aphA6*	NF
40		*aadA1-2-17*	NF
41		*aadB*	NF
42		*armA*	NF
43		*rmtB*	NF
44	Macrolides	*ermB*	NF
45		*mphA*	NF
46	Quinolones	*qnrA*	NF
47		*qnrS*	NF
48		*qnrB1*	NF
49		*qnrB4*	NF
50		*Qep*A	NF

~ 3-8 X10^6 cells was tested. Positive grading criteria: 1+: ≥ 10^1-10^2, 2+: >10^2-10^3 and 3+ = >10^3 positive cells NF = Not found

Tested person: Rattapha Chinli
(Ms. Rattapha Chinli)

Authorized person: Suporn Foongladda
(Assoc.Prof.Dr. Suporn Foongladda)

Tel: 02-4199811, 0819390258 e-mail: suporn.foo@mahidol.ac.th

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PrOme™ - Max

In-Vivo Studies

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Effects of SANZYME BIOLOGIC's, **Prome™ - BS** & **Prome™ - Max** on intestinal integrity and immune response of broiler chickens challenged with *Clostridium perfringens*

Study **Location:** R&D Facility, Thailand

Study **Period:** July - Aug 2016 (42 days)

Experimental Design

Study Groups	**Description**	
T1	Practical Control (Positive control)	PC
T2	Positive Control + *Clostridium perfringens* (CP)	PC + CP
T3	PC + **Prome™-BS** (100g/T) + CP	PC + PBS + CP
T4	PC + **Prome™-Max** (100g/T) + CP	PC + PMX + CP
T5	PC + Competitor *Bacillus subtilis* (100g/T) + CP	PC + CBS + CP
T6	PC + Zinc Bacitracin (335g/T) + CP	PC + ZB + CP

Controlled Inputs

1	T2, T3, T4, T5 & T6 groups were infected with *Clostridium perfringens* (CP) $3x10^9$ CFU/g per bird per day on day 13, 14 & 15
2	T2, T3, T4, T5 & T6 groups were infected with oocysts of *Eimeria spp.* on day 7
3	All groups were induced with a heat stress through temperature scale by 3°C to 4°C
4	**Prome™-BS** is a proprietary strain of *Bacillus subtilis* SAN 144BS • Potency used for experiment = 1 Billion CFU/g
5	**Prome™-Max** is a proprietary blend of *Bacillus subtilis* SAN 144BS + *Bacillus coagulans* SAN 135BC + *Bacillus licheniformis* SAN 136BL • Potency used for experiment = 4 Billion CFU/g
6	Competitor product was a well-known brand of *Bacillus subtilis* • Potency used for experiment = 1 Billion CFU/g

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Growth Performance

FCR			
Groups	**Description**	0-18 day	0-42 day
T1	PC	1.275	1.759
T2	PC + CP	1.358	1.843
T3	PC + PBS + CP	1.313	1.610
T4	PC + PMX + CP	1.412	1.790
T5	PC + CBS + CP	1.330	1.650
T6	PC + ZB + CP	1.321	1.729

Serum Antibody Titers

Groups	**Description**	**Antibody Titers - ND**	**Antibody Titers - IBD**
		18^{th} day	42^{nd} day
T1	PC	2.33	514
T2	PC + CP	1.75	576
T3	PC + PBS + CP	2.20	644
T4	PC + PMX + CP	2.35	657
T5	PC + CBS + CP	1.83	521
T6	PC +ZB + CP	2.17	564

Intestinal Morphological Analysis (Histo-patholology Report)

Groups	**Description**	**V/C Ratio - Jejunum**	**V/C Ratio - Ileum**
		42^{nd} day	42^{nd} day
T1	PC	12.08	7.06
T2	PC + CP	7.99	5.37
T3	PC + PBS + CP	11.93	6.71
T4	PC + PMX + CP	11.98	6.75
T5	PC + CBS + CP	11.27	6.33
T6	PC +ZB + CP	10.45	6.42

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Bactericidal Effect on *Clostridium perfringens*
(Microbiological Assay Report)

Groups	Description	CP - Ileum (x 10^9 CFU/g)	CP - Caecum (x10^9 CFU/g)
		42nd day	42nd day
T1	PC	2.54	3.69
T2	PC + CP	6.23	6.29
T3	PC + PBS + CP	2.77	3.47
T4	PC + PMX + CP	2.65	3.32
T5	PC + CBS + CP	2.97	4.12
T6	PC + ZB + CP	3.88	5.63

Protein Retention Assessment in Gut
(Biochemical Assay Report)

Groups	Description	Protein Retention % (42nd day)
T1	PC	60.64
T2	PC + CP	57.73
T3	PC + PBS + CP	63.49
T4	PC + PMX + CP	64.01
T5	PC + CBS + CP	63.04
T6	PC + ZB + CP	63.24

Conclusions:

1. **Prome™-BS** & **Prome™-Max** have a strong activity against *Clostridium perfringens* as compared tocompetitor BS & AGP
2. **Prome™-BS** & **Prome™-Max** improved immunity significantly as compared to competitor BS
3. **Prome™-BS** & **Prome™-Max** improved overall zootechnical performance and gut integrity in challenged conditions as compared to competitor BS